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PREVALENCE OF SUBCLINICAL MASTITIS, ASSOCIATED RISK FACTORS AND ANTIBIOTIC SENSITIVITY OF *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS* IN CATTLE IN MULTAN, PAKISTAN

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ABSTRACT

Mastitis is the most widespread and economically important disease of dairy sector worldwide. The present research was, therefore intended to establish the prevalence of mastitis in and around the Multan district. A total of 100 dairy cows were randomly sampled during the period from July 2018 to October 2018 from different local dairy farms at Multan. After screening with California Mastitis Test, milk samples from mastitic animals were cultured and subjected to microbiological examination.The overall prevalence of subclinical mastitis in cows was 38% and quarter-wise prevalence of subclinical mastitis was 36.20%.. Mastitis in front left quarters was 34.40%, right front quarters 31.60%, while left rare quarters 41% and right rear quarters 38%. Bacteria *Staphylococcus, Streptococcus, Corynebacterium, Enterococci, Salmonella, Bacillus, Klebsiella,* and *Pseudomonas* were isolated from infected samples. *Staphylococcus aureus* was the top-ranking isolated bacteria followed by *Strept. Agalactiae* and *E. coli*. The lack of proper milking techniques and no teat dipping were the most significantly associated risk factors. In the current study, *E. coli* isolates were sensitive to Gentamycine, Streptomycin, Chlororamphenicol and Kanamycin while resistant to Vancomycin and Penicillin. Similarly, *S. aureus* was extremely susceptible to Chloramphenicol, Vancomycin and Kanamycin but resistant to Tetracycline and Penicillin. It was concluded that subclinical mastitis was widely prevalent in Multan city. This study will help the farmers to adopt effective measures to control mastitis regarding the risk factors.

Keywords: bacteria, gentamycin, prevalence, subclinical mastitis, treatment

INTRODUCTION

Mastitis (inflammatory reaction of the udder tissues) is the most widespread and most important disease of economic interest in the dairy sector worldwide (Biswadeep *et al.,* 2015). At the very least, each fourth dairy buffalo and cow is bothered by this condition (Hoque *et al.,* 2015). Current losses figures due to this infection are not available in Pakistan, although it was assessed in 1978 that in the Punjab province alone the overall losses about Rs.240 million per year due to clinical mastitis (Ashfaq *et al.,* 2015). It is worth noting that the subclinical type of mastitis is 15-40 times more common than its clinical equivalent. These casualties are primarily due to *Streptococcus agalactiae*, produced by *Escherichia coli*, *Staphylococcus aureus,* and *Corynebacterium pyogenes*. The clinical form of mastitis possesses all the five key signs of inflammation like redness, pain, heat, swelling, and loss of milk production, while the sub-clinical mastitis lacks all signs of inflammation. Sub-clinical mastitis can be detected by applying direct (Somatic Cell Count; SCC) or indirect (California Mastitis Test; CMT or others).

Sub-clinical mastitis is responsible for 60-70% of economic losses to the dairy industry of the USA (Huirong *et al.,* 2019). This disease causes annual losses of about US$ 35 billion world-widely. Field studies have also shown that mastitis is among the most significant animal health concerns to become a serious livestock disease in Pakistan (Welderufael *et al.,* 2016; Rohmeier *et al.,* 2020). The higher losses with this disease might be due to a lack of mastitis prevention measures like teat dipping and dry period antibiotic treatment (Dagmara *et al.,*

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2020). It is important to remember that this research did not report losses caused by sub-clinical mastitis, a type of mastitis that is 3-40 times most prevalent than clinical mastitis and causes the highest general decrease in many other dairy farms (Dorota *et al.,* 2020). Subclinical mastitis is confirmed by laboratory analysis of milk or animal tests as there is no noticeable swelling of the udders or milk anomalies. It is a well-known fact that every buffalo or cow develops mastitis before she expires. Mastitis can be controlled more effectively if prevalence is known in the animal population and area. Thus, the current research was designed to examine the animal-wise and quarter-wise occurrence of sub-clinical mastitis, its associated risk factors and antibiotic sensitivity of isolates of *S. aureus and E. coli* in and around Multan city.

MATERIALS AND METHODS

Study Area and selection of animals

Multan city is located on the banks of River Chenab and the center of south Punjab. The geographical location of Multan is 3721 square kilometers. It has three tehsils i.e. Multan, Jalapur peer wala, and Shujaabad. Among all cattle population, Sahiwal and Crossbreed cattle are the main breeds of this region. In this study, a total of 100 dairy cows were randomized sampled during the period from July 2018 to October 2018 from different local dairy farms at Multan. A predesigned questionnaire proforma was administered to owners to collect the information to assess the risk factors associated with mastitis.

Collection and transportation of milk sample

The Procedure for collecting quarter foremilk samples identified by National Mastitis Council Inc., U.S.A. (1990) was observed. The sterile disposable syringes of 5 ml capacity, labelled as LF (left front), LR (left rear) RF (Right front) and RR (Right rear), were used. Every teat was washed with cotton soaked with 70% ethanol. A different pledge was used for each teat. For every teat, a separate pledged was used. About 4-5 ml of milk was collected aseptically after discarding the first few streams. For microbiological analysis, collected samples were rapidly cooled in the icebox and directed to the Laboratory, Department of Clinical Sciences, FVS, BZU, Multan. California Mastitis Test (CMT) was performed to confirm the mastitis (Kashif *et al.*, 2013). Briefly, milk samples from the individual quarter and test solution were mixed in the same quantity in Petri dishes. The change in the consistency of milk ranging from mild flakes to gel formation indicated mastitis while no change in milk consistency indicated mastitis free animal.

Bacteriological examination

CMT positive samples were used for microbiological analysis. Microbiological analysis of milk specimens was started around eight hours after the collection of samples. MacConkey's agar and Blood agar were applied for the growth of bacteria. To achieve a secure dispersion of pathogens, the samples were shaken eight times. By using a platinum-rhodium loop. About 0.01ml of each milk sample was streaked onto the blood agar plate. Milk specimens from all quarters were grown on the 100 mm plate by placing each quarter sample on (one-fourth of an area) of the plate and incubated for 48 hours at 37°C. When five or more identical colonies were present on the plate, the quarter was estimated affected. In order to identify a sample as infected or compromised, National Mastitis Council Inc., (1987) recommendations on the importance of colony numbers in pure or mixed cultures were used.

Identification and Purification of Bacteria

Identification and purification were done by automatic method (Vitek® 2 Compact, Biomérieux, France). Gram negative and positive bacteria were identified using the GN ID and the GP ID card. It was carried out in accordance with the procedures mentioned by the manufacturer.

Antibiotic sensitivity testing

Antibiotic discs were added to the surface of the inoculated agar plates. (Inoculated with the standardization of bacterial suspension using McFarland standards) using aseptic technique. Approximately seven antimicrobials such as Gentamycin, chloroamphenicol, Vancomycin, Penicillin, Tetracycline, Kanamycin and Streptomycin (Oxoid, Hampshire, England) were selected from the main class of antimicrobials and checked for sensitivity. Every disc was pressed down to ensure maximum contact with the agar surface. After measuring the inhibition region, it was classified as sensitive, intermediate, and resistant.

Data analysis

The quarter wise prevalence was determined by dividing the number of positive quarters over the

total number of quarters tested. The chi-square value was used to find the significant difference among risk factors and variables. P-value ≤ 0.05 were considered statistically for all analysis.

RESULTS

Animal-wise prevalence of subclinical mastitis in cows was 38% depicted by Table I and the quarter wise prevalence of subclinical mastitis was 36.20% as shown in Table II. It further showed 34.4 in RF, 31.60% in LF, 41% in RR, and 38% in LF. The results showed that the incidence of subclinical mastitis was higher in the rear teat as compared to the front teat.

Table 1. Animal wise mastitis prevalence in dairy cows

Total No. of animals	CMT Positive animals	Total No. of animals/affected animals	% Prevalence
100	38	38/100	38%

Table 2. Quarters wise mastitis prevalence in dairy cows

Quarters	Total quarters examined	Blind Quarters (showing no. secretion)	Positive no. of quarters/ total quarters examined	% Prevalence
RF	099	01	033	34.04%
LF	098	02	031	31.60%
RR	096	04	040	41.00%
LR	097	03	037	38.00%
Total quarters	390	10	141	36.90%

S. aureus (32.66%) was isolated from cases positive for mastitis in the present study as a top-ranking pathogen, followed by *Strep. agalactiae* (18.36%) and *E. coli* (20.40%) depicted in Table 3.

Table 3. Most prevalent bacteria species isolated from mastitis quarters

Bacteria	Out of total	Prevalence
Staphylococcus aureus	16/49	32.66
Streptococcus agalactiae	9/49	18.36
E. coli	10/49	20.40

Risk factors associatedwere the management factors such as housing, production system, floor type, proper milking techniques, udder washing prior to milking, routine mastitis testing with CMT, and the use of teat dipping. Cow factors included were the breed and history of mastitis. A description of the frequency of all variables of the survey considered in the analysis was given in Table 4. Among the risk factors examined, use of teat dips, udder drying towel for each cow history of mastitis and routine testing for mastitis was significantly correlated with the presence of mastitis ($p< 0.05$). The results showed that the prevalence was higher in the semi-intensive production system (46.6%) than the int ensive production system (34.2%). In relation to floor type, the prevalence was significantly higher in the earthen floor (60%) than concrete type floor (30.6%). Based on the interval in the cleaning of the floor, the prevalence was significantly higher in weekly cleaning (60%) than daily cleaning (32.5%) of the floor. About teat dipping, the infection rate was higher (41.6%) in those animals in which teat dipping was not performed (30%). Concerning the breed of the animal, the prevalence was significantly higher in exotic (40%) than crossbred (65.7%) animals. The association of infection decreased with proper milking techniques. In this research, mastitis was more likely to occur in cows with a prior history of mastitis than in cows without an earlier history of mastitis.

From total positive samples, 10 and 15 isolates of *E.coli* and *S. aureus* respectively were tested for susceptibility to seven different antimicrobial discs. The *E. coli* isolates were sensitive to Gentamycine (100%), Streptomycin (100%), Chlororamphenicol (60%) and Kanamycin (70%). Whereas intermediate to Tetracyclin (40%) and resistant to Chlolorompheniсol (40%), Vancomycin (100%), Kanamycin (30%), Penicillin (100%) and Tetracycline (60%). *S. aureus* isolates were sensitive to Chloramphenicol (100%), Vancomycin (100%), Kanamycin (100%) and Gentamycin (13.3%) whereas intermediate to Streptomycin (20%) and showed resistance to Tetracycline, Penicillin as shown in Table 5.

DISCUSSION

Subclinical mastitis is one of the most significant cattle and buffalo infections that cause economic losses in dairy farming, and the incidence is growing day by day. The bacterial origin is at the forefront of many causes of mastitis. The quarter-wise overall occurrence was 36.2% and animal-wise prevalence was 38% in cows of subclinical mastitis. A previous study has shown the prevalence of clinical and sub-clinical mastitis was 12.5% and 51.8% at cow level and 10.7% and 46.4% at the quarter level by CMT, SFMT, and WST, respectively (Zeryehun and Gerema, 2017). Although herd-mate cows showed a greater rate of mastitis (sub-clinical) in cows (75%) and individually controlled animals at 44.44 percent. (Abebe *et al.,* 2016) revealed the occurrence of 74.7% in cows based on the White Side Test and CMT. (Amer *et al.,* 2018) used cultural analysis and identified 74.7% infection in cows at the herd level. (Patil *et al.,*

2015) recorded incidence (59.2%) and (36.8%) of subclinical mastitis in buffaloes and cows. (Christine *et al.,* 2020) detected occurrence of mastitis 80% in cows, out of which 6.8% was clinical mastitis, while subclinical was 73.1%. (Kavitha *et al.,* 2009) utilized the Ciba-Geigy Mastitis Test and detected that 34.48% buffaloes were affected by sub-clinical mastitis. (Motaung *et al.,* 2017) have investigated an occurrence of 30% in buffaloes after using pH test, white Side test and Strip Cup test. (Rohmeier *et al.,* 2020) also published that total animal-wise occurrence was 44%while the quarter-wise prevalence was 44.1% in cows based on Surf Field Mastitis Test. (Dorota *et al.,* 2020) estimated the total occurrence of sub-clinical mastitis 36% in crossbred cows and 27% in buffaloes by using microbiological testing of milk and the Surf Field Mastitis Test. The animal-wise prevalence was observed to be 45% while the quarter-wise prevalence was 35.25% (Bachaya *et al.,* 2011). Differences in management strategies, detection methods, breeds of animal, conditions at the environment and immune responses of animals may be the cause of the variation in the predominance of subclinical mastitis in current and recent studies. Extreme weather promotes the disease and induces stress to the body, thereby decreasing immunity, contributing to an increased sub-clinical incidence associated with infectious and environmental mastitogens. Several earlier studies (Cha *et al.,* 2011; DeVliegher *et al.*, 2012) demonstrated that great management diminishes the rate of mastitis to a high extent. (Huirong *et al.,* 2019) detailed that the correct method of milking is essential to lessen the occurrence of mastitis. The considered variables were decided as hazard components influencing mastitis as a breed, age, season, environment, management and hygiene (Pitkala *et al.,* 2004; Sharif *et al.,* 2007) recorded microbial growth in 21-33% of milk samples, whereas, just 15.16% identified in dairy buffaloes (Iqbal *et al.,* 2004). This disparity may be due to the climate changes, farm management practices, the location, draught purpose, the difference in handling of samples and the use of antibiotics.

Table 4. Associated risk factors of mastitis in cattle in and around Multan

Variable	Number examined *n*=100	Negative (%)	Positive (%)	P-value
Production system				
Semi-intensive	30	16 (53.3)	14 (46.6)	0.059
Intensive	70	46 (65.1)	24 (34.2)	
Floor type				
Earthen	25	10 (40)	15 (60)	0.402
concrete	75	52 (69.3)	23 (30.6)	
Cleaning of floor				
Daily	80	54 (67.5)	26 (32.5)	0.122
Weekly	20	8 (40)	12 (60)	
Teat dips				
Yes	40	28 (70)	12 (30)	0.01*
No	60	35 (58.3)	25 (41.6)	
Breed				
Crosses	35	23 (65.7)	12 (34.3)	0.124
Exotic	65	39 (60)	26 (40)	
Proper milking techniques				
Yes	84	55 (65.4)	29 (34.5)	0.001*
No	16	7 (43.7)	9 (56.3)	
History of mastitis				
No	68	43 (63.2)	25 (36.8)	0.026*
Yes	32	19 (59.4)	13 (40.6)	

Table 5. Antibiotic sensitivity test for *E. coli* (n=10) and *S. aureus* (n=15)

Antibiotics	Units (µg)	*E. coli*			*S. aureus*		
		S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Chloroamphencol	25	6 (60%)	-	4 (40%)	15 (100%)	-	-
Vancomycin	30	-	-	10 (100%)	15 (100%)	-	-
Gentamycine	15	10 (100%)	-	-	2 (13.3%)	-	13 (86.7)
Kanamycin	30	7 (70%)	-	3 (30%)	15 (100%)	-	-
Streptomycin	15	10 (100%)	-	-	-	3 (20)	12 (80%)
Penicillin	10	-	-	10 (100%)	-	-	15 (100%)
Tetracycline	30	-	4 (4%)	6 (60%)	-	-	15 (100%)

In the present analysis, *S. aureus* was isolated from cases positive for mastitis as the top-ranking pathogen (32.66%). It was also identified as a major pathogen in earlier studies (Ebrahimi *et al.,* 2007; Mpatswenumugabo *et al.,* 2017; Vakkamaki *et al.,* 2017; El-jakee *et al.,* 2019), reported 9.44% *E. coli* and 8.33% *Strep. agalactiae* isolates from subclinical mastitis milk specimens while(Suleiman *et al.,* 2018) gained 42.6 percent *S. aureus* and 30% growth of *Strep. agalactiae* and *Strep. Strep. dysgalactiae*. The primary cause of coliforms is polluted farm environments and they mostly cause clinical infections. (Vakkamaki *et al.,* 2017) obtained 8.33% *Streptococci,* 3.88% CNS other than *E. coli* and 9.44% *Strep*. *Agalactiae* (Botrel *et al.,* 2009) from subclinical mastitis milk samples, 13.7 percent coagulase-negative staphylococci, 30.2% CNS and 9.3% *Strep. dysgalactiae* were tested. The control programmes for mastitis recorded in recent years will need to be examined in accordance with our farming systems and local requirements. In this study, *E. coli* isolates were sensitive to Gentamycine, Streptomycin, Chlororamphenicol and Kanamycin while resistant to Vancomycin and Penicillin. Similarly, *S. aureus* was extremely susceptible to Chloramphenicol, Vancomycin and Kanamycin but resistant to tetracycline and Penicillin. These results were analogous to earlier researches (Seyoum *et al.,* 2018; Kalayu *et al.,* 2020).

It was concluded that subclinical mastitis was widely prevalent in Multan city. The lack of proper milking techniques and no teat dipping were the most significantly associated risk factors. *E. coli* isolates were sensitive to Gentamycine, Streptomycin, Chlororamphenicol and Kanamycin while resistant to Vancomycin and Penicillin whereas *S. aureus* was highly susceptible to Chloramphenicol, Vancomycin and Kanamycin but resistant to Tetracycline and Penicillin. This study will help the farmers to adopt effective measures to control mastitis in cattle.

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AUTHOR'S CONTRIBUTION

T. Ahmed: Designed the study and won the project/grant

M. Kashif: Performed analysis of data and write up

E. Ahmad: Performed the microbiological test and interpreted the data

M. Nadeem: Executed the experiments and analyzed the data

M. Rizwan: Sample collection and performed screening tests

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