



PREVALENCE AND ASSOCIATED RISK FACTORS OF BUFFALOE (*BUBALUS BUBALIS*) MASTITIS IN CENTRAL ZONE OF KHYBER PAKHTUNKHWA, PAKISTAN

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Abstract

The bubaline mastitis is the inflammation of the parenchyma of the mammary gland of buffaloes either may be traumatic, infectious or toxic in nature and have economic importance in the dairy industry worldwide. This loss is mostly due to the severity of the infection, management practices, regional conditions, reduced milk production, increased treatment costs, and potential premature culling of affected animals. Mastitis can be categorized as clinical and subclinical type on the bases of clinical sign and symptoms. Clinical mastitis (CM) was detected by milk composition and morphology of both fore and hind quarters. Subclinical mastitis (SCM) was recognized by various standard tests.

The current research study was designed to identify the prevalence, bacteriology and both extrinsic and intrinsic risk factors in three buffaloes breed from five selected districts of Khyber Pakhtunkhwa, Pakistan. In the present study, five hundred (500) milk samples were collected randomly from Nili-Ravi, Kundhi and Azakheli buffalo's breeds. The duration of sample collection was January 2022 to January 2023. For the detection of Subclinical mastitis (SCM), various field tests such as Surf Field Mastitis Test (SFMT), California mastitis test (CMT), Ultrasonic milk analyzer, electric conductivity (EC) and somatic cell counts SCC (cell/ml) and various biochemical test such as Indole, Methyl Red, Voges-Proskauer, Citrate (IMViC) tests and catalase test were performed. In result total ten bacterial species were isolated as *Staphylococcus aureus*, *E.coli*,

Streptococcus agalactiae, *K. pneumoniae*, *Streptococcus dysgalactiae*, *Bacillus cereus*, *Streptococcus uberis*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Streptococcus pyrogenes*. The highest prevalence of bacteria was of *Staphylococcus aureus* which was the most frequently recovered bacterial species in all buffaloes breed Nili Ravi, Kundhi and Azakheli with percentage of 25.86%, 23.80% and 23.07% respectively, followed by *E.coli*, with 20.86%, 21.95% and 17.94%. *Streptococcus agalactiae* 16.36%, 17.07% and 20.52%. *Streptococcus pyrogenes* was founded only in Nili-Ravi breed. Period wise prevalence in all breeds was noted as dry group, (36%), the lactating group was 24% and period before weaning or milking was 40%. Area wise prevalence of *Staphylococcus aureus* was high in Peshawar (46%), followed by Charsadda (20%), Nowshera (13%), Mardan (13%) and Swabi (06%). The overall quarter wise prevalence was accounted 53%, 42% and 33.33% in Nili Ravi, Kundhi and Azakheli breeds respectively. Month wise prevalence was founded more in summer follow by winters, spring and autumn all these prevalence results based on field test and biochemical tests. The values of electric conductivity (EC) and somatic cell counts (SCC) variables were transformed into base 10 logarithms. The relationship of the electric conductivity (EC) and somatic cell counts (SCC) variables with fixed effects was analyzed by using one-way ANOVA (Analysis of Variance) and the mean differences were determined by the Schaffer's test.

It is concluded from our study that statistical analysis of intrinsic risk factors revealed significant differences ($p < 0.05$) in the prevalence of mastitis among breed, stage of lactation and body condition score. Similarly, production system, previous mastitis exposure, hygiene practice and type of floor were extrinsic risk factors significantly associated with the occurrence of subclinical mastitis (SCM) in different breeds of buffaloes in district Peshawar, Charsadda, Nowshera, Mardan and district Swabi, Khyber Pakhtunkhwa (KP), Pakistan.

Key words: Subclinical mastitis(SCM), buffaloes, Surf Field Mastitis Test, Nile-Ravi, Kundhi, Azakheli.

1. Introduction

Mastitis is eminent across the globe as serious and the most expensive disease of mulching animals. It is defined as inflammation of the mammary gland in response to traumatic injury and infection. It is characterized by five signs: swelling, redness, heat, pain, and loss of function (Singha *et al.*, 2023). It can be classified as clinical and subclinical type depends on the manifestation of the disease (Ali *et al.*, 2018). When the milk appears abnormal with a physical and chemical change, we called it Clinical mastitis (CM). The mammary gland also may exhibit change in its structure and morphology. The subclinical mastitis (SCM) is the non-observable form of mastitis (Cobirka *et al.*, 2022). As compared to the clinical type, subclinical mastitis is 15–40 times more common and long lasting and very difficult to identify. The world faces approximately 35 billion US dollars loss caused by mastitis alone. Ratafia in 1987 stated that losses might be much more in Pakistan due to mal practice of teat dipping and dry period antibiotic therapy as a preventive measurement (Arshad *et al.*, 1995).

Throughout the world buffalo population is estimated to be approximately 185.29 million, with 179.75 million (97%) of the buffaloes found in Asia (Mittal *et al.*, 2019). Pakistan is the country with the second largest buffalo population (27.3million heads) in the world after India with approximately 108.7 million buffaloes (Minervino *et al.*, 2020). There is a large population of cattle's 46.1 million and buffaloes 38.8 million (GOP 2018). The population of buffaloes in Pakistan has been reported to be increased by 1.6 times between 1986 and 2006 (Ali *et al.*, 2021).

There are five breeds of buffalos in Pakistan are Nili, Ravi, Nili-Ravi, Kundhi and Azakheli (Pen *et al.*, 2022). Until the 1960s, when the two breeds merged to produce the Nile-Ravi breed, Nile and Ravi were classified as distinct breeds (Tahir *et al.*, 2019). Nili-ravi breeds is also known as "Black Gold" due to their high milk production (10-20 liters milk per day) and economic value (Jaiswal *et al.*, 2021).

In Pakistan the Punjab province has the highest buffalo population, particularly in the central region with Faisalabad, Sheikhupura and Jhang being the most populated districts. Nili-Ravi and Kundhi breeds are the main buffalo breeds with 10.4 million and 6.7 million populations respectively (Zaman *et al.*, 2022). Nili-Ravi is predominantly found in Punjab while Kundhi are mostly located in Sindh province. Large population of Azakheli buffaloes exist in the Khyber Pakhtunkhwa (KP) province of Pakistan (Pan *et al.*, 2022). Pakistani buffalo breeds are known globally for their milk production (Anas *et al.*, 2023).

Buffaloes play a leading role in the national economy by producing milk, meat and draught power (Ali *et al.*, 2021). In milk production buffalo contributes about 68 %, followed by cattle 27% and sheep/ goat/camel 05% (Sikandar *et al.*, 2020). Due to high fat contents of buffalo milk is the most preferred species in Pakistan and also has 32% share in red meat production and 2% in draught power (Rehman *et al.*, 2017). Buffalo milk has a great nutritional value because it contains 87% water, 3.4% proteins, 4.8% lactose, 13% total solids, 4% fats and 0.8% minerals. Buffalo milk and milk products are white because of lack of carotene (Ijaz *et al.*, 2019).



Figure 1: Nili-Ravi (a), Kundhi (b), and Azakheli breed (c)

Throughout in Pakistan the field surveys of various diseases delineated that sub clinical mastitis (SCM) is an important health hazard as compared to clinical mastitis and in ranking it is considered as the number one pathological disease of dairy farm animals affecting all breed of buffaloes (Khan *et al.*, 2023). Besides its desperate effects on economy it is important from milk processing and zoonotic point of view because the mastitic milk harbors different organism which are potentially pathogenic for humans and processing of affected milk results in poor quality byproducts so raw milk direct consumption is not advised because of the crucial risk of microbial contamination (Pierezan *et al.*, 2022). Therefore pasteurization of milk is essential for guaranteeing of milk safety and extending its shelf life (Kanaan *et al.*, 2023).

Mastitis is a multi-etiological complex disease, which is caused by over 150 species of micro-organisms (PAL *et al.*, 2020). Based on different characteristics of distribution and interaction with teats and ducts. These causative pathogenic microorganisms are classified as contagious and environmental pathogens. The major causes of contagious mastitis which could be buffaloes-associated pathogens, includes *Staphylococcus aureus* and *Streptococcus agalactiae* while, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Escherichia coli* and *Klebsiella pneumonia* are the main causes of environmental mastitis (Shoaib *et al.*, 2021).

‘Risk Factor’ means any factors that elevate the probability or risk of a disease occurrence. (Algammal *et al.*, 2020). Subclinical mastitis (SCM) raised by interaction of different factors associated with the host, (buffaloes) pathogen (bacteria) and the environment. Furthermore, due to subclinical mastitis the increased somatic cell count (SCC) and decline fat, protein and lactose levels were experienced in affected milk which results in reduced shelf life of processed milk and milk products. Susceptibility of subclinical mastitis to cattle and buffaloes differs between both species and buffaloes are more susceptible than other cattle’s.

Sub clinical mastitis (SCM) causes a tremendous increase in the number of white blood cells (WBC) in the milk. In the context of milk, these cells are called milk somatic cells. Somatic cells are part of the natural defense mechanism and include lymphocytes, polymorph- nuclear cells, macrophages, and some epithelial cells. Somatic cell count (SCC) can be measured quantitatively by California mastitis test (CMT). It is a simple, easy and low cost screening test for detection of

subclinical mastitis in dairy farms. Validity of CMT in diagnosis of infected quarters was established in various Milking stages (Tezera *et al.*, 2021).

In sub-clinical mastitis (SCM) no obvious clear clinical signs like swelling and hardness of quarters as well abnormality of milk is observed. it can only be diagnosed by various laboratory test and a common farmer is not aware with different techniques and sophisticated test. The present study was designed to investigate the prevalence of sub-clinical mastitis in three buffaloes breed; to identify various intrinsic and extrinsic risk factors; and to isolate different types of bacterial species associated with sub clinical mastitis(SCM) in buffaloes kept under field conditions in selected districts of Khyber Pakhtunkhwa (KP), Pakistan.

2. Materials and Methods

2.1. Ethical approval

This study was approved by the ethical review committee, department of Zoology, Abdul Wali Khan University Mardan. Milk samples were obtained from buffaloes as per the guidelines of Veterinary Research Institute (Research), Peshawar, Khyber Pakhtunkhwa Pakistan with the prior consent of the owners and without any harm to the animals.

2.2. Study areas

The research work was carried out during January 2022 to January 2023 in College of Veterinary Sciences and Animal Husbandry (CVS & AH), Abdul Wali Khan University Mardan. The study samples were collected from district Peshawar, Charsadda, Nowshera, Mardan and district Swabi, Khyber Pakhtunkhwa (KP), Pakistan.

2.3. Dairy animals

Total five hundred (500) lactating buffaloes from 50 dairy buffaloes farms were collected and examined for the presence of sub clinical mastitis (SCM). The dairy farms were using both machine-milking and manual method for milk collection. Machine-milking helped in detecting increase or decrease in milk

2.4. Data collection

A preformed questioner with close ended questions was used to collect data about various buffaloes and quarter level factors thought to impact the occurrence of mastitis in the dairy buffaloes. The **intrinsic factors** (animal age, breed, parity, stage of lactation and body condition score (BCS)) as well as **extrinsic factors** (floor type, hygienic practice, production system, tick infestation and previous clinical mastitis history) were assessed to investigate their association with the occurrence of mastitis. Age of animals was determined by counting the number of rings in the horn of buffaloes and dentition characteristics and categorized as young adults (3– 6 years), adults (6 to ≤ 10 years) and old (>10 year).

Stage of lactation was categorized as early (1-100days) or (1– 4 months), middle (100-190days or >4 – 8months), and late (>190 days) or (>8 months to the beginning of dry period). Parity was also categorized as few (with ≤ 3 calves), moderate (4– 7 calves), and many (>7 calves). Body condition score of buffaloes (BCS) was categorized as poor (if the dairy buffaloes are emaciated with visible ribs from distance) and good (if the dairy buffaloes were well nourished with at least no visible ribs from distance). The floor type was grouped into muddy (floor which was not well managed) and concrete (floor which is well managed).

Milking hygiene practice was categorized into good (if there is a practice of washing and drying udder with separate towels, milking healthy and young animals first) and poor (If washing and drying of udder with a separate towel and milking with order is not practiced) and production system was also categorized into intensive (if the farms have feed reserves sufficient for a year, operated by a hired labor and thus, buffaloes were managed with intensive feeding and watering) and extensive (if the farms have not feed reserves for a year, operated mainly by family labour and

thus, buffaloes were allowed to graze in the field extensively.

2.5. Milk sample collection

Five hundred milk samples were randomly collected from lactating buffaloes. Milk samples were taken before milking from each quarter. Information on the buffaloes, age, parity, breed, lactation stage, body condition score (BCS) production system, milking hygienic practice, floor type, tick infestation and previous mastitis history were also collected at the time of sampling using preformed data recording excel sheet.

2.6. Diagnosis of subclinical mastitis (SCM)

Sub clinical mastitis (SCM) did not show any visible abnormalities in udder, teat and milk and very difficult to identify so it was detected by various field tests such as Surf Field Mastitis Test (SFMT) California mastitis test (CMT), Ultrasonic milk analyzer, electric conductivity (EC), somatic cell counts SCC (cell/ml) and gram staining. Biochemically it was detected by various standard tests such as IMViC tests (Indole, Methyl Red, Voges-Proskauer, and Citrate tests) and catalase test.

2.6.1. Surf Field Mastitis Test (SFMT)

This test has been named as Surf Field Mastitis Test (SFMT) due to use of reagents Surf Excel Powder (Lever Brothers Pak Ltd.). It is the most common and easy test for detection of ailments because surf excel powder are easily available in every village for a farmers.

For the purpose of Study, 03 % surf solution was made by adding 3gram of common detergent surf excel powder in 100 ml of water. Equal volume of surf solution and milk samples were mixed in a petri-dishes, change in consistency or gel formation indicated mastitis milk while healthy samples result no gel formation. On the basis of severity, the mastitis is graded in to four categories from higher to lower intensity as, more severe P3 (+++), severe P2 (++), moderate P1 (+) and doubtful T ($\pm$) respectively. When milk samples mixed with detergent, rupture of somatic cells or white blood cells (WBCs) in milk occurs and released deoxyribonucleic acid (DNA) from cell along with other cellular contents. Detergent and DNA combined to forms gel, gel consistency depends upon the number of somatic cells.

2.6.2. California mastitis test (CMT)

The test is called the California Mastitis Test (CMT) because it was developed in California by two American scientists, Schalm and Noor lander in 1957(Chandra *et al.*, 2019). The CMT is a simple, inexpensive, and quick test used to assess the somatic cell count (SCC) in milk as an indicator of mastitis. This test has same protocol as Surf Field Mastitis Test (SFMT) instead of surf solution California mastitis reagent such as sodium lauryl sulphate (SLS) was used. In this test the milk was collected from all four quarters of buffaloes in four shallow cups on the CMT paddle tray labeled as fore right(FR), fore left(FL) rare right(RR) and rare left(RL) and an equal amount of reagent was added to each cup. A gentle circular motion was applied to the mixtures in a horizontal plane for 15sec. The coagulation or gel shows the presence and severeness of the infection (Austin *et al.*, 2023).

2.6.3. Somatic Cell Count (SCC)

It is the foremost methods used for the diagnosis of subclinical mastitis (SCM) and used as indicator to find out the status, stages and severeness of disease in buffaloes. Somatic cell counting was done by using methylene blue staining after staining the numbering of somatic stained cell count through microscope. Less than 200,000 cells per milliliter was considered as normal milk when a milk somatic cells concentration exceeding 200,000 cells/ml generally indicates that buffaloe breeds have SCM (Youssif, *et al.*, 2021). The analysis of variance (ANOVA) test was conducted to test the possible significance ($P \leq 0.05$) among different mean values using SPSS,

version 25.

2.6.4. Electrical Conductivity (EC)

The presence of pathogens or bacteria in milk significantly increases the electrical conductivity and somatic cell counts(SCC) in milk. There is a moderate positive relationship between SCC and EC. The existence of pathogens in buffaloes' milk significantly decreases lactose quantity and increased protein content in milk as a result, EC of milk has increased (Uhrinčat' *et al* 2019). The lactometer was used to check EC of milk in all buffaloe breeds. Generally normal milk has an electrical conductivity between 4.0 and 5.5 mS/cm (milli Siemens per centimeter) while mastitic milk, due to changes in milk composition, has a higher EC i.e; >5.5 mS/cm.

2.7. Data management and Statistical analysis

Collected data were first entered into Microsoft Excel spreadsheet and analysed using Statistical Package Graph pad prism. Descriptive measures like proportions and percentages were used to summarize and present the collected data. The prevalence of mastitis was calculated as the number of lactating buffaloes tested positive by California mastitis test (CMT) or buffaloes showing symptoms of mastitis, divided by the total number of tested population. The existence of association between independent variables with the occurrence of dependent variables (mastitis) was verified using Pearson Chi- square (χ^2) statistical test. The independent variables assessed were production system, floor type, hygienic practice, breed, age, body condition score (BCS), parity, stage of lactation, tick infestation and previous clinical mastitis history. For this study, the level of significance was set at 0.05 with 95% confidence interval. The results of this study were considered statistically significant when $p < 0.05$.

3. Results

3.1. The frequency of microbial agents isolated from subclinical mastitis (SCM)

The percent prevalence of various bacterial species in cases of subclinical mastitis(SCM) isolated from Nile-ravi, Kundhi and Azakheli buffaloe breeds was examined (**Table 1**). Total ten identified genera of bacteria was identified in which the most common infectious bacteria noticed were as *Staphylococcus aureus* (25.86%,23.80%,23.07%) followed by *Escherichia coli* (18.06%,21.95%,17.94%), *Streptococcus agalactiae* (15.51%,17.07%,20.51%), *K. pneumoniae* (12.06%,12.19%,10.25%), *Streptococcus dysgalactiae* (10.34%,7.31%,7.69%), *Bacillus cereus* (6.89%,2.43%,2.56%), *Streptococcus uberis* (5.17%,4.87%,7.69%), *Micrococcus luteus* (3.44%,2.43%,2.56%), *Pseudomonas aeruginosa* (1.72%, 2.43%, 5.12%) and no species of *Streptococcus pyrogenes* were found in Nile-ravi, Kundhi and Azakheli buffaloe breeds respectively (**Table 1**).

Table 1: The frequency of microbial agents isolated from Nile-ravi, Kundhi and Azakheli breeds

Bacteria	Total cases of Nili-Ravi, Kundhi and Azakheli breed(300)			Total positive cases			No of isolates			Frequency		
	NR	K	A	NR	K	A						
<i>S. aureus</i>							15	10	9	27.27	23.8	23.68
<i>E.coli</i>	-	-	-	-	-	-	12	9	7	21.81	21.95	18.42
<i>S. agalactiae</i>	-	-	-	-	-	-	9	7	8	16.36	17.07	21.05
<i>K. pneumoniae</i>	-	-	-	-	-	-	7	5	4	12.72	12.19	10.52
<i>S. dysgalactiae</i>	-	-	-	-	-	-	6	3	3	10.9	7.31	7.89
<i>B. cereus</i>	-	-	-	-	-	-	3	1	1	5.45	2.43	2.63
<i>S. uberis</i>	-	-	-	-	-	-	2	2	3	3.63	4.87	7.89
<i>M. luteus</i>	-	-	-	-	-	-	1	1	1	3.63	2.43	2.63
<i>P.aeruginosa</i>	-	-	-	-	-	-	1	1	1	3.63	2.43	2.63
<i>S. pyrogenes</i>	-	-	-	-	-	-	0	0	0	0	0	0
Total	100	100	100	55	41	39	55	41	38	55%	41%	38%

Note: (NR)Nile-Ravi; (K) Kundhi; (A) Azakheli.

3.2. Prevalence of subclinical mastitis (SCM) in different periods

The data presented in (table.2) reveals that the total number of animals inside the farm was classified as a dry group, total 180 buffaloes with (36%), were heifer; lactating group was 120 buffaloes with a percentage of 24% and period before weaning or milking was 40%.

Table 2: Period wise prevalence in Nile-ravi, Kundhi and Azakheli breed.

S. No	Group	Total cases	Nile-ravi	Kundhi	Azakheli	Percentage(%)
1	Dry period(60-150 days)	180	110	47	23	36
2	Lactating period(320days)	120	88	22	10	24
3	Period before weaning	200	120	50	30	40

3.4. Area-wise prevalence of subclinical mastitis (SCM)

The prevalence of subclinical mastitis was examined based on area, with the distribution pattern of bacterial species identified in milk samples collected from various locations, namely Peshawar, Newshehra, Charsadda, Mardan and Swabi, summarized in table.3. In all regions, *Staphylococcus aureus* emerged as the primary pathogenic bacterium additionally, other significant bacterial species such as *Streptococcus agalactiae*, *Escherichia coli* and *Streptococcus dysgalactiae*, were also identified, exhibiting a relatively consistent incidence distribution. Specifically, the occurrence of *Staphylococcus aureus* was high in Peshawar(46%), follow by Charsada (20%), Newshehra(13%), Mardan(13%) and Swabi(06%) detected in Nili-Ravi buffaloes breed, over all area wise result of all buffaloes breed are shown in table.03

Table 3: Area-wise prevalence of subclinical mastitis(SCM) in nili Ravi breeds

Bacteria	Total isolates from Nili-Ravi	Percent (%) Prevalence in Five Districts. n(%)				
		Peshawar	Charsadda	Noshehra	Swabi	Mardan
<i>S. aureus</i>	15	07(46)	03(20)	02(13)	1(06)	2(13)
<i>E.coli</i>	11	04(36)	02(18)	01(09)	01(09)	03(27)
<i>S. agalactiae</i>	9	03(33)	01(11)	02(22)	02(22)	01(11)
<i>K. pneumoniae</i>	7	03(42)	01(14)	02(28)	0	01(14)
<i>S. dysgalactiae</i>	6	02(33)	01(16)	01(16)	01(16)	01(16)
<i>B. cereus</i>	4	03(75)	0	01(25)	0	0
<i>S. uberis</i>	3	02(66)	0	01(33)	0	0
<i>M. luteus</i>	2	01(50)	01(50)	0	0	0
<i>P.aeruginosa</i>	1	01(100)	0	0	0	0
<i>S. pyrogenes</i>	0	0	0	0	0	0
Total	58	44.82%	15%	17%	6.89%	12%

Table 4: Area-wise prevalence of subclinical mastitis in Kundhi breed

Bacteria	Total isolates from Kundhi	Percent (%) Prevalence in Five Districts. no(%)				
		Peshawar	Charsadda	Noshehra	Swabi	Mardan
<i>S. aureus</i>	10	04(40)	02(20)	02(20)	01(10)	01(10)
<i>E.coli</i>	9	03(33)	02(22)	02(22)	0	02(22)
<i>S. agalactiae</i>	7	02(28)	01(14)	02(28)	01(14)	01(14)
<i>K. pneumonia</i>	5	02(40)	01(50)	01(50)	0	01(50)
<i>S. dysgalactiae</i>	3	01(33)	01(33)	01(33)	0	0

<i>B. cereus</i>	1	01(100)	0	0	0	0
<i>S. uberis</i>	2	01(50)	0	01(50)	0	0
<i>M. luteus</i>	1	01(100)	0	0	0	0
<i>P.aeruginosa</i>	1	01(100)	0	0	0	0
<i>S. pyrogenes</i>	0	00(00)	0	0	0	0
Total	41	39.02%	17.07%	21.95%	4.87%	12.19%

Table 5: Area-wise Prevalence of Subclinical Mastitis (SCM) in Azakheli breeds

Bacteria	Total isolates from Azakheli	Percent (%) Prevalence in Five Districts. no (%)				
		Peshawar	Charsadda	Noshehra	Swabi	Mardan
<i>S. aureus</i>	9	03(33)	01(11)	02(22)	01(11)	01(11)
<i>E.coli</i>	7	02(28.7)	01(14.28)	01(14)	01(14)	02(28)
<i>S. agalactiae</i>	8	04(50)	01(12.5)	01(12)	01(12)	01(12)
<i>K. pneumoniae</i>	4	02(50)	01(25)	01(25)	0	0
<i>S. dysgalactiae</i>	3	01(33)	01(11)	01(11)	0	0
<i>B. cereus</i>	1	01(100)	0	0	0	0
<i>S. uberis</i>	3	01(33)	01(11)	01(11)	0	0
<i>M. luteus</i>	1	01(100)	0	0	0	0
<i>P.aeruginosa</i>	2	01(50)	0	01(50)	0	0
<i>S. pyrogenes</i>	1	01(100)	0	0	0	0
Total	39	43.58%	15.38%	20.51%	7.69%	10.25%

3.5. Quarter wise prevalence of mastitis

Staphylococcus aureus was shown to be substantially more prevalent bacterial pathogen in the rare right (RR) and rare left (RL) quarters of buffaloes with clinical mastitis as compared to other bacterial species. Similarly, the incidence was noticeably lower in the fore left (FL) and fore right (FR) quarters. In the same way, *Escherichia coli* and *Streptococcus dysgalactiae* were the next significant bacterial species found in animal quarters (**Figure 2**).

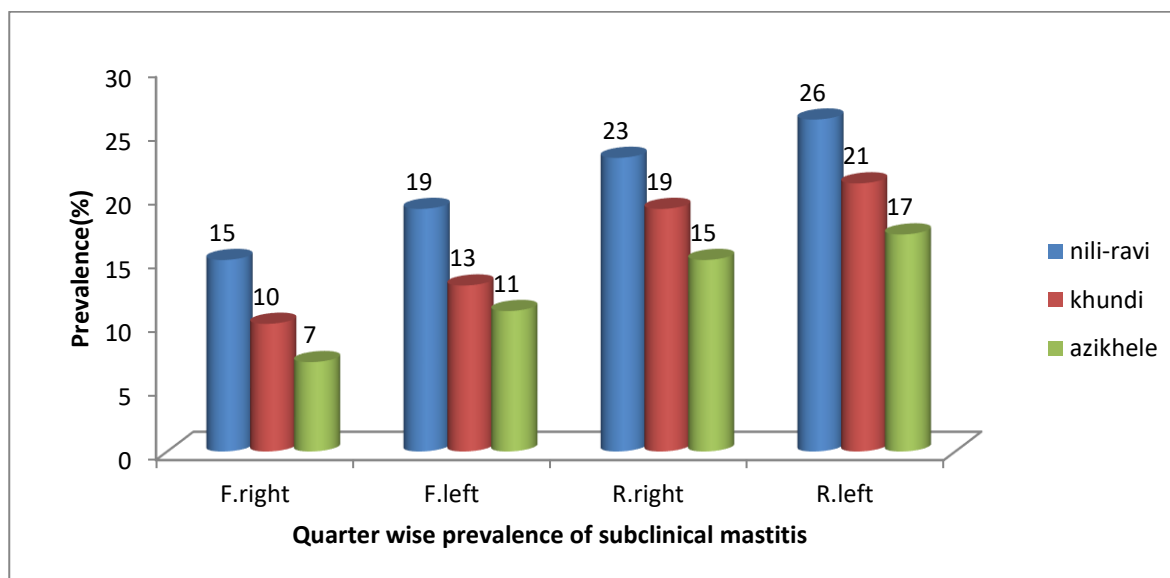


Figure 2: Quarter wise prevalence of Subclinical Mastitis (SCM) in buffaloes breed

3.6. Month wise prevalence of Subclinical Mastitis (SCM) in buffalo's breeds

According to the research, the highest prevalence of subclinical mastitis was founded in the summer

seasons in which *S. aureus* was most dominant species and *Escherichia coli* and *Streptococcus dysgalactiae* were found to be the second and third most prevalent bacterial species, respectively. In buffaloes, the summer muggy season of July, August and September had the highest occurrence of subclinical mastitis. Nevertheless, the dry summer months had the lowest prevalence of mastitis in buffaloes. In the dry-off period as an important time when pathogenic bacteria might start their offense to the mammary gland. The findings about the persistency of the bacterial species in the dry period were recorded in (Table 5). More cases of subclinical mastitis (SCM) were founded in summer hot and humid month (June-august) followed by winter (Dec-Feb), autumn (sep-nov) and spring (march-may).

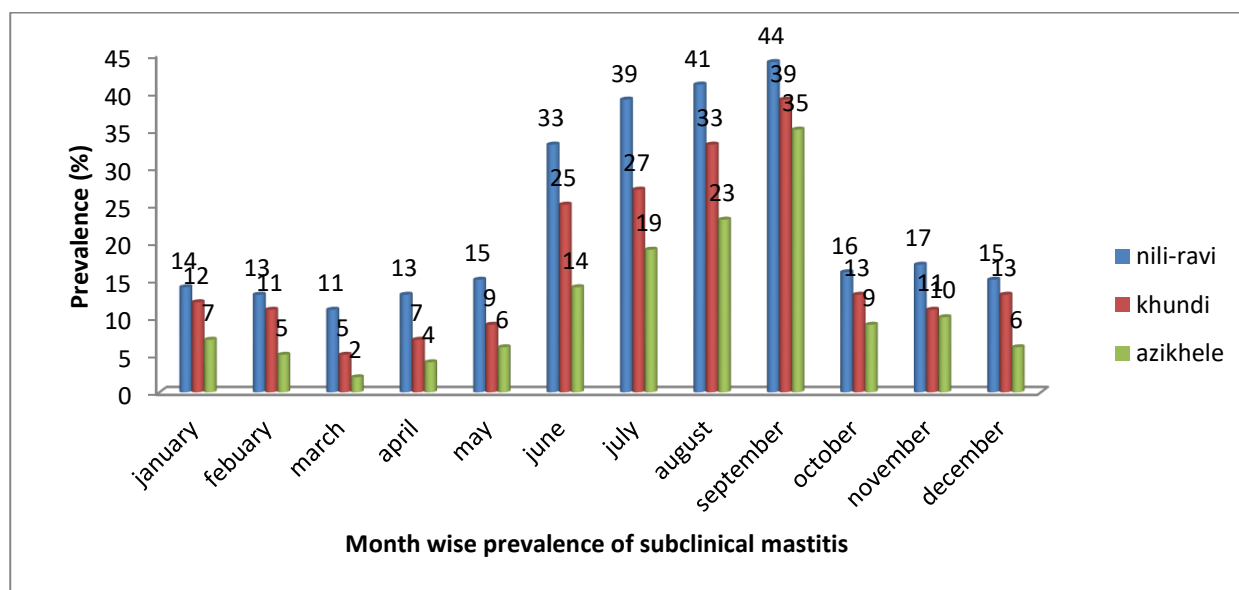


Figure 3: Month wise prevalence of Subclinical Mastitis (SCM) in buffalo's breeds

Table 6: Total number of affected quarters in the farm based on CMT score

S. No	CMT in the examined farm	Total No of quarters	No of affected quarters	Percentage (%)
1	Dry period(60-150 days)	180*4=720	544/720	75.55
2	Lactating period(320days)	120*4=480	206/480	42.91
3	Period before weaning	200*4=800	150/800	18.75

Table 7: Statistical analysis of SCC (cell/ml) and EC (mS/ cm) of the examined subclinical mastitis (SCM) milk samples.

S. No	Parameters	No. of Samples	Min	Mix	Mean $\pm$ SD
1	SCC	500	2.5×10^5	2.7×10^6	$5.6 \times 10^5 \pm 9.8 \times 10^4$
2	EC	500	5.50	10.83	8.044 ± 2.219

3.7. Risk Factors

This study revealed significantly higher prevalence of mastitis 55% (n = 250) in cross breeds as compared to local breeds 44.% (n =200; p <0.0184; Table 8). In comparing prevalence among production systems, a statistically significant (p <0.001) peak prevalence was observed in buffaloes reared under intensive production system 64% (n = 288) than extensive production system 36% (n = 162; s.no,8). In this study, buffaloes affected with clinical mastitis previously, were found to be more prone to mastitis than non- exposed ones with prevalence of 77% (n = 350) and 22% (n = 100), respectively, and the result is statistically significant (p <.05;s.no 10). Lactation stage was considered as risk factor for the occurrence of mastitis in this report. Buffaloes during early stage of lactation had a higher mastitis prevalence 46% (n = 210) than middle 16% (n = 75) and late 36.67% (n = 165) stage of lactation and the difference was statistically significant (p <.05; s. no 5).

Table 8: Prevalence of mastitis in milking buffaloes based on intrinsic risk factors

S. No.	Various stages of intrinsic risk factors		No of positive cases(n=450)	Percentage (%)	Chi square test(χ^2)	P-value
1	Age	2-3years	47	10.44	306.8	<0.0001
		4-5years	56	12.44		
		6-7years	64	14.22		
		8-9years	95	21.11		
		≥ 10 years	188	41.77		
2	Breed	Local	200	44	5.556	0.0184
		Cross	250	55		
3	Season	winter	140	31.22	34.89	<0.0001
		spring	70	15.56		
		summer	145	32.11		
		Autumn	95	21.11		
4	Parity	1 st	60	13.33	170.5	<0.0001
		2 nd	165	36.67		
		3 rd	98	21.78		
		4 th	64	14.22		
		5 th	35	7.77		
		≥ 6 th	28	6.22		
5	Lactation stages	Early	210	46.67	63	<0.0001
		Mid	75	16.67		
		Late	165	36.67		
6	Body condition	Good	300	66.60	50	<0.0001
		Poor	150	33.30		

Table 9: Prevalence of mastitis in milking buffaloes based on various extrinsic risk factors and significance differences between each risk factor stage

S. No	Various stages of extrinsic Risk factors		No of positive Cases(n=450)	Percentage (%)	Chi-square-test χ^2	P-value
1	Floor type	Concrete	280	62.22	26.89	<0.0001
		Muddy	170	37.78		
2	Production system	Extensive	162	36	35.28	<0.0001
		Intensive	288	64		
3	Hygienic practice	Good	290	64	37.56	<0.0001
		Poor	160	35		
4	Mastitis history	Yes	350	77	138.9	<0.0001
		No	100	22		
5	Tick infestation	Yes	306	68	58.32	<0.0001
		No	144	32		

4. Discussion

Mastitis is the inflammation of mammary glands and throughout the world it is one of the most prevalent disease condition of dairy farm animals with economic importance (Ali *et al.*, 2021). Most of European and Asian nations, the dairy products and buffaloes' milk have grown in popularity in recent years (Konuspayeva *et al.*, 2023). Statistics of Food and Agriculture

Organization (FAO) indicates that the buffalo (*Bubalus bubalis*) is the world's second-largest milk producer, with a projected milk production capacity of 134 billion liters in 2020, or 15% of global milk production (Tezera, *et al.*, 2021). About 74% of the world's buffalo population resides in south Asian nations which producing more than 99% of the world's buffalo milk these Asian nation are Bangladesh, Bhutan, Nepal, Sri Lanka, china, India and Pakistan (Mervino *et al.*, (2020).

Mastitis is the outcome of various factors associated with the host (buffaloes), environment and the pathogens (bacteria). Among pathogens, bacteria are the most frequently associated etiological agent of the ailments (Ali *et al.*,2021). Various bacterial genera causes contagious mastitis includes *Streptococcus agalactiae* and *Staphylococcus aureus* while, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Escherichia coli* and *Klebsiella pneumonia* are the main causes of environmental mastitis (Shoaib *et al.*, 2021).

These results are in agreement with our findings because animal surroundings such as bedding and manure are source of common contagious pathogens, these pathogens may be present in air, soil and as environment and are considered the basic cause of mastitis. Milker's hands, towels, cloth and flies spread these pathogenic bacteria to clean udders during the milking process and responsible for most of the subclinical mastitis(SCM).

In the present study, a total of 135isolates (55 from Nili-Ravi buffaloes, 41 from Kundhi buffaloes and 39 from Azakheli buffaloes) were found positive in which ten different microbial species were recovered. *Staphylococcus aureus* was the most frequently recovered bacterial species in Nili Ravi, Kundhi and Azakheli accounting 27.27%, 23.86% and 23.07% respectively of all isolates, followed by *Escherichia coli* ,*streptococcus agalactiae*, *Bacillus cereus*, *Streptococcus uberis*, *Micrococcus luteus* ,the least recover bacteria species was *Pseudomonas aeruginosa* with3.63%, 2.48% and 2.63% respectively as shown in (Table 01).

From this study we find out that local breeds like Kundhi and Azakheli are well adapted to harsh environmental conditions, including rugged terrain and poor quality forages, which can reduce stress and improve overall health and often show more resistant to diseases, including mastitis, as compared to exotic Nili Ravi breed due to high milk production this breed are more susceptible to mastitis.

Most recent study conducted in Sindh showed their incidences in subclinical mastitis milk samples Recovered *Staphylococcus aureus* (27.97%), *Escherichia coli* (17.95%), *Streptococcus agalactia* (13.77%), *Bacillus cereus*, *Streptococcus dysgalactiae* (10.43%), *Streptococcus uberis* (9.18%), *Micrococcus luteus* (7.39%) and *Pseudomonas aeruginosa* (2.29%) respectively (Baloch *et al.*, 2024) This variation may be due to manage mental conditions at the farm, season, area transportation conditions, difference in sample handling in the laboratory and use of antibiotics. In another study *S. aureus* was founded in Nili-Ravi and Kundhi buffaloes was isolated as top ranking pathogen from positive cases of mastitis (Ali *et al.*, 2021). This considerable increase of diseases in buffaloes is alarming, which might be because dairy farming is rapidly growing in Khyber Pakhtunkhwa (Sarba & Tola, 2017). In Jammu and Kashmir region 81% cases of SCM was reported in Nili-Ravi buffaloes (Bath *et al.*, 2017). This variation in studies may be due to the number of milk samples processed and areas under study in the present study were considerably more than the other studies. In this present study the quarter wise prevalence was found to be 57%, 42% and 33% respectively. *Staphylococcus aureus* was shown to be substantially more prevalent bacterial pathogen in the right (RR) and left rare (RL) quarters of buffaloes as compared to other bacterial species (Table 3). Area wise prevalence of mastitis was found in Nili-Ravi breed as 44% in district Peshawar, 15% in Charsadda, 17% in Nowshera, 12% in Mardan and 6% in district Swabi. Similarly, 43%,15,20,7 and 10% were recorded in Azakheli breed while the prevalence in Kundhi breed were documented as 39%,17,21,4 and 12% respectively. Month wise prevalence of SCM was reported more in summer hot and humid month (June-August) follow by winter dry periods (Dec-Feb), autumn (Sep-Nov) and spring (March-May).

There are several factors which affects the findings and results of other researchers may be different due to method of detection, breeds of animals, immune response, management, climatic condition,

hygienic practice, animal health condition, immunity stress etc, extreme weather conditions and deficiency of fodder create stress to the body; decrease immunity, leading to increased sub-clinical incidence associated with contagious and environmental mastitogens. Inflammation of mammary glands increases the number of somatic cells in milk. When milk from sub-clinically mastitis quarters is mixed with anionic detergent solutions such as California Mastitis Test or Surf Field Mastitis Test reagent, a Chemical reaction causes the gel formation Ideally milk from all four quarters should be tested separately. Mastitis milk is not good for human consumption due to the presence of bacteria and their toxins, as well as high number of white blood cells. Non-infected animals should be milked ahead of infected ones. Using a simple test like Surf Field Mastitis Test, farmers should test their dairy animals before purchasing, if positive; avoid buying milk of such animals (buffaloes).

Cross breed buffaloes (Nili-Ravi) are more likely affected by mastitis than that of local (Kundhi and Azakheli) breed due to their adaptation with local environment. The occurrence of bubaline mastitis and lactation stage was significantly ($p<0.05$) associated with each other. Higher infection in buffaloes in early lactation stage followed by late and medium lactation stages, the early lactation stage infection might be due to the carryover of infection from dry period. In dairy buffaloes most new infections occur during the early part of the dry period and in the first two months of lactation. The current findings showed significant higher prevalence of buffalo's mastitis in intensive production systems (54.4%) when compared with extensive production systems (33.3%). This could be attributed to the variation in hygienic standards of dairy environment and milking conditions. In most dairy farms buffaloes kept in intensive system maintained in a dirty and wet area which could favour the proliferation and transmission of mastitis causing organisms. The prevalence of mastitis in buffaloes kept under poor hygiene and with good hygienic status was found to be 49.6% and 35.1%, respectively. Prevalence of mastitis was significantly ($p<0.05$) associated with milking hygienic practice. Dairy buffaloes with poor milking hygiene standard are severely affected than those with good milking hygiene practices. This might be due to absence of udder washing, milking of buffaloes with common milker's and using of common udder cloths, which could serve as a vehicle to spread especially for contagious mastitis.

5. Conclusion

It is concluded that mastitis, particularly sub-clinical type, is a more prevalent disease condition of dairy animals like buffaloes in the central zone of Khyber Pakhtunkhwa Pakistan. The major bacterial species causing both clinical and subclinical mastitis in all breed of buffaloes detected were *Staphylococcus aureus*, *Escherichia coli*, *Pneumoniae*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Micrococcus luteus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Streptococcus uberis*, *Bacillus ubtilis* and *Streptococcus Pyogenes*. It was observed that bacterial species, especially pathogenic bacteria, remained persistent in the animal quarters throughout the dry period followed by summer season. Majority of the risk factors were noted to be the main reasons for the observed high prevalence of mastitis in the study area.

The cases of mastitis can be reduced to an appreciable extent and the production can be increased by adopting managerial measures and practices on priority basis. The calf should be prevented to cause any injury to the quarters; Full hand milking should be practiced; wet and dirty floors should be discouraged; Pacca floor must be even and properly bedded; more attention should be given to cross breed than local breeds. Detailed epidemiological, microbiological and economic research studies are suggested at national level to devise strategies for the control and prevention of mastitis.

Acknowledgments

The authors would like to extend their gratitude to the Higher Education Commission(HEC), Islamabad, Pakistan for funding this research work through HEC-NRPU Project No. 8633.

Author's Contribution

Tahira Tayyeb: Investigation, Writing-original draft preparation; **Asad Ullah:** Supervision & Conceptualization; **Sadeeq ur Rahman:** Methodology & Resources; **Imad Khan:** Project administration; **Raheela Taj & Shumaila Gul:** Software; **Rainaz Begum & Maryam Begum:** Writing-review and editing; **Ali Gohar:** Validation; **Muhammad Aslam:** Formal analysis; **Sulha Syed & Muhammad Owais Khan:** Data Curation; **Umar Hayat & Mansoor Ahmad:** Visualization.

Conflict of interest

The author(s) declared no potential conflicts of interest with respect to research, authorship, and/or publication with the work submitted.

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