ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt
International Journal of Applied Sciences and Biotechnology
(IJASBT)

Research Article

Antibiogram Study of Bacteria Isolated from Subclinical Mastitis in Dairy Cattle of Kirtipur and Chandragiri Municipalities, Kathmandu, Nepal

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Article Information

Received: 18 June 2023
Revised version received: 06 September 2023
Accepted: 10 September 2023
Published: 29 September 2023

Cite this article as:
M. Paudel et al. (2023) Int. J. Appl. Sci. Biotechnol. Vol 11(3): 135-142. DOI: 10.3126/ijasbt.v11i3.58875

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Abstract

The dairy sector in Nepal has a high potential for growth. Unfortunately, its progress is impeded by diseases such as mastitis. The purpose of the current investigation was to identify the predominant bacterial species in mastitis affected cattle milk and to ascertain the most efficacious antibacterial treatment against the bacterial infections. The study was conducted from 17th August 2022 to 12th February 2023 in lactating cattle of the Kirtipur and Chandragiri municipalities of Kathmandu district, Nepal. A total of 640 milk samples from 160 cattle were collected randomly from cattle farms of both the municipalities. Initial screening for mastitis was conducted on collection sites by the California Mastitis Test (CMT). Further bacterial isolation and identification were carried out at the Central Veterinary Laboratory, Kathmandu using standard bacterial techniques and the antibiogram were evaluated based on CLSI guidelines. Among 640 samples collected from 160 cattle, 3.91% (25/640) tested CMT-positive at the individual quarter level, whereas the prevalence at the cow level was 15.62% (25/160). The major bacterial species isolated, in subsequent culture of CMT positive samples, were *Staphylococcus spp.* (10, 40%), E. coli (6, 24%), *Streptococcus spp.* (5, 20%), *Enterococcus spp.* (2, 8%), *Bacillus spp.* (1, 4%), and *Klebsiella spp.* (1, 4%). Antibiogram study reveals that major gram-positive bacteria such as *Staphylococcus spp.*, *Streptococcus spp.*, *Enterococcus spp.*, and *Bacillus spp.* were highly sensitive to Ampicillin, Florfenicol, and Gentamicin. On the other hand, the major gram-negative bacteria; *E. coli*, and *Klebsiella spp.* were highly sensitive to Florfenicol, Gentamicin, Trimethoprim/Sulfamethoxazole and Ceftriaxone. Early diagnosis and careful monitoring of antimicrobial susceptibility before therapeutic intervention are essential to prevent high economic losses due to mastitis and to mitigate the potential risk of antimicrobial resistance in the livestock population.

Keywords: Subclinical mastitis; Dairy cattle; California mastitis test; Antibiogram; Kathmandu

Introduction

Nepal is predominantly an agriculture and livestock-based country, where approximately 66% of the population is engaged in the agricultural sector, contributing to one-third of the nation's GDP. Notably, livestock plays a significant role, contributing to 32% of the agricultural GDP and 11.17% of the total country's GDP (LIVESTOCK STATISTICS OF NEPAL, 2022). The contribution of cattle and buffaloes to the livestock GDP is crucial, and the contribution of milk is at the top, which alone shares 11.05% of the total AGDP (LIVESTOCK STATISTICS OF NEPAL, 2022). Cattle is one of the major livestock commodities reared with an estimated population of 7466841 contributing 1060487 metric tons of milk per annum (LIVESTOCK STATISTICS OF NEPAL, 2022). Despite the figures, milk production frequently fails to meet the nation's demand owing to various factors, among which mastitis occurrence holds a prominent position. Mastitis-related costs encompass reduced milk yield, milk condemnation owing to antibiotic residues, veterinary expanses, the removal of chronically infected cows, and sporadic fatalities (Lamey *et al*., 2013).

Burangohain and Dutta (1994) have reported a 30 to 50 percent decrease in milk production from the affected quarter and 15 percent from an affected cow. Additionally, mastitis poses a significant zoonotic risk through the excretion of bacteria and their toxins in milk. (González and Wilson, 2003). Bovine mastitis is characterized by an inflammatory response in the mammary gland tissue, triggered by either physical trauma or microbial infections (Gomes and Henriques, 2016). The extent of inflammation and clinical characteristics distinguish bovine mastitis into three categories: clinical, sub-clinical, and chronic mastitis (Khan and Khan, 2006). Clinical mastitis is further classified as per-acute, acute, and sub-acute based on the severity of the inflammation (Kibebew, 2017). Subclinical mastitis (SCM) is marked by physical, chemical, bacteriological, and cytological alterations in milk, as well as pathological changes in the mammary gland, and it is reported to be 15-40 times more prevalent than the clinical form of mastitis (Abd-Elrahman, 2013). Mastitis results from a diverse range of pathogens and is categorized based on epidemiological factors into contagious and environmental mastitis (Cervinkova *et al*., 2013). The primary reservoir for contagious pathogens is the udders of infected cows, and they are primarily transmitted from cow to cow during milking. These pathogens often result in chronic sub-clinical infections, with periodic outbreaks of clinical episodes. Examples of contagious pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma spp*., and *Corynebacterium bovis* (Radostits *et al*., 2006). Similarly, environmental mastitis refers to intra-mammary infections caused by pathogens that primarily originate from the environment in which cows are housed (Smith *et al*., 2001). Environmental pathogens, such as *E. coli*, *Klebsiella spp.*, *Streptococcus dysgalactiae, and Streptococcus uberis*, primarily cause sub-clinical and then clinical infections of shorter durations (Harmon, 1994).

Mastitis not only accounts for 70% of economic losses and is a significant impediment to milk production but also poses a zoonotic risk for the transmission of major diseases such as tuberculosis, brucellosis, leptospirosis, and streptococcal sore throat to humans, as noted by (Bachaya *et al*., 2011; Birhanu *et al*., 2017). The economic impact, effect on animal productivity, implications for international trade, and concerns regarding animal welfare associated with mastitis highlight its significant importance to the agricultural industry (Thomson, 2000). Therefore, mastitis has become a critical area of focus in the field of veterinary clinical practice globally, necessitating the development of rapid, sensitive, and cost-effective diagnostic tools to identify cases of intramammary infection.

Although the specific pathogens responsible for subclinical mastitis (SCM) may differ across countries and studies, the frequently identified microorganisms include *Staphylococcus aureus*, *Staphylococcus intermedius*, *Staphylococcus hyicus*, *Streptococcus spp.*, *Escherichia coli*, *Klebsiella spp.*, and *Corynebacterium spp.*(Shrestha and Bindari, 2012; Khanal and Pandit, 2013; Gaire and Karki, 2016; Birhanu *et al*., 2017). In recent years, multidrug-resistant (MDR) E. coli strains have been detected in cases of SCM, potentially endangering public health when unpasteurized milk is ingested (Ombarak *et al*., 2019). Therefore, the objective of this study was to determine the prevalence of mastitis among lactating cattle, identify the major causative agent, and provide insights for the development of effective mastitis control strategies in the Kathmandu area.

Materials and Methods

Site of Study

This study was conducted in the Kirtipur municipality and Chandragiri municipality area of Kathmandu district, which is regarded as the pocket sites of milk production. The samples collected were subjected to laboratory analysis at the Central Veterinary Laboratory (CVL), which is located in Tripureshwor, Kathmandu, Nepal.

Site Profile

Situated at an elevation of 1300 meters above sea level, Kathmandu district is located in the mid-hill region of the country and is known for its fertile soil. With an area spanning 395 square kilometers, it is positioned between 27° 27' North and 27° 49' North latitude, and 85°10' East and 85°32' East longitude, as per CBS 2006 records. (Fig. 1).

Sample Size Determination

A total of 640 quarters milk samples were screened for the CMT from 160 dairy cattle. Samples were collected from each farm using a probability-based proportional method, and animals were selected systematically using random sampling techniques. Those animals presently undergoing antibiotic treatment were excluded in this study. And the CMT-positive samples were subjected to a bacterial culture, gram staining, and Antimicrobial Sensitivity Test.

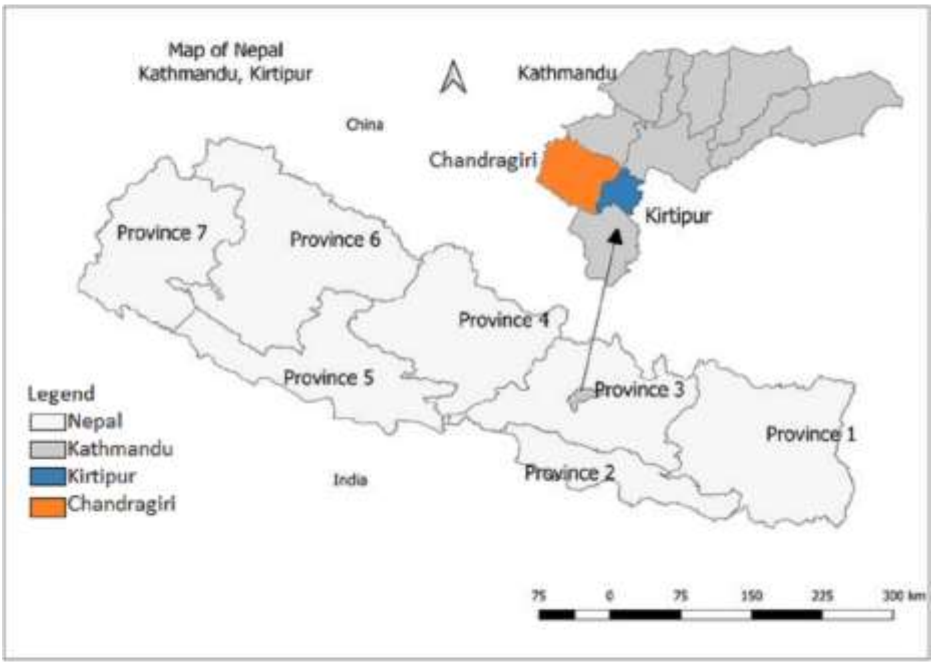

Fig. 1: Map of Nepal, the Kathmandu district, and the area where the samples were collected.

Data Collection by Questionnaire Survey

A questionnaire was designed to collect detail information from the farmer/farm owners. The questionnaire was focused on some possible determinants of subclinical mastitis (SMC) such as previous exposure to clinical mastitis, parity, breed, status of farm, management practices etc. In order to evaluate the potential impact of management practices on SCM, the questions were asked to each farmer during the sample collection and information regarding individual cattle characteristics, their production history and other relevant details were recorded.

Sampling Technique and Processing of Sample

5 ml of milk samples from each quarter of an individual cattle were collected and tested for subclinical mastitis by CMT. The CMT positive samples were subsequently transported to the Central Veterinary Laboratory (CVL) in a cool box for further testing. All the transported samples were collected in a sterilized, screw-capped test tube following an aseptic procedure with correction labelling of and the positions viz., left front (LF), left hind (LH), right front (RF), and right hind (RH) for each cattle sample., The collected samples were then cultured aerobically on sheep blood agar, nutrient agar, and McConkey's agar for a period of 24-96 hours at 37℃. Individual colonies were sub-cultured as indicated by (Alaidarous *et al*., 2017). The isolated microorganisms were identified by using gram staining and the biochemical testing's (Versalovic and American Society for Microbiology, 2011). The isolated bacteria were cultured on Mueller Hinton agar and subjected to antibiotic sensitivity testing using 14 different antibiotics through the disc diffusion method (Bauer *et al*., 1966) as per Clinical Laboratory Standard Institute (CLSI) guidelines. The antibiotics used for sensitivity testing were Gentamicin(10mg), Ampicillin(10mg), Trimethoprim/Sulfamethoxazole (7.25/23.75)mg, Tetracycline(30mg), Levofloxacin (5mg), Florfenicol (30mg), Ceftriaxone (30 mg) In accordance with the guidelines of the (Clinical and Laboratory Standards Institute, 2023), the inhibition zones of these antibiotics were measured and recorded as sensitive, intermediate, or resistant.

Data Analysis

Data analysis was done by using a Microsoft Excel worksheet. Percentages were used to express the prevalence and the proportion of the isolates of the different genera of bacteria causing mastitis in the area of this study.

Results

Altogether, 640 samples were collected from each quarter of 160 dairy cattle. Out of 640 samples, 25 (3.91%) were found to be positive in the California Mastitis Test (CMT) as described in Table. 1.

Table 1: Results of CMT

Result of CMT	Occurrence rate
CMT Positive (n=25)	3.91%
CMT Negative (n=615)	96.09%
Total sample	640

All the CMT-positive samples (n= 25) were subjected to bacterial culture for bacterial identification and isolation. Among the isolated bacteria, 72% (n=18) were gram +ve while 28% (n=7) were gram –ve. *Staphylococcus spp., E. coli, and Streptococcus spp. were* the major bacteria isolated from milk samples. Table. 2 below presents the number of bacterial isolates that were obtained in milk microbial culture.

Table 2: Bacterial species isolated from milk samples of Kirtipur and Chandragiri municipalities.

Bacterial species	Number (n), (%)
Staphylococcus spp.	10, (40%)
E. coli	6, (24%)
Streptococcus spp.	5, (20%)
Enterococcus spp.	2, (8%)
Bacillus spp.	1, (4%)
Klebseilla spp.	1, (4%)
Grand Total	25, (100%)

Antimicrobial Susceptibility Test (AST)

Antimicrobial susceptibility test reveals that *Staphylococcus spp*.(n=10) were found to be highly sensitive to Ampicillin and Florfenicol (n=8), followed by Gentamicin (n=7), Trimethoprim/Sulfamethoxazole (n=6), Levofloxacin (n=6). Ceftriaxone was found to have the very least sensitivity against *Staphylococcus spp.*, (Fig.2). *E. coli* isolated (n=6) were found to be highly sensitive to Florfenicol (n=5), followed by Gentamicin (n=4), Trimethoprim/Sulfamethoxazole (n=4), and Ceftriaxone (n=4), while Ampicillin followed by Tetracycline and Levofloxacin, were found to be very least sensitive as depicted in (Fig.3). Against *Streptococcus spp*, the average sensitivity of Ampicillin, Tetracycline, Florfenicol, and ceftriaxone, were higher than other antimicrobials as depicted in (fig.4). Against *Enterococcus spp*, the average sensitivity of Gentamicin, Ampicillin, Trimethoprim, and Florfenicol were higher than other antimicrobials as depicted in (Fig.5).

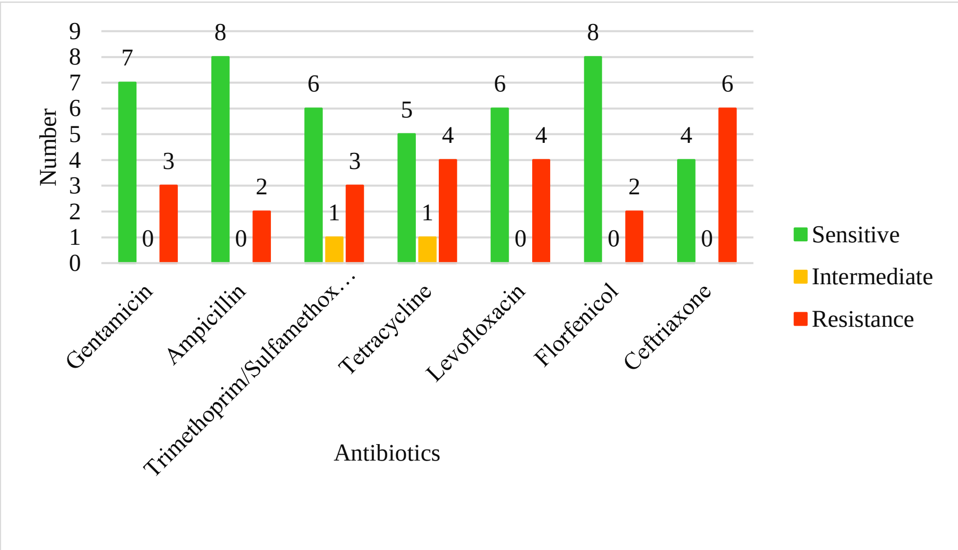

Fig. 2: Bar Graph representing Antimicrobial susceptibility pattern against *Staphylococcus spp.*

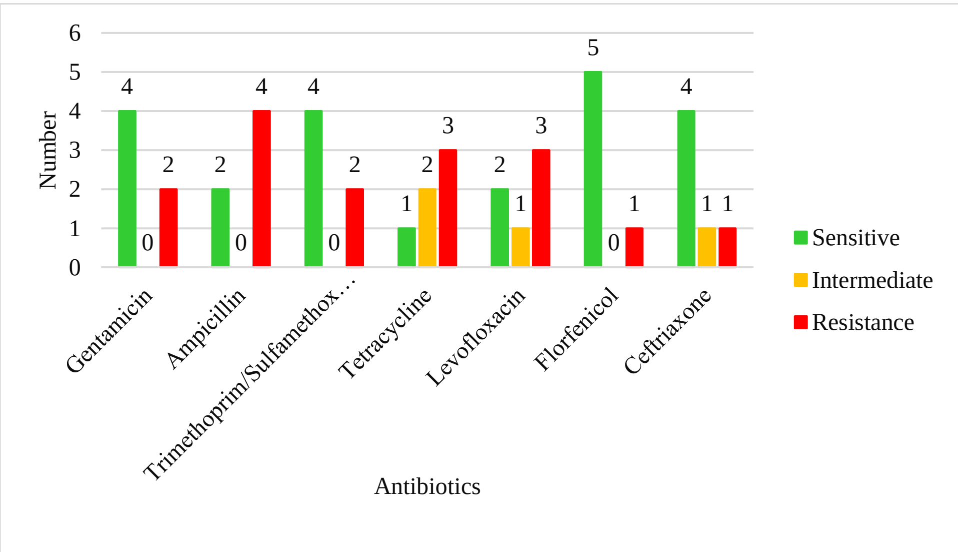

Fig. 3: Bar Graph representing Antimicrobial Susceptibility pattern against *E. coli.*

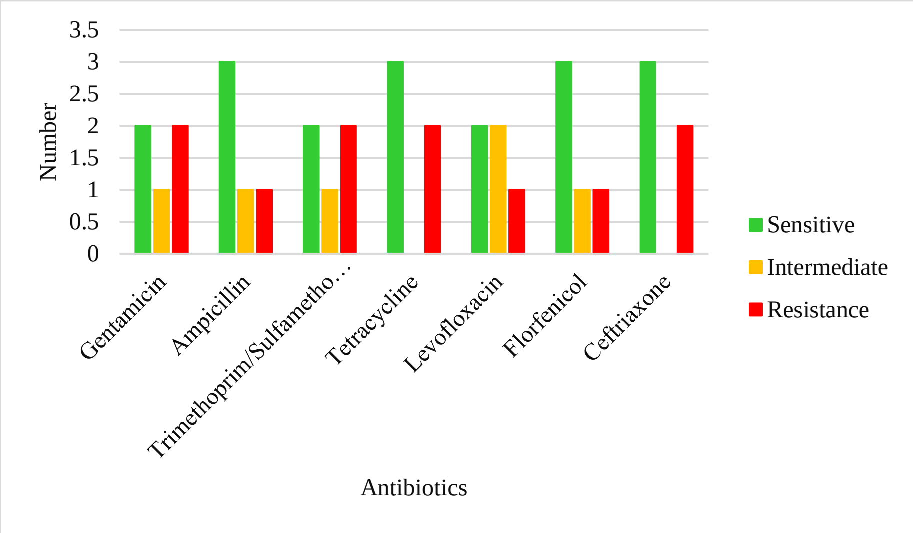

Fig. 4: Bar Graph representing Antimicrobial Susceptibility pattern against *Streptococcus spp.*

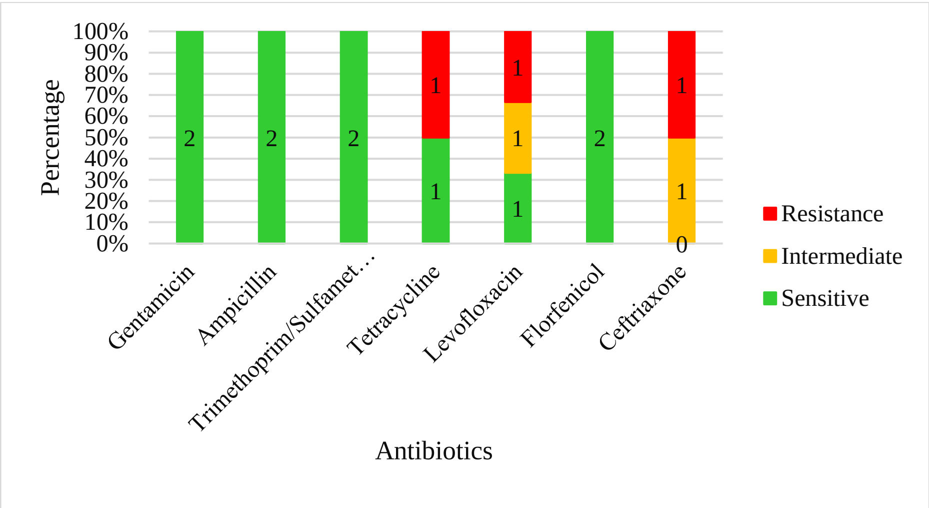

Fig. 5: Bar Graph representing Antimicrobial Susceptibility pattern *against Enterococcus spp.*

Discussion

The findings of this study provide useful insights into clinical and subclinical mastitis. The prevalence of subclinical mastitis at the individual quarter level was 25/ 640 (3.91%) and the cow level prevalence was 25/160 (15.62%) while the Khakural (1996) reported the prevalence rate of 17.2% in Kathmandu Valley by CMT test which was more than our findings. The decreased prevalence of mastitis might be attributed to increased farmer knowledge and consciousness regarding the cleanliness of the farm floor, a major risk factor for mastitis. The present study revealed that *Staphylococcus spp.* (40%), *E. coli* (24%), and *Streptococcus spp.* (20%) were the major pathogens responsible for mastitis infections in cattle. These findings align closely with the results of a previous study conducted by Tenhagen *et al*. (2006; Kateete *et al*. (2013; Khanal and Pandit (2013; Boireau *et al*. (2018), where they also identified *Streptococcus spp.*, *E.coli*, and *Staphylococcus spp*. as the predominant pathogens. In the context of Nepal, Shrestha and Bindari (2012) studied 200 milk samples from 50 dairy cows in the Bhaktapur district and found 52% of animals with subclinical mastitis with *Staphylococcus spp* as the most prevalent one. Similarly Khanal and Pandit, (2013) detect 46.1% of the sample positive for SCM at Chandreshwor and Archalbot VDCs of Lamjung district which on cultured found that 11.1% was streptococcal mastitis, 9.5% was coliform mastitis and 7.9% was *Staphylococcal* mastitis. A study conducted by Gaire and Karki (2016) revealed that 63.3% of the tested cattle in Madhyapur Thimi Municipality and Balkot of Bhaktapur were diagnosed with subclinical mastitis. According to the current study, 72% of the 25 isolated bacteria were gram-positive, while the remaining were gram-negative. This result is consistent with previous research by Langerhuus *et al*. (2013), who reported that gram-positive strains were predominant in cases of mastitis in dairy cattle. (Dhungel *et al*., 2019) revealed that the presence of *Staphylococcus spp.* in milk samples could be an indicator of inadequate farm sanitation and improper hygiene practices among farmers. Here, in this case, the situation might match. Similarly, the isolation of *E coli* in milk might be due to fecal contaminations in the udder or the water used for the milking (Hinthong *et al*., 2017; Dhungel *et al*., 2019). Present research align with the finding of Sorge *et al*. (2021) where they insists that *Streptococcus spp.* and the *Enterococcus spp*., are closely related species that may be isolated in milk . Though *Bacillus* was isolated from the milk samples but we could not confirm that they were either normal flora of the milk or environmental contamination.

One potential mechanism contributing to the resistance of *Staphylococcus spp.* to tetracycline could involve two mechanisms: (i) Active efflux occurs due to the acquisition of plasmid-borne genes, namely tetK14–16 and tetL, and (ii) ribosomal protection is facilitated through transposon-located or chromosomal tetM or tetO determinants (Nesin *et al*., 1990; Schwarz *et al*., 1998). While resistance to aminoglycosides like gentamycin might arise from (i) a reduction in drug uptake and/or its accumulation within bacteria, and (ii) the expression of bacterial enzymes that modify the antibiotic, rendering it inactive (Shaw *et al*., 1993; Davies and Wright, 1997).The current study reveals a notable degree of antimicrobial resistance (AMR) to Ceftriaxone, suggesting a potential prevalence of β-lactamase enzyme-producing *S. aureus* strains in Nepal (Shrestha *et al*., 2021); however, it's crucial to highlight the absence of molecular investigations into the precise characteristics and resistance mechanisms of these strains.

Present research revealed that Ampicillin and Florfenicol are two antimicrobial agents that are effective against *Staphylococcus spp*. These results are consistent with the findings of Müller-Premru *et al*. (2005) and Kehrenberg and Schwarz (2006), which also reported a significant efficacy of Ampicillin and Florfenicol, respectively, against methicillin-susceptible Staphylococcus aureus (MSSA) strains. Gentamicin, Trimethoprim/ Sulfamethoxazole, and Levofloxacin are also commonly used antimicrobial agents for the treatment of *Staphylococcus spp.* infections. Studies have shown varying levels of susceptibility to these agents among different *Staphylococcus spp.* strains and in different geographic regions (Al-Talib *et al*., 2015) .

In this study, we found out that *E. coli* isolated were found to be highly sensitive to Florfenicol, followed by Gentamicin, Trimethoprim, and Ceftriaxone while Ampicillin followed by Tetracycline and Levofloxacin were found to be very least sensitive. Comparing with our finding Kahlmeter, (2003) found that *E. coli* isolates from human urinary tract infections were highly susceptible to Nitrofurantoin, Fosfomycin, and Mecillinam, while susceptibility to Trimethoprim/Sulfamethoxazole, Ciprofloxacin, and Gentamicin varied. Consistence with our finding, other studies on *E. coli* by Xiao *et al*. (2022). Paudel *et al*. (2023) showed high susceptibility to Imipenem and Amikacin, but lower susceptibility to Ampicillin, and Levofloxacin. The presence of resistance bacteria to these antibiotics is concerning, as these drugs are considered critical for treating drug-resistant pathogens in humans, as per the WHO (2017). Therefore, the occurrence of such resistance bacteria in milk in Nepal, where unpasteurized milk is consumed, presents a significant public health risk.

Conclusion and Recommendation

The current research investigated the prevalence of mastitis in cattle by CMT and microbial culture techniques, and evaluate the antibiotic sensitivity patterns in common bacterial isolates. In field settings, CMT was discovered to be a cost-effective and convenient diagnostic method for mastitis. Present study revealed mastitis be a significant economic burden for dairy farmers in the region, with an overall prevalence of 3.91% recorded in cattle. The most commonly associated bacteria with mastitis were *Staphylococcus spp., E. coli, and Streptococcus spp*. Antibiotic susceptibility testing showed high sensitivity of gram-positive bacteria (*Staphylococcus spp., Streptococcus spp., Enterococcus spp., and Bacillus spp.*) to Ampicillin, Florophenicol, and Gentamicin, and of gram-negative bacteria (*E. coli and Klebsiella spp.*) to Florophenicol, Gentamicin, Trimethoprim/Sulfamethoxazole, and Ceftriaxone. The resistance of bacteria to certain antibiotics presents a public health hazard to consumers of fresh milk. Therefore, there is a pressing requirement to mandate milk pasteurization for farmers before distribution and consumption. The prevalence of subclinical mastitis was higher than that of clinical mastitis, highlighting the importance of regular screening at the farmer's level and the imperative need for appropriate treatment following antibiotic sensitivity testing. Hence, it is recommended to conduct awareness programs and provide training to farm owners regarding the proper and timely screening of subclinical mastitis, as well as farm sanitation, to mitigate the significant economic losses associated with mastitis. This finding might be helpful on understanding the distribution of pathogenic bacteria and choosing effective antibiotics in studied area.

Authors' Contribution

M. Paudel & B. Bohara designed the research plan; M. Paudel, B. Bohara & T.R. Gompo performed experimental works, collected the required data & analysed the data; M. Paudel & B. Bohara, prepared the manuscript., T. Prasai & S. Bhattarai, critically revised and finalized the manuscript. Final form of manuscript was approved by all authors.

Conflict of Interest

The authors declare that there are no conflict of interest.

Acknowledgements

The authors sincerely thank the directors of Central Referral Veterinary Hospital (CRVH), and the Central Veterinary Laboratory (CVL), Kathmandu, Nepal for providing the laboratory facilities to carry out the research work. We are grateful to all the respondents and helping hands from Kirtipur, and Chandragiri municipalities of Kathmandu district. We are also thankful to Dr. Binita Tamang, Dr. Kham Bahadur Khatri, Dr. Sandesh Chapagain, and for their support in this research.

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