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Prevalence, Microbial and Molecular identification of Mastitis causing pathogens at district Khairpur Sindh, Pakistan.

Majeeda Ruk[1]. Javed Ahmed Ujan[2*], Sham Lal[3]

[1,2*]Department of Zoology, Shah Abdul Latif University, Khairpur Sindh Pakistan
[3]Institute of Microbiology, Shah Abdul Latif University, Khairpur Sindh Pakistan

Email: javed.ujan@salu.edu.pk

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ABSTRACT

Bovine mastitis is an infectious disease of the mammary glands of dairy cows. It has a high incidence in cows worldwide. It is usually caused by bacteria entering the nipple tube and spreading to the breast tissue. In recent years, molecular diagnostic technology has become the gold standard for diagnosing mastitis. They provide qualitative, quantitative, and large-scale rapid diagnosis. Its diagnostic function can identify infections at the subspecies level, which is essential for epidemiological investigations. They are increasingly used in the management of mastitis to identify vaccine candidates and select dairy cow breeds that are resistant to mastitis.The present study revealed that Staphylococcus aureus had the most significant relative prevalence, followed by Escherichia (15 percent), Streptococci (12 percent), Pseudomonas (10 percent), Klebsiella (7 percent), and Bacillus (5 percent).Furthermore, results of this current research identified a total of 02 mutations in the gap gene of Staphylococcus aureus, 01 mutations in T13 samples of Tehri and 01 mutation in G13 samples of Gambat through the use of PCR gel electrophoresis and DNA sequencing techniques under three different environments of district Khairpur namely Tehri, Gambat and Khairpur. According to the genetic code, these mutations are classified as missense mutations because they alter the codons and their final amino acids. Furthermore, our results revealed the substitution of TTC codon at bp 79 that was changed into TTA codon, where the former one was coding for Phenylalanine amino acid, which is classified as neutral and nonpolar due to the inert and hydrophobic nature of the benzyl side chain, and the changed codon codes for Leucine amino acid, which is also an essential amino acid. Whereas the second nucleotide change was found at 35bp of the Gap gene, where the TGT codon was converted into TGG, the primary codon was coding for Cysteine amino acid, which is a semi-essential amino acid with a thiol side chain that frequently participates in enzymatic reactions as a nucleophile, which was changed into Glutamic acid, which is also a non-essential amino acid which is an excitatory neurotransmitter with one more methylene group in its side chain than aspartic acid. In contrast, no mutation was found in the other samples included in our investigation. Our findings show that Staphylococcus is highly adapted to the TEHRI environment, as indicated by identifying required amino acids in the acquired sequence but not the GAMBAT environment. Our findings can be used as a reference and guide for selecting extremely diversified and well-adapted bacteria environments that cause mastitis in cattle.

INTRODUCTION

Mastitis is an inflammatory condition of the breast that is defined as physical, chemical, and normal biological abnormalities in breast milk, as well as pathological changes in glandular tissues, regardless of the cause. It is the most serious and expensive illness that affects dairy cows (Lightener et al., 1988). Mastitis is one of Pakistan's most critical health concerns, according to field assessments of major livestock diseases (Cady et al., 1983; Ajmal, 1990).

In the dairy sector, mastitis is the costliest illness. Direct expenses such as a temporary/permanent decrease in milk supply, a decrease in milk quality owing to increased somatic cell populations in subclinical instances, and milk rejection in clinical cases of mastitis or due to antibiotic residues are among the economic losses sustained. Indirect losses, such as (1) premature slaughter of cows and early replacement expenses, (2) low cow sales prices, (3) extra veterinarian and pharmaceutical costs, and (4) diagnostic/laboratory costs, are also taken into account.

The Mastitis is classified as subclinical or clinical based on the degree of inflammation (Viguier *et al* 2009). Asymptomatic circumstances make subclinical mastitis (SCM) challenging to diagnose. Nonetheless, SCM accounts for two-thirds of overall milk production losses (Radostits 2000 and FAO 2014). Routine barn-side testing may help identify and treat SCM. An increase in inflammatory and desquamated epithelial cells entering milk during mastitis reduces milk quality and quantity (Schukken 2003 andCeron-Munoz 2002). SCC is used in conjunction with milk's electrical conductivity (EC), the BTB test, and the California mastitis test (CMT). It shows udder infection. Unlike BTB, CMT detects nucleic acid released from somatic cells after lysis

by a detergent (Sandholm1995). This enzyme is secreted into milk during inflammation and may be used to identify SCM (Pyorala 2003).

A perfect diagnostic test should be sensitive, specific, quick, repeatable, and cost-effective. Worldwide, most conservative labs continue to regard bacterial isolation and culture as the (Gold Standard) for diagnosing mastitis. The dispute over whether culture remains the gold standard or if PCR has supplanted it and become the contemporary gold standard is ongoing since both approaches have their strengths and weaknesses (Ghorbanpoor*et al* 2007 and Tatayet al 2015).Therefore, present study was aimed at molecular diagnosis of mastitis causing pathogens at district Khairpur Sindh Pakistan.

METHODOLOGY

Selection and sampling of animals

The cows employed in this research were of various parties, and smallholder farmers at various phases of lactation were randomly picked from several talukas in District Khairpur.All methods for collecting the milk samples from dairy cattle were undertaken and approved following the norms and recommendations established by the Canadian Council on Animal Welfare.For this study, milk with an SCC value of less than 100,000 cells/ml is considered a healthy cow, on the contrary, an SCC value of more than 100,000 cells/ml is considered a diagnosis of subclinical mastitis (Sharma et al., 2011). Table 1 provides detailed information about cow selection.

Design of Studies and Selection of Sample Sizes

This study uses a cross-sectional design. The sample size is determined as follows: n = (1,962) (Pexp) (1 Pexp) / d2, where n is the sample size, 1.96 is the Z statistic, and the confidence level is 95%, Pexp is the expected prevalence, and d is the absolute required The accuracy is equal to 5%. (0.05). The frequency is 54.2% (Gitau et al. 2014), and the estimated sample size is 381 animals; however, 400 animals were sampled instead.

California Mastitis Testing

According to Schalm et al. 1971 and NMC 2017 regulations, the California Mastitis Test (CMT) was performed on the farm. A total of 400 cows were inspected, and samples were taken from a quarter of the milk. Check the udders and nipples of each cow. Check the breasts and nipples for inflammation, animal fever, clots, blood and milk scales [11]. After the physical examination, the California Mastitis Test (CMT) was used to detect SCM. As explained by NMC, CMT results are evaluated as negative, positive, or minimal (Sue et al. 2014). If the cow's CMT reading is (1+, 2+, or 3+), it is considered positive for SCM, while negative and micro readings are considered negative. The cows are then classified as celiac disease or non-celiac disease. If the CMT test is positive for at least one quarter, the cow is considered positive for mastitis. Next, milk samples are collected from CMT-positive and negative cows.

Prior to examining, the bosoms were totally cleaned and dried with water. In the wake of cleaning the areola with a 70% ethanol swab and extricating the milk 4 to multiple times, gather 5 to 10 ml of milk from every quadrant aseptically and place it in a different general jug set somewhat evenly to limit bosoms Pollution (Mbindyo et al. 2020 and NMC 2017). From that point onward, the example vial is appropriately fixed and marked. The examples were shipped to the Institute of Microbiology of Shah Abdul Latif University in Khairpur, where they were kept in a fridge in cool sacks. The examples are brooded quickly or put away in a fridge at 4°C for as long as 1 day.

Staphylococcus aureus isolation and identification

Bacterial cultures were performed on milk samples in accordance with the guidelines of the National Mastitis Committee (Hogan 1999). Mix 3 ml of milk with tryptic soy broth containing 7.5% NaCl and incubate at 37°C for 18-24 hours. Next, pour a total of 10 ml of culture onto Baird-Parker agar plates containing tellurite and 5% egg yolk and incubate at 37°C for 18-24 hours. The two possible colonies from each sample were transferred to tryptic soy agar and plated for DNA capture. In addition, the growth characteristics of Staphylococcus aureus were confirmed, and catalase, tubular coagulase and mannitol salt agar were used to

identify Staphylococcus species (Oxoid, England). The catalase test and growth parameters in Edward's medium (Oxoid, England) were used to identify the Streptococcus genus, and the CAMP test was used to differentiate the groups. Gram-negative bacteria are detected by colony morphology and lactose fermentation and oxidase, sugar fermentation and IMViC tests on MacConkey.

DNA Extraction

For each staphylococcal isolate based on biochemical testing, two separate colonies from an overnight culture at 37°C were used for DNA extraction. According to the manufacturer's instructions, use a genomic DNA purification kit (Thermo Fisher Scientific, Driesch, Germany) to extract the genome of the Staphylococcus isolates. Use Thermo Scientific Gene Jet # K0781 Complete Genomic DNA Extraction Kit (www.thermofisher.com) to extract DNA. According to Hernández et al. 2019, it is stored in a refrigerator at -20°C.

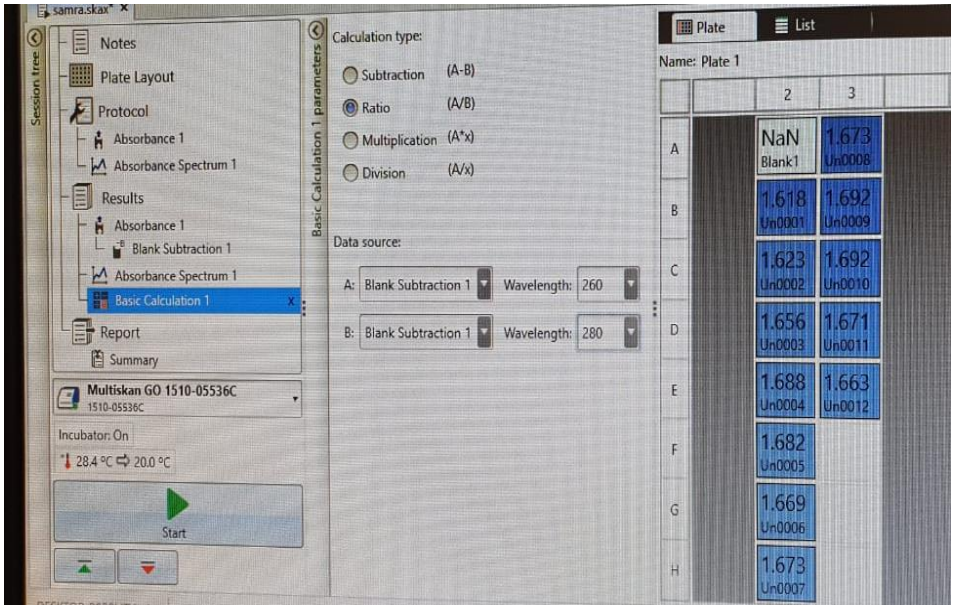
Polymerase Chain Reaction (PCR)

PCR is a technique that uses sequence-specific oligonucleotide primers and thermostable polymerases to amplify unique, organism-specific target DNA sequences in vitro. The primers were developed online using the Primer 3 web application (https://primer3.ut.ee/) and were commercially synthesized by the Korean company Macrogen (https://dna.macrogen.com/). The primers used in this work are listed in Table 1. After this step, the Gap gene of Staphylococcus aureus was amplified by PCR, gel electrophoresis, and sequencing (Gao et al., 2011). The PCR reaction was carried out in a volumetric flask containing 3 L of genomic DNA (30-50 ng/L), 1 L of each primer (10 mol), and 12.5 L of TaqTM Mix (1, 25 units/25 L). Reaction) and 7.5 L ddH2O. Use the following thermal cycler program: 94°C for 10 minutes; 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, 35 cycles; 72°C for 7 minutes.

Table 1Detailed information about primers

Gene (Accession No.)	Oligonucleotide sequence (5' to 3')	Amplicon size (bp)	Annealing temperature (°C)
NC-007622.1	5'-ATGGTTTTGGTAGAATTGGTCGTTTA3'	200	58
	3'-GACATTTCGTTATCATACCAAGCTG5		

Graph 1 Showing results of DNA Quantification through Nanodrop spectrophotometer



Data analysis.

Data input and administration were carried out using Microsoft Excel 2016, while bioinformatic analysis was carried out utilizing online bast on the ensemseble.org website of genome browser to detect mutations among various samples. Prevalence was determined as the percentage of ill cattle in the overall population studied.

RESULTS AND DISCUSSION

Prevalence of Bacterial isolates through biochemical testing

There are 100 isolates in total. The relative prevalence of Staphylococcus aureus is the highest (50%), followed by Escherichia (15%), Streptococcus (12%), Pseudomonas (10%), Klebsiella (7%) and Bacillus (5%). These findings are consistent with those of Shireen (1984), who observed the prevalence of Staphylococcus. Staphylococcus aureus, streptococcus. Without Lactobacillus, Escherichia coli and Corynebacterium. In cattle, they were 55.26%, 18.42%, 15.78%, and 3%. Razzaq (1998) found that the prevalence of Staphylococcus was 53%. Staphylococcus aureus in water buffalo, which is consistent with the current research results. Khan (2002) found that the prevalence of streptococcus was 23%. Buffalo's milk-free, the current research results confirm this. Mei Meng et al. (1999) A higher incidence of Staphylococcus was observed. Staphylococcus aureus, followed by Streptococcus and Escherichia coli, supports the results of the current study.

MolecularIdentification of Gap gene of S.aureus

The biochemical differentiation of S. aureus was validated by amplifying the Gap gene,where a 200 bp amplicon was identified in S. aureus (Fig 1A). The sequencing blast result of the mutation is presented in Fig IB. Using PCR-Gel Electrophoresis and DNA methods, this research found a total of 02 mutations in the gap gene of Staphylococcus aureus, 01 in T13 (Tehri) and 01 in G13 (Gambat) samples. According to the genetic code, these mutations are classed as missense mutations because they alter the codons and their final amino acids.Furthermore, our data indicate that polymorphisms were identified only in T13 samples, while mutations were identified in G13 samples, and no mutations were identified in any of the other ten samples included in our study. Our findings indicate that Staphylococcus aureus is well adapted to the environment of THRHRI (T13) samples due to the presence of necessary amino acids in the sequence retrieved but is not well adapted to the environment of GAMBAT (G13). This variance may be attributable to the season, farm management circumstances, geographic location, transit conditions, laboratory sample processing procedures, and antibiotic usage.

Tables 3A and 3B include thorough information regarding the mutation type, change in codon structure, and matching amino acid coding for the Gap gene.

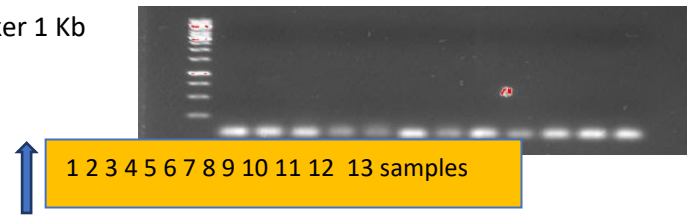
Table 3A and 3B showing theinformation regarding the mutation in Gap gene

Sample location	Position of mutation	Original codon	Changed codon	Original Amino acid	Changed Amino Acid	Type of point mutation
T13	79	TTC	TTA	Phenyl alanine (Essential)	Leucine (Essential)	Missense point mutation
G13	35	TGT	TGG	Cysteine (Non Essential)	Glutamic acid (Non Essential)	Missense Point Mutation
Whereas no any mutation was found from other 10 samples of three locations namely Gambat, Tehri and Khairpur taluka						

Table 3B ***Frequency of gap gene mutation among three talukas of khairpur***

Name of Gene	Sample ID	Found SNPs	Percentage Formula	Percentage
Gap	T13	1	1/68×100	1.4%
	G13	1	1/120×100	0.83%

M: Marker 1 Kb



Results of PCR amplification of Gap gene amplification are shown in fig. 1A

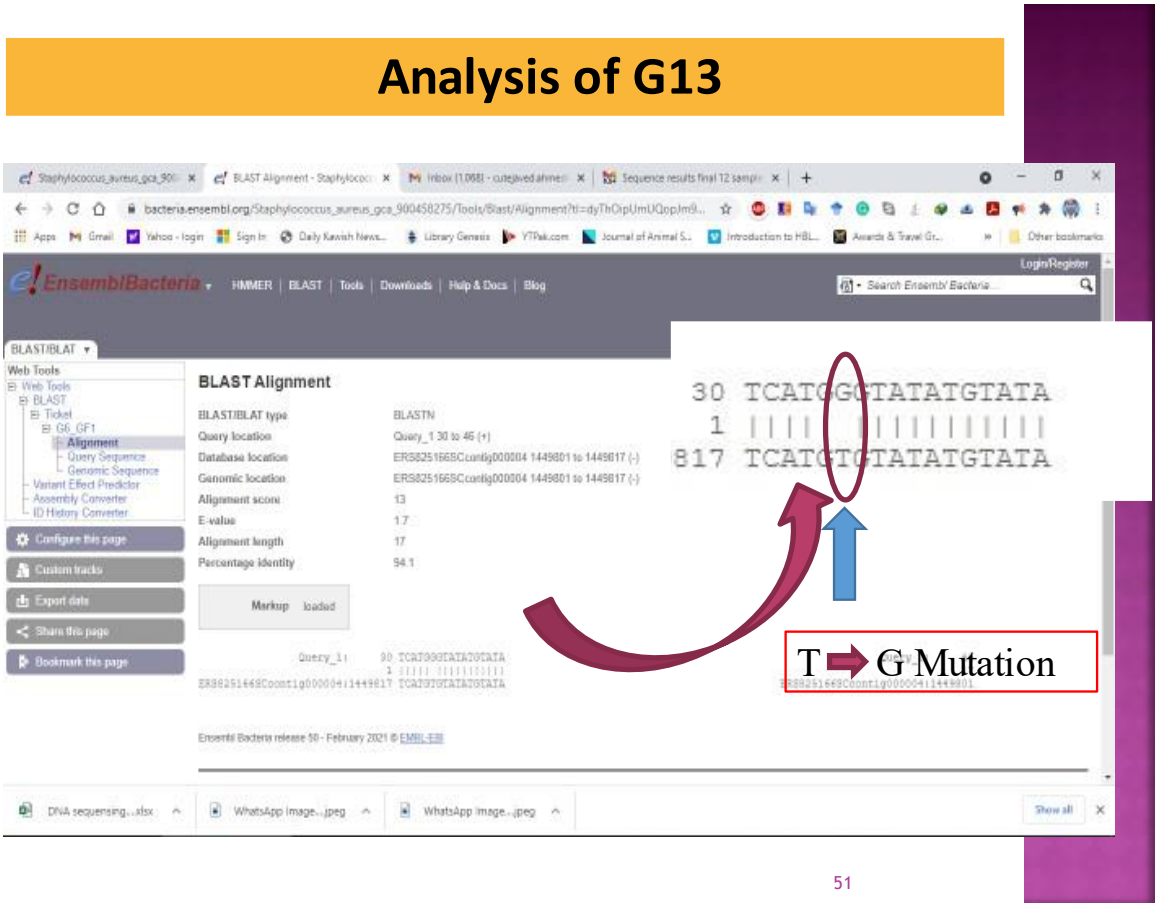


Fig. 1 B DNA Blast of Sequence data of Gap 1 gene

Antibiotic susceptibility

Each of the 100 isolates was tested against five widely used antibiotics. The findings indicated that the isolates' average sensitivity to Enrofloxacin, Chloramphenicol, Gentamycin, Oxytetracycline, and Amoxicillin was declining. In this research, enrofloxacin was shown to be the most efficient antibiotic against a variety of mastitis bacteria, whereas amoxicillin was the least effective. The susceptibility of several mastitis pathogens to various medications is summarized in Table 2.

Table 2 Antibiotic sensitivity testing of bacterial isolates in vitro

Bacterial isolate	**% of sensitive isolates against**				
	Amoxillin	Enrofloxacin	Gentamycin	Chloramphenicol	Oxytetracycline
S.aureus	38	79	97	85	70
Step.agalactiae	37	81	90	81	55
E.Coli	56	100	86	70	70
Strep.dysagalactiae	0	100	51	100	50
C.bovis	100	100	00	00	00

CONCLUSION

The present study revealed that Staphylococcus aureus had the most significant relative prevalence, followed by Escherichia (15 percent), Streptococci (12 percent), Pseudomonas (10 percent), Klebsiella (7 percent), and Bacillus (5 percent).Furthermore, our data indicate that polymorphisms was identified only in T13 samples, while mutations were identified in G13 samples, and no mutations were identified in any of the other ten samples. PCR and PCR based tests have replaced culture as the gold standard in diagnosing mastitis in farm animals due to fast and continual molecular tool development. They help in mastitis diagnosis and assist mastitis control programmes in many ways.

References

1. Ajma~dM. 1990. Livestock health of Pakistan. In: Proc. 3 Int. Congo Pakistan. Veterinary Medical Association, Univ. Grants Commission, Islamabad, Pakistan.
2. El-Sayed, A., Awad, W., Abdou, N. E., & Vázquez, H. C. (2017). Molecular biological tools applied for identification of mastitis causing pathogens. *International journal of veterinary science and medicine*, *5*(2), 89-97.
3. Cady, R.A., S.K. Shah, E.C. Schermerhorn and R.E. McDowell. 1983. Factors affecting performance of Nili-Ravi buffaloes in Pakistan. J. Dairy Sci. 66: 578-586.
4. Ceron-Munoz M, Tonhati H, Duarte J, Oliveira J, Munoz-Berrocal M, Jurado-Gamez H. Factors affecting somatic cell counts and their relations with milk and milk constituent yield in buffaloes. J Dairy Sci. 2002; 85: 2885–2889. PMID: 12487456
5. Food and Agricultural Organization. Impact of mastitis in small scale dairy production systems. Animal Production and Health Working Paper. No. 13. Rome; 2014. Avaiable: http://www.fao.org/3/a-i3377e.pdf.
6. GhorbanpoorM,Seyfiabad,ShapouriM,MoatamediH,JamshidianM,GooraninejadS.ComparisonofPCRandbacterialculturemethodsfordiagnosisofdairycattle'ssubclinicalmastitiscausedbyStaphylococcusaureus.JVetRes2007;62:87–91.
7. Gitau, G. K., Bundi, R. M., Mulei, C. M., &Vanleeuwen, J. (2014). Mastitogenic bacteria isolated from dairy cows in Kenya and their antimicrobial sensitivity. Journal of the South African Veterinary Association, 85(1), 1-8.
8. Gao J, Ferreri M, Liu XQ, Chen LB, Su JL, Han B. Development of multiplexpolymerase chain reaction assay for rapid detection of Staphylococcusaureus and selected antibiotic resistancegenes in bovine mastitic milksamples. J Vet Diagn Investig. 2011;23(5):894–901.
9. Hogeveen H. Mastitis is an economic problem. Proceedings of the BritishMastitis Conference, Institute for Animal Health/The Dairy Group/ADASStoneleigh, UK, p 1-13; 2005; Available under: http://www.britishmastitisconference.org.uk/BMC2005Proceedings.pdf
10. Hogan JS, National MC. Laboratory handbook on bovine mastitis. USA: National Mastitis Council; 1999.
11. Lightener, I.K., G. Miller, W.o.Haneston and C.R Dorn. 1988. Estimation of the costs of mastitis using milk somatic cell count data. J. American Vet. Med. Assoc. 192: 1410-1413.
12. Hernández-Triana, L. M., Brugman, V. A., Nikolova, N. I., Ruiz-Arrondo, I., Barrero, E., Thorne, L., ... &Fooks, A. R. (2019). DNA barcoding of British mosquitoes (Diptera, Culicidae) to support species identification, discovery of cryptic genetic diversity and monitoring invasive species. ZooKeys, 832, 57.
13. Khan, AZ. 2002. Comparative aspects of prevalence of mastitis in buffaloes and crossbred cows and antibiotic susceptibility profiles of isolates. M.Sc. (Hons.) thesis, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan.
14. Memon, M.I., K.B. Mirbathar, M.R. Memon, N. Akhtar, S..A. Soomoro and P. Dewani. 1999. A study on etiology of subclinical mastitis in buffaloes. Pak. J. Agric. Eng. Vet. Sci. 15: 34-36.
15. Pyorala S. Indicators of inflammation in the diagnosis of mastitis. Vet Res. 2003; 34: 565–578. PMID: 14556695
16. Razzaq, A. 1998. Comparative efficacy of Vetimast, Tetra-Delta and Akamycin-D in mastitis of bUff~loes in and around Lahore. M.Sc. (Hons.) thesis, Department of Clinical Medicine and Sur~ery, College of Veterinary Sciences, Lahore, Pakistan.

17. Radostits OM, Arundel JH. Veterinary medicine: a textbook of the diseases of cattle, sheep, pigs, goats and horses. 9th ed. London: Saunders Press; 2000.
18. Schukken YH, Wilson DJ, Welcome F, Garrison-Tikofsky L, Gonzalez RN. Monitoring udder health and milk quality using somatic cell counts. Vet Res. 2003; 34: 579–596. PMID: 14556696
19. Sandholm M. Bovine udder and mastitis. Helsinki: University of Helsinki; 1995.
20. Steele N. Mastitis pathogen identification using polymerase chain reaction inNew Zealand milk samples (Master thesis). New Zealand: Massey University;2015. Available under: https://mro.massey.ac.nz/bitstream/handle/10179/6892/02_whole.pdf.
21. Shireen, N. 1984. Bacteriological survey of chronic mas~itis in cattle and buffaloes. M.Sc. (Hons.) the.sls, .Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan.
22. Sue, M. J., Yeap, S. K., Omar, A. R., & Tan, S. W. (2014). Application of PCR-ELISA in molecular diagnosis. *BioMed research international*, *2014*.
23. Mbindyo, C. M., Gitao, G. C., &Mulei, C. M. (2020). Prevalence, etiology, and risk factors of mastitis in dairy cattle in Embu and Kajiado Counties, Kenya. *Veterinary medicine international*, *2020*.
24. National Mastitis Council (NMC), Laboratory Handbook onBovine Mastitis, Rev. ed. National Mastitis Council Inc., NewPrague, MN, USA, 2017.
25. Sharma N, Singh NK, Bhadwal MS. Relationship of somatic cell count andmastitis: An overview. Asian-Australas J Anim Sci. 2011;24(3):429–38.
26. Schalm, O. W., Carroll, E. J., & Jain, N. C. (1971). Bovine mastitis. Bovine mastitis.
27. Tatay-DualdeJ,SánchezA,Prats-vanderHamM,Gómez, MartínA,AternaA,CorralesJ,etal.SensitivityoftwomethodstodetectMycoplasmaagalactiaeingoatmilk.IrishVetJ2015;68:21–5
28. Viguier C, Arora S, Gilmartin N, Welbeck K, O'Kennedy R. Mastitis detection: current trends and future perspectives. Trends Biotechnol. 2009; 27: 486–493. doi:10.1016/j.tibtech.2009.05.004 PMID: 19616330