WWW.IJRPR.COM

International Journal of Research Publication and Reviews

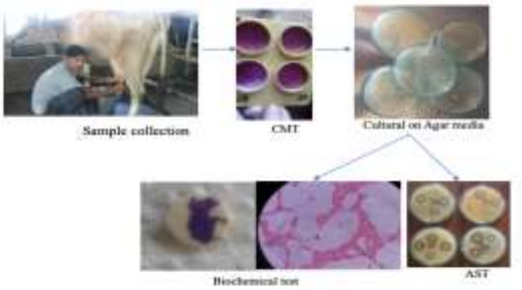
Journal homepage: www.ijrpr.com ISSN 2582-7421

Prevalence and Antibiogram of *Escherichia Coli* and *Staphylococcus* Species in Subclinical Mastitis among Dairy Cows in Western Chitwan, Nepal

***Gita Pandey*[1], *Chet Raj Pathak*[2], *Pratik Hamal*[1]**

[1]National Cattle Research Program, Rampur, Chitwan, Nepal
[2]Agriculture and Forestry University (AFU), Rampur, Chitwan, Nepal
E-mail: gitapandeyk@gmail.com

Graphical abstract



ABSTRACT

Mastitis is a major economically important disease manifested in clinical and subclinical forms among dairy animals. This study aimed to isolate the mastitis-causing bacteria, their prevalence, and associated risk factors of subclinical mastitis (SCM) with their antibiogram. This study was conducted from July 2017 to May 2019 in Western Chitwan, Nepal. Approximately 10 ml milk samples were collected from each quarter in a sterilized bottle from 340 cows. The California Mastitis test (CMT) was performed for screening of mastitis, which was followed by immediate bacteriological culture in both Nutrient Agar (NA) and Selective media [Mannitol Salt Agar (MSA), Eosin Methylene Blue (EMB) Agar, Mac Conkey Agar], Grams' staining, and confirmatory biochemical test for respective organisms. The results from CMT revealed that the prevalence of SCM was 41.47% (141/340). The biochemical cultural characteristics found that the most common *Staphylococcus* (39.01%) followed by *E. coli* (23.40%), *Streptococcus* (17.73%), and others unidentified (19.85%). The statistical analysis shows a non-significant association between breed, stage of lactation, milk yield, farming system, and prevalence of SCM among dairy cattle. Antibiogram profile indicated that tetracycline (80.3%) was the most effective drug followed by ceftriaxone (70.5%), Gentamicin (59.0%), and Enrofloxacin (44.3%). In conclusion, there was a high prevalence of subclinical mastitis in western Chitwan. Mastitis pathogens develop resistance against ampicillin. Further studies are required to develop alternative control strategies for mastitis in Nepal.

Keywords: Antimicrobial-resistant, CMT, Mastitis, Risk factor, *Streptococcus*

Introduction

Mastitis is one of the most common and serious problems in the dairy industry ruining the economy of the globe (Azooz et al., 2020). Mastitis is an inflammation of the mammary gland caused by various infectious organisms that leads to abnormal and reduced milk production in dairy cattle(Cheng & Han, 2020). The mastitis accounts for nearly, a 10% deficit in the total worth of the milk industry (Khanal & Pandit, 2013). The economic loss is mainly due to reduced milk quality and quantity, increased labor and treatment costs, and increased culling rates (Lamey et al., 2013). Depending on the severity of the inflammation and clinical manifestations, the disease can be categorized as either subclinical or clinical mastitis. The clinical forms of mastitis can be easily recognized as they manifest pathological and chemical changes in the mammary gland and milk with reduced production, whereas subclinical mastitis (SCM) is an asymptomatic condition and is more difficult to diagnose. The SCM is 15 to 40 times more common than clinical mastitis (Seegers et al., 2003). In the case of SCM in dairy cattle reduced milk production may lead to losses of about 70% (Sargeant et al., 2001). The prevalence of SCM in dairy animals ranges from 13.6 to 60 % in different parts of Nepal (Kharel et al., 2023).

The various groups of pathogens are responsible for causing mastitis with bacteria being the most common (Dalanezi et al., 2020). The most common isolated organisms were *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus* sp., *Escherichia coli*, *Klebsiella* sp., and *Corynebacterium* sp. (Sharma et al., 2012). Epidemiologically, mastitis pathogens are divided into three categories: infectious (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis*, *Mycoplasma* species), environmental (*Escherichia coli*, *Klebsiella*, *Streptococcus uberis*, *Streptococcus bovis* and *Streptococcus dysgalactiae*) and other (Sharma et al., 2012). Coagulase-negative s*taphylococci* (CNS), *Serratia* spp., *Pseudomonas aeruginosa*, *Nocardia asteroids*, *Candida* spp, and *Arcanobacterium pyogenes*. The problem of fungal mastitis has increased in recent years (Kurt & Eski, 2021). The most common pathogens causing fungal mastitis are *Candida* spp., *Trichosporon* spp., *Cryptococcus* spp., *Saccharomyces* spp., and *Aspergillus* spp. (Kurt & Eski, 2021).

Multiple and mixed groups of different pathogens, proper diagnosis, and specific treatment and control of mastitis are considered challenging and complicated (Taponen et al., 2017). In addition, the antimicrobial resistance of bacterial pathogens makes the control of mastitis even more critical (Melesse & Minyahil, 2019). Consequently, pathogen isolation and antibiogram testing have become essential for the successful treatment and control of mastitis among dairy cattle.

It has been reported that antibiotic treatments for mastitis cases in Nepal are usually challenging and fail to overcome the problems (Shrestha et al., 2021)The growing concern about the unsuccessful treatment of this disease could be due to the development of antibiotic resistance. The development of antibiotic-resistant bacterial species poses a major threat to treatment protocol and public health. Thus, this study shed light on the prevalence and risk factors associated with subclinical mastitis and the antibiotics susceptibility testing (AST) of the different isolated bacteria to different antibacterial drugs in western Chitwan, Nepal.

Material and methods

Research site and sample size

The study was conducted in the milk pocket area of western Chitwan including Rampur and Sharadanagar. The sample size was calculated using a formula provided by (Daniel, 1999)

Sample size (n) = $[Z^2*P\ (1-P)]/d^2]$

Where, **Z**: Z statistic for a level of confidence; (95.0%, the Z value is 1.96, **P**: expected prevalence; P is considered 0.33 (33%), the prevalence of SCM (Sharma., 2015), **d**: precision (0.05)

Using this formula, calculated the sample size (n) = **340**.

Sample collection

Dairy herds were randomly selected for sample collection. The samples were collected from July 2017 to May 2019. Before sample collection, the handler's hands and udders of animals were cleaned properly and dry. The first few streams of foremilk were discarded, and then about 10 ml of milk from each quarter was collected in a sterilized bottle. They were numbered and labeled as left front (LF), left hind (LH), right front (RF), and right hind (RH). The samples were stored in a cool box and taken to the National Cattle Research Program laboratory for further analysis.

California Mastitis Test (CMT)

The samples were tested by CMT immediately after arrival at the laboratory. A total of 3 ml of the milk sample and 3 ml of the CMT reagent were kept in each of the four cups of CMT paddle from each quarter individually and the mixture was rotated in a circular motion for a few seconds. The change in milk viscosity or gel formation was recorded. The CMT results were scored as 0,1,2, 3, and 4 based on gel formation. The interpretation Score 0: Negative (no evidence of precipitation), Score 1: Slight precipitation with no tendency towards gel formation, Score 2: Weak positive (distinct precipitation with no tendency towards gel formation), Score 3: Distinct positive (mixture thickened immediately with gel formation), Score 4: Strong positive (Gel formation immediately).

Pathogen isolation and identification

The CMT-positive samples were immediately subjected to bacteriological culture. Milk samples were streaked on Nutrient agar (NA), Mac Conkey agar, Mannitol Salt agar (MSA), and Eosin Methylene Blue (EMB) agar (Hi-Media). They were incubated at 37°C for 24 hours. The culture isolates were identified and confirmed by colony characteristics, Grams' staining, and specific biochemical tests such as oxidase test, catalase test, indole test, and MR-VP test.

Antimicrobial susceptibility testing (AST)

The bacteria isolated from the infected samples were also subjected to in vitro antibiotic susceptibility testing using various antimicrobial agents by disk diffusion method (Bauer 1966). The commonly used antimicrobials gentamicin (10 μg), ampicillin (10 μg), ceftriaxone (30 μg), enrofloxacin (5 μg), and tetracycline (10 μg)) were used for the antibiotic susceptibility test.

Muller Hinton Agar (MHA) (Hi-Media, India) was used for the Antibiotic Sensitivity Test (AST). The isolated pure bacterial colonies as taken using a sterile cotton swab, streaked on MHA, and incubated at 37°C for 24 hours. The antimicrobial disks were placed widely on the surface of the MHA plate using sterile forceps. The plates were incubated overnight at 37°C. The zone of inhibition was measured and then classified as sensitive, intermediate, and resistant according to the interpretation table provided according to the CLSI standard.

Data Analysis

Chi-square statistics were used to assess the association between risk factors and the prevalence of SCM. The P-values of <0.05 were considered statistically significant. The EpiTools Epidemiological Calculators (Ausvet) were used for statistical analysis.

Results

Prevalence of subclinical mastitis

A total of 340 samples were screened for subclinical mastitis (SCM) by the California Mastitis Test (CMT). The CMT results showed that the overall prevalence of subclinical mastitis was 41.47% (141/340). The result of bacterial isolates showed that the highest prevalence was of *Staphylococcus* spp. (39.01%), followed by followed by *E. coli* (23.40%), *Streptococcus* (17.73%), and others unidentified (19.85%).

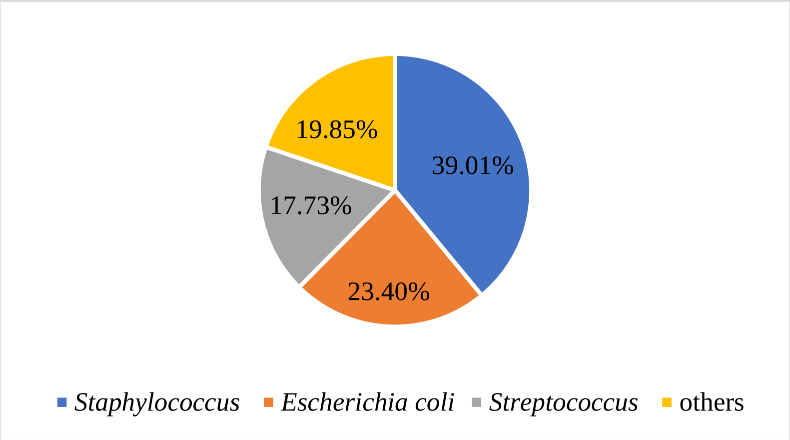


Figure 1. Prevalence of different pathogens in subclinical mastitis in dairy cattle.

The prevalence of SCM was higher in dairy cows in late lactation (43.93 %) than in dairy cows in early lactation (38.02 %). The chi-square test ($\chi 2$= 1.19; p=0.28) showed that the association between the stage of lactation and the prevalence of mastitis was not significant. Similarly, there was no significant association between the breed of the animal and the prevalence of SCM.($\chi 2$= 0.02; p=0.88). However, the prevalence of SCM was higher in Holstein cattle (46.70%) than in Jersey cattle (34.26%). The prevalence of subclinical mastitis was higher in cattle with high yields than in cattle with low yields but there was no significant association between milk yields and mastitis prevalence. ($\chi 2$= 2.07; p=0.15).The prevalence of SCM was higher in commercial farming systems (44.92 %) than in conventional (35.82%) but there was no significant association. ($\chi 2$= 2.78; p=0.09).

Table 1. Subclinical mastitis association with risk factors.

Risk Factors	**Classification**	**Positive**	**Total**	**P- Value**
Lactation (stage)	Early	54 (38.02%)	142	0.28
	Late	87 (43.93%)	198	
Breed	Jersey	60 (34.26%)	143	0.88
	Holstein	81(46.70%)	197	
Milk yield	High (>10lt)	82(45.05%)	182	0.15
	Low (<10lt)	59 (37.34%)	158	
Farming System	Conventional	48 (35.82%)	134	0.09
	Commercial	93 (44.92%)	207	

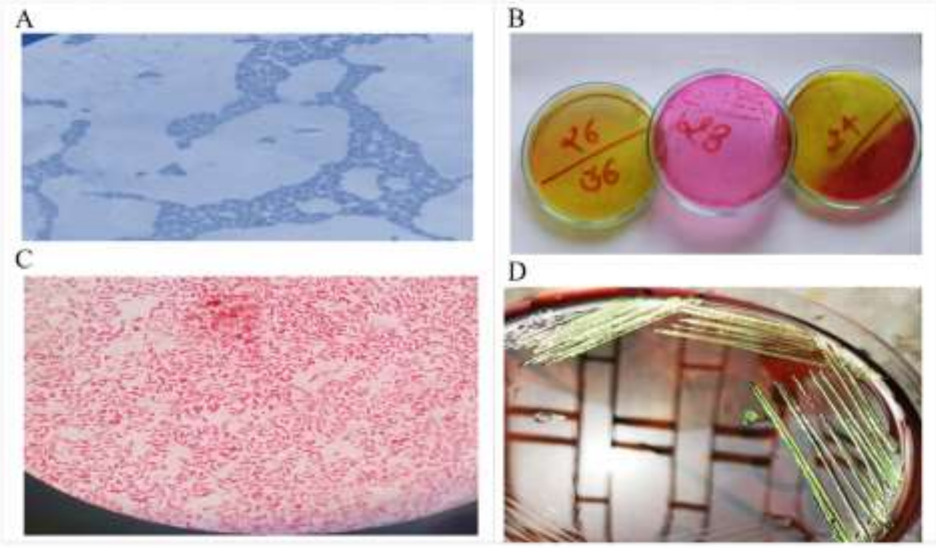


Figure 2. Gramm staining and culture photos of microorganisms in different agar media. A, Gram's staining smear of *Staphylococcus* sp.; B, *Staphylococcus* spp. cultural characteristic in MSA; C, Gram's staining smear of *Escherichia coli*; D, *E. coli* cultural characteristic in EMB;

Antibiotic sensitivity results of isolates

Antibiogram profile indicated that tetracycline(80.3%) was the most effective drug followed by ceftriaxone (70.5%), Gentamicin (59.0%), Enrofloxacin (44.3%), and ampicillin (0)was found to be the least effective antibiotics against bacterial isolates. The data on the zone of inhibitions of different antibiotics for isolates was done according to an interpretative chart (CLSI standard).

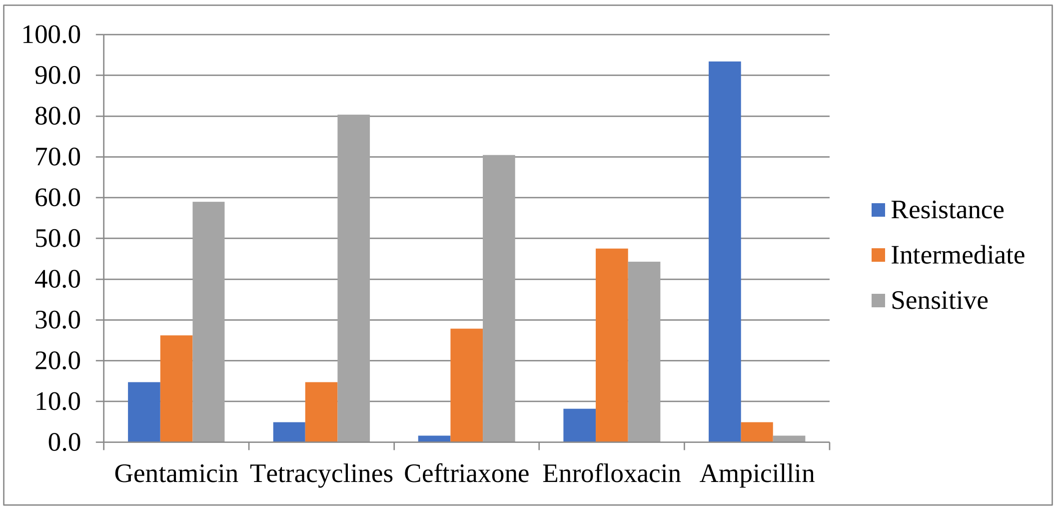


Figure 5. Antibiogram susceptibility pattern of isolates pathogens

Discussion

Subclinical mastitis (SCM) is tough to diagnose as there are no inflammatory changes in the udder and no changes are observed in the milk. However, there are many biochemical and cellular changes in the milk with subclinical infection. These changes can be easily screened by laboratory tests such as CMT and other routine tests. A study conducted by Khanal & Pandit, (2013) reported that the prevalence of subclinical mastitis was 46.1% which is like the present findings. The research performed by Shrestha & Bindari,(2012) found a 52% prevalence of SCM in Bhaktapur, and Dhakal, (2002) showed a prevalence rate of SCM of 56%, which was higher than the present findings. The differences in environmental conditions, management, and hygiene practices adopted by farmers could be the reasons.

In contrast, Dhakal & Tiwari, (1992)reported that the prevalence of SCM in Western Chitwan was 30% which was lower than our findings. Similarly, Khakural (1996) and Dhakal (2007) reported a prevalence of 17.2% and 21.7%, respectively. The difference could be due to the variability in seasons of the research and the different farming practices.

In this study, the prevalence of subclinical mastitis was higher in late lactation (43.93%) than in early lactation (38.02%), but there is no association between stage of lactation and the prevalence of mastitis, which is consistent with the findings of Khanal & Pandit, (2013). However, the contrast results reported by Dhakal et al., (2007) in which they found that mastitis was more common in early lactation than in late lactation, which contrasts. The difference could be due to the different practices and breeds of animals.

In our results, the prevalence of subclinical mastitis was higher in high-yielding cattle as compared to low-yielding which is concurrent with the results of Kader et al., (2003). They reported that the prevalence of SCM was higher in the high milk yielders (87.5%) than in the low milk yielders (33.33%). The prevalence of SCM was higher in commercial farming systems (44.92 %) than in conventional (35.82%) but there is no significant association. Our findings are similar to those of Rahman et al., (2004)and Genfors, (2018). The high prevalence in commercial farms may be due to a large number of cattle and improper hygienic conditions and the chance of disease spread is high.

In this study, the common isolated bacteria were *Staphylococcus* followed by *E. coli*, *Streptococcus*, and others. The bacterial isolates from earlier studies in Nepal by Khakural, (1996);Dhakal et al., (2007); Shrestha & Bindari, (2012) have a similar pattern. However, Dhakal and Subedi, (2002) found that Coliform mastitis was the most common type followed by Staphylococcal mastitis in Chitwan. Khanal & Pandit, (2013) reported that *Streptococcus* was the most frequent bacteria followed by Coliform and then *Staphylococcus* in the Lumjung district which is also different from our study. The difference in the prevalence of isolates may be due to the difference in sample collection time and different practices adopted by the farmers.

Antibiogram profile indicated that tetracycline was the most effective drug followed by ceftriaxone, gentamicin, and enrofloxacin. (Dhakal et al., 2007)found a higher percentage of sensitivity with the drug enrofloxacin, followed by gentamicin, tetracycline, and chloramphenicol. At this time the organism showed decreased sensitivity to enrofloxacin. The improper use of antibiotics leads to decreased effectiveness of antibiotics.

Conclusion

In the present study, the prevalence of subclinical mastitis in cattle is investigated using CMT, microbial culture techniques, and antibiogram of the common isolates. The prevalence of SCM in western Chitwan was high, and the common cause is *Staphylococcus* followed by *E. coli* and *Streptococcus* species. Subclinical mastitis is higher in the late lactation stage than in the early stage and is higher in commercial farming than in conventional farming. However, there is no significant association between breeds, stages of lactation, milk yield, farming systems, and mastitis prevalence. The AST result shows that tetracycline was the most effective drug followed by ceftriaxone, gentamicin, and enrofloxacin. Inappropriate use of drugs leads to drug resistance, so proper use of antibiotics is necessary to control antibiotic resistance problems.

Acknowledgements:

The authors would like to thank the Nepal Agricultural Research Council (NARC), Nepal, for providing the funds to conduct this research. We would like to thank all the staff of the National Cattle Research Program for their support throughout the research period. I would like to thank all the farmers who supported us during the sample collection.

References

Azooz, M. F., El-Wakeel, S. A., & Yousef, H. M. (2020). Financial and economic analyses of the impact of cattle mastitis on the profitability of Egyptian dairy farms. *Veterinary World*, *13*(9), 1750.

Cheng, W. N., & Han, S. G. (2020). Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments — A review. In *Asian-Australasian Journal of Animal Sciences* (Vol. 33, Issue 11, pp. 1699–1713). Asian-Australasian Association of Animal Production Societies. https://doi.org/10.5713/ajas.20.0156

Dalanezi, F. M., Joaquim, S. F., Guimarães, F. F., Guerra, S. T., Lopes, B. C., Schmidt, E. M. S., Cerri, R. L. A., & Langoni, H. (2020). Influence of pathogens causing clinical mastitis on reproductive variables of dairy cows. *Journal of Dairy Science*, *103*(4), 3648–3655. https://doi.org/10.3168/jds.2019-16841

Daniel, W. W. (1999). Biostatistics: A foundation for analysis in the health sciences. . *7th Edition, John Wiley & Sons, Inc., Hoboken.*

Dhakal, I. P. and S. K. (2002). Clinical Mastitis in different breeds of cattle and buffaloes at Chitwan, Nepal. *Journal of Institute of Agriculture and Animal Science.23:65-69.*

Dhakal, I. P. , & Tiwari, K. R. (1992). Epidemiological investigation of subclinical mastitis in western Chitwan. Nepal. *Vet. Review, 7, 6-10*.

Dhakal, I. P., Dhakal, P., Koshihara, T., & Nagahata, H. (2007). Epidemiological and bacteriological survey of buffalo mastitis in Nepal. *Journal of Veterinary Medical Science*, *69*(12), 1241–1245.

Genfors, E. (2018). *Udder health in dairy cows in Tajikistan–prevalence of mastitis and bacteriological findings*.

Kader, M. A., Samad, M. A., & Saha, S. (2003). Influence of host level factors on prevalence and economics of subclinical mastitis in dairy milch cows in Bangladesh. *Indian Journal of Dairy Science*, *56*(4), 235–240.

Khakural GP. (1996). Study on prevalence of subclinical mastitis in Kathmandu valley. *Proceedings of the 1st National Workshop on Livestock and Fisheries Research in Nepal, Khumaltar, Lalitpur, 7-9 May, 1996, Pp 185-188.*

Khanal, T., & Pandit, A. (2013a). Assessment of sub-clinical mastitis and its associated risk factors in dairy livestock of Lamjung, Nepal. *International Journal of Infection and Microbiology*, *2*(2), 49–54. https://doi.org/10.3126/ijim.v2i2.8322

Khanal, T., & Pandit, A. (2013b). Assessment of sub-clinical mastitis and its associated risk factors in dairy livestock of Lamjung, Nepal. *International Journal of Infection and Microbiology*, *2*(2), 49–54. https://doi.org/10.3126/ijim.v2i2.8322

Kharel, M., Timisina, K. P., Adhikari, S. P., Dhakal, C., Khanal, D. R., & Paudel, T. P. (2023). Does mastitis cause economic loss in dairy cattle in Nepal? *Nepal Agriculture Research Journal*, *15*(1), 55–65. https://doi.org/10.3126/narj.v15i1.51064

Kurt, S., & Eski, F. (2021). Pathogen isolation and antibiogram analysis in dairy cows with clinical mastitis in Adana region, Turkey. *Etlik Veteriner Mikrobiyoloji Dergisi*, *32*(1), 20–26. https://doi.org/10.35864/evmd.906990

Lamey, A., Mohamed Ammar, A., Rezk allah Zaki, E., Khairy, N., Saad Moshref, B., & Kamal Refai, M. (2013). Virulence Factors of Escherichia coli Isolated from Recurrent Cases of Clinical and Subclinical Mastitis in Buffaloes. *International Journal of Microbiological Research*, *4*(1), 86–94. https://doi.org/10.5829/idosi.ijmr.2013.4.1.71103

Melesse, E., & Minyahil, T. (2019). Prevalence of bovine mastitis, risk factors, isolation and anti-bio gram of major pathogens in Mid Rift valley, Ethiopia. *International Journal of Livestock Production*, *10*(1), 14–23. https://doi.org/10.5897/ijlp2018.0517

Rahman, M. A., Rauf, S. M. A., & Ahmed, M. U. (2004). *Prevalence of mastitis in cows as dairy and subsistence farming systems*. MS Thesis. Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensing.

Sargeant, J. M., Leslie, K. E., Shirley, J. E., Pulkrabek, B. J., & Lim, G. H. (2001). Sensitivity and specificity of somatic cell count and California Mastitis Test for identifying intramammary infection in early lactation. *Journal of Dairy Science*, *84*(9), 2018–2024. https://doi.org/10.3168/jds.S0022-0302(01)74645-0

Seegers, H., Fourichon, C., & Beaudeau, F. (2003). Production effects related to mastitis and mastitis economics in dairy cattle herds. *Veterinary Research*, *34*(5), 475–491.

Sharma, N., Rho, G. J., Hong, Y. H., Kang, T. Y., Lee, H. K., Hur, T.-Y., & Jeong, D. K. (2012). Bovine mastitis: an Asian perspective. *Asian Journal of Animal and Veterinary Advances*, *7*(6), 454–476.

Sharma P. (2015). Prevalence, anti-biogram and risk factors associated with Staphylococcus aureus in lactating dairy cattle of Chitwan, Nepal. . *(M. V. Sc. Thesis). Agriculture and Forestry University (AFU), Rampur, Nepal.*

Shrestha, A., Bhattarai, R. K., Luitel, H., Karki, S., & Basnet, H. B. (2021). Prevalence of methicillin-resistant Staphylococcus aureus and pattern of antimicrobial resistance in mastitis milk of cattle in Chitwan, Nepal. *BMC Veterinary Research*, *17*, 1–7.

Shrestha, S., & Bindari, Y. R. (2012). *International Journal of Agriculture and Biosciences Prevalence of Sub-Clinical Mastitis among Dairy Cattle in Bhaktapur District, Nepal*. www.ijagbio.com

Taponen, S., Liski, E., Heikkilä, A. M., & Pyörälä, S. (2017). Factors associated with intramammary infection in dairy cows caused by coagulase-negative staphylococci, Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Corynebacterium bovis, or Escherichia coli. *Journal of Dairy Science*, *100*(1), 493–503. https://doi.org/10.3168/jds.2016-11465