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Research Article

The Prevalence and Associated Risk Factors of *Coa* Gene (Coagulase Positive *Staphylococcus aureus)* from Bovine Milk

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Abstract

The study describes prevalence of coagulase positive *Staphylococcus aureus,* a zoonotic pathogen, and rule out key risk factors associated with the spread of this pathogen. A total of 900 milk samples of animals (n = 450 cattle and n = 450 buffaloes) from three subdistricts of Faisalabad were collected and processed microbiologically for staphylococci identification while, PCR for coa gene identification. Surf field mastitis test was used to screen the samples for subclinical mastitis at milking. Chi-square test was used to assess association of risk factors with mastitis. The overall prevalence of subclinical mastitis was 55% comprising 54 and 56% in cattle and buffalo's milk, respectively. A significant difference ($P<0.05$) was seen in the prevalence of subclinical mastitis among the cattle from different cities. The prevalence of c*oa* gene presented 39.33% from total samples. Highest prevalence was found in tehsil Samundari followed by Jaranwala and Faisalabad presenting 43%, 39%, and 36%, respectively. The quarter based prevalence of subclinical mastitis was found 32% while 5.58% of quarters were blind. Right side quarters and teats were more affected. The risk factors except body condition and parity presented significant association with mastitis. The study concluded that bacterial coa gene, was prevalent in milk, and determinants were strongly associated with higher prevalence.

Keywords: *Coagulase, Staphylococcus aureus, Coa gene, Risk Factors and subdistricts*

İnek Sütünde Koagulaz Pozitif Staphylococcus aureus *Coa* Geni Prevalansı ve İlgili Risk Faktörleri

Özet

Bu çalışma; zoonotik bir patojen olan koagülaz pozitif *Staphylococcus aureus*'un prevalansını ve bu patojenin yayılmasında rol oynayan risk fakörlerini tanımlamaktadır. Faisalabad'ın 3 ayrı bölgesindeki hayvanlardan toplanan toplam 900 adet süt örneğinde (n = 450 sığır ve n = 450 manda) *Staphylococci* identifikasyonu amacıyla mikrobiyolojik analiz ve coa geninin tespiti amacıyla PCR gerçekleştirildi. Subklinik mastitisin taranmasında sağım esnasında sörf mastitis testi uygulandı. Risk faktörlerinin mastitisle ilişkisini değerlendirmek amacıyla Ki-kare testi kullanıldı. Sığır ve manda sütlerinde subklinik mastitis prevalansı sırasıyla %54 ve %56'sı olarak hesaplanırken toplamda %55 olarak bulundu. Farklı şehirlerden örneklenen sığırlar arasında subklinik mastitis prevalansı yönünden anlamlı bir fark tespit edildi ($P<0.05$). *Coa* geninin prevalansı toplam örneklerde %39.33 olarak saptanmıştır. En yüksek prevalans oranı %43 olarak Samundari'de saptanırken bunu sırasıyla %39 ile Jaranwala ve %36 ile Faisalabad izlemektedir. Subklinik mastitisin meme loblarındaki prevalansı %32 olarak saptanırken meme loblarının %5.58'inde ise körelme belirlendi. Sağ taraftaki meme lobları ve meme başlarının daha fazla etkilendiği saptandı. Vücut kondisyonu ve doğum sayısı dışındaki risk faktörleri mastitis ile anlamlı ilişki içeriyordu. Çalışma, bakteriyel coa geninin sütte yaygınlığını ve göstergelerin yüksek prevalans ile kuvvetli bir ilişkisi olduğunu göstermektedir.

Anahtar sözcükler: *Koagülaz, Staphylococcus aureus, Coa geni, Risk faktörleri ve alt bölge*

INTRODUCTION

Milk quality and quantity deteriorates with bacterial invasion to milk producing glands that resultantly increases somatic cell count making milk unfit for consumers. This deterioration accounts for annual losses in dairy industry reaching to approximately 2 billion dollars in USA and 526 million dollars in India where subclinical mastitis gives

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its contribution to approximately 70% of these losses [1]. Among bacterial contaminants in bovine mastitis, *Staphylococcus aureus (S. aureus)* are overwhelming to control and are well-known to cause subclinical mastitis. The pathogen is a Gram positive organism with ability to survive in high salt and high temperature range appearing as an opportunistic pathogen and frequent colonizer of the epithelium causing severe diseases in humans and animals. The organism is of zoonotic importance that becomes more serious in its resistant form against antimicrobial agents [2,3]. The bovine *S. aureus* transferred to humans may lead to outbreak in humans and animal population [4].

The lower cure rate of bovine *S. aureus* mastitis, evading immune response of the host, and extensive use of beta-lactam antibiotics add to *S. aureus* nuisance [5,6] which influences the pathogenesis of *S. aureus* in mastitis. Coagulase positive *S. aureus* has ability to yield toxins such as; exfoliative, toxic shock syndrome toxin and panton-valentine leucodin. Subclinical mastitis has dynamic importance in its association with zoonotic impacts as milk serves as vehicle of pathogens transmission [7].

Intramammary infections caused by *S. aureus* damage the alveolar physiology; decrease in milk production, and impart harmful effects on milk composition [8,9]. *S. aureus* inflicted intramammary infections are long term and chronic as bacteria has the ability to hide itself in mammary epithelial cells and host phagocytes. Concurrent resistance of bacterial species to antimicrobials of different structural classes is increasing in multitude complicating therapeutic management of infections [10].

The traditionally infectious agents are identified on the basis of cultural examination that sometimes fails in case of non-cultivable strains thus creating ambiguity in identification. Polymerase Chain Reaction (PCR) works as an effective technique to diagnose coagulase positive *S. aureus* from human and animal origin [11,12]. Adding to this, scarce epidemiological studies regarding *S. aureus* prevalence in bovine milk may unleash zoonotic spread of pathogenic *S. aureus*.

Keeping in view the animal and human health hazard anchored with this pathogen, the current study was designed to estimate prevalence of *Coagulase* positive *S. aureus* from cattle and buffalo milk obtained from various private and public dairy farms of Pakistan and to rule out key risk factors associated with spread of this pathogen.

MATERIAL and METHODS

The district Faisalabad, 3rd largest city in Pakistan situated 184 meter higher to sea level with altitudes between 31°20′-31°33′ N and 73°13′-72°55′ [13]. The sole livelihood of people in and around the jurisdiction of district Faisalabad is agro based where lives of locals are directly linked with livestock rearing. Cattle and buffalo are kept as live banks by families in that mostly bovine serve as only income generator for house hold expenses. Three tehsils of district Faisalabad i.e. Faisalabad, Jaranwala and Samundari were selected as representative of bovine population of district. The bovine populations were approached using convenient technique of sampling as described by Thrusfield [14] keeping in view constraints of farm owner's willingness and access to the laboratory. The milk samples collected from bovine were divided into three categories; (1) 40-90 bovine, (2) 91-200 bovine and (3) >200 bovine in such a way that ten farms from each tehsil each with 30 milk samples from each farm (n=15 cattle, n=15 buffalo) totaling 900 milk samples (n=450 cattle, n=450 buffalo) were taken. Subclinical form of mastitis was assessed by Surf Field Mastitis Test (SFMT) proposed by Muhammad et al.[15].

Milk samples (n=900) were streaked on blood agar and later based on colony characters were sub-cultured on Mannitol Salt Agar. Gram staining and catalase test [16] were performed to differentiate organisms as Staphylococci. The Staphylococci were processed to DNA extraction using bacterial DNA extraction kit (GF-1 Bacterial DNA extraction kit, Vivantis Technologies Sdn. Bhd, Malaysia). The extracted DNA was quantified by Nano-Drop technique (NanoDrop 2000, Thermo-Scientifics, NanoDrop products, 3411 Silverside Rd, Bancroft Building, and Wilmington, DE 19810 USA).

The specification of PCR reaction included reaction mixture of 25 µL having, 12.5 µL of master mix (2x master mixes, Accuprime TM super mix11), 1.5 µL (10 pmol) from each primer, 8 µL distilled water, and 1.5 µL DNA sample. The primers, Coag 2 (CGA GAC CAA GAT TCA ACA AG), Coag 3 (AAA GAA AAC CAC TCA CAT CA) were used with product size of 970bp. The reaction of PCR was run in thermocycler (Eppendorf, Mastercycler®5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, and Germany) with following specification; initial denaturation at 94°C/10 min for once, and denaturation at 94°C/45 seconds, annealing at 54°C/1 min and extension at 72°C/2 min, respectively for 30 cycles each. Final extension at 72°C/10 min were given at the end for single time. The PCR product was run on 1.5% agarose gel stained with ethidium bromide [17] that was photographed under ultraviolet illuminator *(Fig. 1)*.

Risk factors assumed in dichotomous questionnaire to check their association with mastitis included information like specie, breed, age, diet, season, system of rearing, presence of ticks, body health, use of teat dips, feed and water, udder consistency and condition, animal physiological status either lactating or dry, parity, milking system, hygiene and milker's care during milking, use of antibiotics for general ailments, mastitis purpose, call for veterinary professionals, and call for veterinary consultancy.

Statistical Analysis

The data was subjected to statistical analysis using appropriate designs on SPSS version 22.0 computer program. Prevalence of mastitis was calculated as per formula described by Thrusfield [14] and chi-square test was used to statistically correlate the risk factors with prevalence at 5% probability.

RESULTS

Subclinical form of mastitis is type in which there are apparently no abnormal signs except that screening test detects invisible abnormality in milk. The current study found 55% (495/900) subclinical mastitis in bovine from district Faisalabad with highest percentage noted in tehsil Samundari (67.67%) followed by Jaranwala (52.33%) and Faisalabad (45%) *(Table 1)*. There was a significant difference (P<0.05) of subclinical mastitis among different tehsils on overall bovine subclinical mastitis basis. However, subclinical mastitis in cattle from different tehsils indicated a non-significant difference (P<0.05) presenting 62.67%, 50.67%, and 48.67% of subclinical mastitis from tehsil Samundari, Jaranwala, and Faisalabad, respectively. Similar pattern was observed in case of buffalo milk subclinical mastitis from different tehsils of district Faisalabad. The comparison of specie showed a non-significant difference (P>0.05) of subclinical mastitis in cattle and buffalo from different cities of district Faisalabad with 48.67% versus 41.33%, 50.67% versus 54%, and 62.67% versus 72.67% of subclinical mastitis between cattle and buffalo in tehsil Faisalabad, Jaranwala, and Samundari was noted, respectively.

Coa gene *(Staphylococcus aureus)* presented 39.33% in bovine from district Faisalabad from total collected milk samples *(Table 2)*. The comparison of *S. aureus* prevalence from different tehsils showed 43%, 39%, and 36% from tehsil Samundari, Jaranwala, and Faisalabad, respectively. Subclinical mastitis among different tehsils of district Faisalabad presented a non-significant difference (P>0.05) with 34%, 36%, and 40% of prevalence from tehsil Faisalabad, Jaranwala, and Samundari, respectively. Similar pattern was noted in case of buffalo milk subclinical mastitis from different tehsils of district Faisalabad.

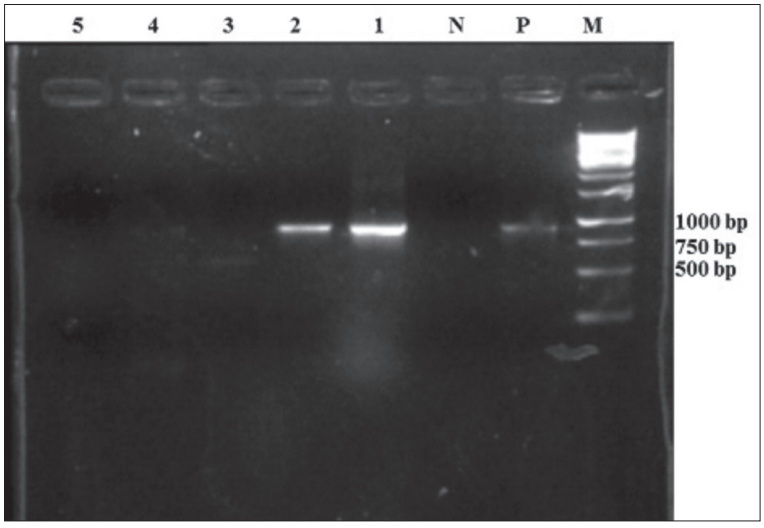

Fig 1. PCR results of *coa* gene, M = 1kb marker (product brand), P = positive control (coa gene, Momtaz et al.[18]), N = neative control, 1-2 positive samples at 907bp, 3-5 negative samples

The number of blocked quarters were found 5.58% (201/3600) with highest percentage of blocked quarter noted in case of front right (FR) followed by rear right (RR), front left (FL), and rear left (RL) with 6.89%, 6.56%, 4.67%, and 4.22%, respectively *(Table 3)*. The study found right side suffering higher mastitis compared to left side quarters with a non-significant difference (P>0.05). The quarter based prevalence was

Table 1. *Prevalence of subclinical mastitis in bovine from District Faisalabad*

Sampled Area	Cattle				Buffalo				Overall (Cattle and Buffalo)			
	No. Milk Samples	No. Positive (%)	C.I	*P-value*	No. Milk Samples	No. Positive (%)	C.I	*P-value*	No. Milk Samples	No. Positive (%)	C.I	*P-value*
Faisalabad	150	73 (48.67)	39.98-56.02		150	62 (41.33)	33.42-9.24		300	135 (45)	39.36-50.64	
Jaranwala	150	76 (50.67)	41.97-58.03	0.022	150	81 (54)	46.00-2.00	0.001	300	157 (52.33)	46.67-58.00	0.001
Samundari	150	94 (62.67)	54.90-70.43		150	109 (72.67)	51.41-0.60		300	203 (67.67)	62.36-72.96	
Total	450	243 (54)	48.94-58.16		450	252 (56)	65.50-9.82		900	495 (55)	51.75-58.25	

C.I: *Indicates confidence interval set at 95%; P<0.05 indicate significant difference*
** Between specie comparison: cattle versus buffalo from Faisalabad P=0.246, Jaranwala, P=0.488, Samundari P=0.064, and Total district P=0.461*

Table 2. *Prevalence of coa gene (Staphylococcus aureus) from bovine milk of District Faisalabad*

Sampled Area	Cattle				Buffalo				Overall			
	No. Milk Samples	No. Positive (%)	C.I	*P-value*	No. Milk Samples	No. Positive (%)	C.I	*P-value*	No. Milk Samples	No. Positive (%)	C.I	*P-value*
Faisalabad	150	51 (34)	26.40-41.61		150	57 (38)	30.21-45.80		300	108 (36)	30.56-41.44	
Jaranwala	150	54 (36)	28.29-43.71	0.547	150	63 (42)	34.07-49.92	0.373	300	117 (39)	33.47-44.53	0.212
Samundari	150	60 (40)	32.13-47.87		150	69 (46)	38.00-54.00		300	129 (43)	37.39-48.61	
Total	450	165 (36.67)	32.20-41.12		450	189 (42)	37.43-46.56		900	354 (39.33)	36.14-42.52	

C.I: *Indicates confidence interval set at 95%; P<0.05 indicates significant difference*
** Between specie comparison: cattle versus buffalo from Faisalabad P=0.470, Jaranwala P=0.287, Samundari P=0.294, and Total district P=0.101*

Table 3. *Prevalence of teat blockage and quarter based prevalence*

	Prevalence of Blocked Quarters				Prevalence on Quarter Basis			
Quarters	Total Quarter Examined	No. Blocked (%)	CI	P-value	Total Quarter Examined	No. Blocked (%)	CI	P-value
FR	900	62 (6.89)	5.23-8.55		838	290 (34.61)	31.38-37.83	
FL	900	42 (4.67)	3.29-6.05	0.028	858	252 (29.37)	26.32-32.42	0.001
RR	900	59 (6.56)	4.94-8.18		841	311 (36.98)	33.72-40.25	
RL	900	38 (4.22)	2.91-5.53		862	235 (27.26)	24.29-30.23	
Total	3600	201 (5.5)	4.83-6.33		3399	1088 (32.0)	30.35-33.48	

CI: *Indicates confidence interval set at 95%*

found 32% (1088/3399) from bovine. The right side quarters presented higher prevalence with 36.98% and 34.61% in rear right (RR) and front right (FR) compared to left side quarters with 29.37% and 27.26% in front left (FL) and rear left (RL), respectively. The difference of quarter based prevalence among different quarters showed significant difference (P<0.05).

The study found non-significant association of mastitis with age in case of cattle whereas age of buffalo was found significantly associated with subclinical mastitis *(Table 4)*. The breeds of cattle showed significant association with mastitis occurrence among them Frisian cattle had higher prevalence of subclinical mastitis (66.67%) than to Sahiwal (40%) and non-descript cattle (48.83%). However, breeds factor in case of buffalo subclinical mastitis did not have a significant (P>0.05) association with mastitis occurrence. Higher number of calving (>4 calving in life), lack of teat dipping, higher tick infestation, udder pathology, lactation status, higher milk yield, higher milking frequency, unhygienic milker's hands, use of beta-lactam drugs for general ailment, lack of veterinary professional services for disease treatment and prevention proved potential risk factors associated with this disease.

DISCUSSION

The prevalence of *S. aureus* mastitis in current study was calculated from milk samples irrespective of screening test. The findings of Farooq et al.[19] reporting 44% of from subclinical mastitis was in line with current study. Contrary to current study Ali et al.[20] stated 8.32% *S. aureus* prevalence from bovine milk. Highest Staphylococcal species in bovine milk had been reported by various studies [21,22]. Higher prevalence of *S. aureus* was also noted by Khan and Muhammad [23] presenting 45% on subclinical mastitis basis, respectively. The higher prevalence in current study might be due the significant association of assumed risk factors with mastitis. The discrepancies in *S. aureus* prevalence included its stay and survival in keratin of healthy animal's teat canal, and formation of biofilm that may give rise to lower than exact isolation of *S. aureus* during *in vitro* culturing. The prevalence of *S. aureus* varies specie to specie [24], breed, geographic zones, and farm management conditions. The surrounding of animal such as bedding and manure are true source of contagious microorganisms. These microbes may be present in soil as well as in air as environmental microorganisms. Milker's hands, towels and flies spread these pathogenic bacteria to clean udders during the

Table 4. *Bivariate analysis of risk factors associated with subclinical mastitis in cattle and buffalo from District Faisalabad*

Parameters	Levels	Cattle			Buffalo		
		Total Examined	Diseased (%)	*P*-value	Total Examined	Diseased (%)	*P*-value
Age	3-5 years	240	51.25	0.447	180	46.66	0.001
	>5 years	219	54.79		261	64.36	
Breed	* Sahiwal/**Nili-ravi	150	40.00	0.001	141	55.31	0.339
	* Frisian/**Kundi	180	66.67		129	53.48	
	*Scrub/** Scrub	129	48.83		171	61.40	
Rearing system	Open	270	61.11	0.001	273	63.73	0.001
	Confined	189	41.26		168	46.42	
No. of Calving	2-4	240	56.25	0.137	204	55.88	0.659
	>4	219	49.31		237	58.22	
Body health	Normal	240	53.75	0.748	216	55.55	0.659
	Thin	120	50.00		129	60.46	
	Emaciated	99	54.54		96	56.25	
Use of teat dips	Yes	153	29.41	0.001	144	41.66	0.001
	No	306	64.70		297	64.64	
Presence of ticks	Yes	285	60.00	0.001	267	67.41	0.001
	No	174	41.37		174	41.37	
Feed & Water	Well fed	390	56.15	0.001	198	45.45	0.001
	Underfed	69	34.78		243	66.66	
Udder consistency & condition	Normal	288	46.87	0.001	252	48.80	0.001
	Inflamed	51	76.47		66	68.18	
	Painful	120	57.50		123	68.29	
Lactation Status	Dry	198	42.42	0.001	171	42.10	0.001
	Lactating	261	65.43		270	66.66	
If Lactating then milk yield	<1	54	16.66	0.001	30	10.00	0.001
	2-5	216	55.55		114	44.73	
	5-10	114	57.89		177	64.40	
	>10	75	64.00		120	70.00	
No. of Milking if lactating	Once/day	54	16.66	0.001	30	10.00	0.001
	Twice/day	405	57.77		411	60.58	
Milker's hands hygiene during milking	Yes	156	30.76	0.001	144	41.66	0.001
	No	303	64.35		297	64.64	
Hygienic condition during milking	Yes	177	33.89	0.001	138	23.91	0.001
	No	282	64.89		303	72.27	
Generally used antibiotics	B-lactam group	390	56.15	0.001	381	61.41	0.001
	Others	69	34.78		60	30.00	
Antibiotics used in mastitis	B-lactam group	435	53.79	0.001	360	65.00	0.001
	Others	24	37.50		81	22.22	
Call for veterinary professional	Self-treatment	165	58.18	0.039	165	63.63	0.008
	Few day post self- treatment	183	54.09		186	56.45	
	Only emergency	30	30.00		21	28.57	
	Always on call	45	53.33		45	60.00	
	Always veterinarian	36	41.66		24	37.50	
Veterinary professional consultancy	Only veterinary assistant	231	68.83	0.001	240	70.00	0.001
	Only veterinarian	81	29.62		63	28.57	
	Both	147	40.81		138	47.82	

*P<0.05 indicate significant association, * Cattle breeds, ** Buffalo breed*

milking process and are responsible for most of the mastitis cases [25].

Subclinical mastitis serves as reservoir of infectious pathogens and together with other forms of mastitis piles up to 70% of economic losses [1]. The rate of subclinical cattle mastitis (54%) in current study was in line with findings of Singh and Baxi [26] who described 54% prevalence of subclinical mastitis in India while higher bovine subclinical mastitis was noted as 64% and 86.3% by Mureithi and Njuguna [27] and Abrahmsen et al.[28], respectively. Whereas, lesser to the current study was reported as 44% and 34.4% from Pakistan and Kenya [29,30] respectively.

The prevalence of subclinical mastitis in buffalo in present study is in agreement with findings of Getahun et al.[31] and Mustafa et al.[32] who reported 54.7% and 59.64% subclinical buffalo mastitis, respectively. Higher prevalence in buffalo might because of enriched nutrients in milk that favors comparatively higher growth of microbes. In addition to this pendulous udder and longer teats of buffaloes make it prone to mastitis. Contrary to the finding of current study lower prevalence was found to be 32.85% [33] and 23.18%. The discrepancies in prevalence are also linked with different climatic zones, management trends, and exposure to microbial environment.

The higher prevalence of subclinical mastitis in cross bred cattle was in line with findings of Sanotharan et al.[34] and Alemu et al.[35] who reported significant association ($P<0.05$) of crossbred animals with subclinical mastitis. The significant association ($P<0.05$) of breed with mastitis occurrence was also reported by Alebachew and Alemu [36] and Lakew et al.[37]. The difference in anatomical, genetic, production status may predispose occurrence of this disease [38]. Researchers have noticed native breed resistant to mastitis [39] and this could be due to genetic resistance and adaptation to the environment [40]. Higher prevalence of mastitis in late lactation was in agreement with results of Dego and Tareke [9]. However, Mungube et al.[41] reported higher prevalence of mastitis in early stage of lactation. The variations in the effect of stages of lactation among different studies could be related probably to disparities in age, parity and breed of the sampled animals as indicated by Getahun et al.[31].

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Conflict of Interest Statement

The authors declared that they have no conflict of interest.

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