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# Prevalence of subclinical mastitis and antibiogram of *Escherichia coli* in cow milk of western Chitawan

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**Abstract**
Mastitis, a management-related disease affecting cow production efficiency, was studied in 90 dairy cattle in Western Chitwan. Subclinical mastitis (SCM) prevalence was determined using the CMT test, with 31.09% (111 out of 357) of milk samples testing positive. No significant difference was found in quarter-wise prevalences of SCM. Commercial farms had a higher SCM prevalence (39%) than conventional farms, a statistically significant finding. The CMT test demonstrated a sensitivity of 95% in identifying SCM. Bacteriological culture and biochemical tests revealed *E. coli* in 16.25% (18 out of 106) of samples. Among the antibiotics tested on Muller-Hilton Agar using CLSI 2012, Ciprofloxacin, Norfloxacin, and Tetracycline were most effective, while Amoxyclav was completely resistant. Furthermore, 83.33% of isolates displayed a Multiple Antibiotic Resistance (MAR) index exceeding 0.2, indicating the need for rational antibiotic use. The study highlights a concerning SCM prevalence of 31.09% in Chitwan's dairy cattle, with 16.98% of SCM cases attributed to *E. coli*. These findings indicate emerging management issues affecting animal health and economic losses. The study emphasizes the importance of farm and personal hygiene to mitigate the risk of *E. coli* infection. Additionally, prudent antibiotic use and public awareness are crucial to control the unregulated antibiotic usage. Overall, the study underscores the significance of managing mastitis in dairy cattle through effective practices and preventive measures. Reducing SCM prevalence can enhance production efficiency and economic outcomes for farmers in the region.

**Keywords:** Subclinical mastitis, CMT, *E. coli*

## Introduction
Nepal, a developing country heavily reliant on agriculture, has approximately 65.6% of its population engaged in this sector. Agriculture contributes about 32% to the total GDP, with livestock playing a vital role. Traditionally, livestock farming in Nepal was predominantly sustainable, but commercialization has gradually modified this approach. The rise of commercial cattle farms in Chitwan has been notable, with 365 registered farms and several others seeking registration. Despite the economic importance of livestock, farmers often face challenges due to poor management practices and hygiene, resulting in reduced productivity. Mastitis, a prevalent disease worldwide, significantly affects dairy animal production and health, leading to decreased milk yield and higher somatic cell counts. Mastitis can be classified as clinical and subclinical.

## Subclinical mastitis in cattle
Mastitis affects cattle and is brought on by a variety of infectious agents, which are typically categorized into those that cause contagious mastitis, which are spread from infected quarters to other quarters and cows, those that are common inhabitants of the teat skin and cause opportunistic mastitis, and those that cause environmental mastitis, which are typically found in the cow's environment and enter the teat from that source (Radostits, 2006) [30]. The Gram-negative bacteria *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *A. pyogenes* are among the environmental coliforms by Corbirka *et al*, 2020. Some pathogens involved in mastitis are Contagious like *Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma bovis,* and *Corynebacterium bovis* some are teat skin opportunistic pathogens *coagulase-negative*

*staphylococci*, furthermore, environmental pathogens are environmental *Streptococcus* spp., including *Streptococcus uberis* and *Streptococcus dysgalactiae*, which are the most prevalent; less prevalent is *Streptococcus equinus* (formerly referred to as *Streptococcus bovis*) (Radostits, 2006) [30]. Environmental coliforms include the Gram-negative bacteria *Escherichia coli, Klebsiella* spp., *Enterobacter* spp., and *Arcanobacterium* (formerly *Actinomyces) pyogenes* (Radostits, 2006) [30].

**Uncommon pathogens:** many, including *Nocardia* spp., *Pasteurella* spp., *Mycobacterium bovis, Bacillus cereus, Pseudomonas* spp., *Serratia marcescens, Citrobacter* spp., anaerobic bacteria species, fungi, and yeasts (Radostits, 2006) [30]. A total of about 140 microbial species, subspecies, and serovars have been isolated from the bovine mammary gland (Radostits, 2006) [30]. Detecting subclinical mastitis requires special diagnostic tests since there are no visible milk abnormalities. The somatic cell count (SCC) is commonly used to identify inflammatory changes, with a higher SCC indicating greater tissue inflammation. And bacteriological culture can also identify the pathogens involved in the development of subclinical mastitis (Chakrabarti, 2012) [5]. This problem is worldwide. Etiologies for mastitis are like bacteria, mycoplasma, fungus, and viruses, but among them, bacteriological origins are the most common. The sources of infection included an infected cow, contaminated bedding and manure, vaginal and uterine infections, the milker's hand, a suckling calf, the milking machine, and house flies (Chakrabarti, 2012) [5]. Many articles have claimed that the worldwide prevalence of SCM is high and is a major concern regarding the prevention of mastitis. If we can treat or decrease the incidence of SCM, then we can minimize the risk of mastitis. Many studies have been done in different countries to calculate the prevalence of SCM, and some of the findings are: in Chitwan, the prevalence of SCM was found to be 33.33% (Sharma, 2015) [42]. An Epidemiological investigation of subclinical bovine mastitis in western Chitwan, Nepal, by Dhakal (1993) [10] has found a 30% prevalence of SCM in cattle. Khakural (1996) [23] found 17.2% of SCM in the Kathmandu Valley. Shrestha and Bindari (2012) [45] analyzed 200 milk samples collected from 50 dairy cows in Bhaktapur. They found 52% of animals suffering from subclinical mastitis. A study was conducted by Sudhan (2005) [41] to determine the prevalence of sub-clinical mastitis and the pathogen associated with sub-clinical mastitis in India. His findings suggest that the prevalence of SCM is 14.43% in cattle.

### *E. coli* in cattle with SCM

*E. coli* is responsible for the development of coliform infections, both clinical and subclinical. They are generally found in bedding, manure, and the digestive tract and cause environmental mastitis. Many studies have been done to find out the prevalence of *E. coli* in SCM. Shrestha and Bindari (2012) [45] analyzed 200 milk samples collected from 50 dairy cows in Bhaktapur. They isolated 10% *E. coli* from the total bacterial growth of a subclinical mastitis-positive sample. A similar study done by Hamal (2016) found that 6.89% of the *E. coli* in the sample was positive for SCM found 1.72% *E. coli* in an SCM-positive sample. And this research was done on organized farms only. A study conducted by Hameed *et al*. (2008) [15] in Pakistan to study microorganisms associated with mastitis in cattle found *E. coli* (16%).

### Pathogenesis of *E. coli* mastitis

Coliform bacteria, such as *Escherichia coli*, *Enterobacter aerogenes, Klebsiella pneumoniae*, and *Serratia marcescens*, are common pathogens responsible for causing mastitis. These bacteria are natural inhabitants of soil, digestive tracts, manure, and bedding materials. Contaminated bedding, with coliform numbers reaching 1,000,000 or more per gram, increases the likelihood of udder infections and clinical mastitis. When the ends of the teat come into contact with coliform bacteria, they enter the udder through the teat sphincter. Coliform bacteria have the ability to either quickly develop inside the mammary gland or stay dormant. Coliforms emit endotoxins into the body of the cow as the immune system tries to eliminate them. These endotoxins alter the permeability of the blood vessels, causing edema and acute enlargement of the gland as well as a considerable rise in neutrophil levels in the milk. The number of neutrophils may rise by 40–250 times, effectively reducing *E. coli*'s ability to survive. This excessive migration of neutrophils is linked to the pronounced systemic leukopenia and neutropenia seen in cases of peracute coliform mastitis (Radostits, 2006) [30]. Clinical signs of coliform mastitis are primarily caused by the endotoxins produced by the bacteria. Infected cows display a high fever, a depressed appetite, rapid weight loss, abnormal milk production, and decreased milk production. Seasonal trends, such as hot temperatures, plenty of rain, and erratic weather, frequently lead to the development of new clinical illnesses. Early in lactation, severe instances are more frequent in older, high-yielding cows. Coliform bacteria are pervasive in the environment and have variable degrees of impact on all dairy herds. Although they account for fewer than 5% of all infected quarters in a herd at any given time, they are the primary cause of a high percentage of acute clinical cases. In some cases, the release of sufficient endotoxin can lead to seriously ill cows and even death. Coliform bacteria cause numerous cases of acute clinical mastitis in dairy cows. High temperature, udder inflammation, decreased appetite, dehydration, diarrhea, decreased milk output, and abnormal milk are among symptoms of affected cows. Although the milk may appear watery and have clots, these characteristics may not always point to a particular mastitis infection. Typically, only one quarter of cows are clinically infected, although coliforms can also cause persistent subclinical infections. Treating these infections is generally not effective, as the majorities are eliminated by the cow's immune system.

### Antibiotic sensitivity test for *E. coli*

It has been debatable how to treat coliform mastitis in cattle because the endotoxin lipopolysaccharide endotoxin, which is released by *E. coli* when it is killed by the cow's immune system, is what causes the majority of the clinical symptoms to appear. Antibiotics act to kill the bacteria, and in this case, these infections would result in the production of endotoxin, which is fatal to cow health (Petersson-Wolfe, 2011) [29]. But sometimes the infection becomes systemic and severe; in such cases, the administration of antibiotics through the perenteral route, followed by an intramuscular infusion, fluid therapy, and electrolyte therapy, is recommended (Radostits, 2006) [30]. Unregulated uses of antibiotics lead to the development of *Extended Spectrum beta-lactamase* enzymes producing *E. coli* (ESBL in *E. coli*) and multiple antibiotic-resistant strains of *E. coli*. Hence, proper drug selection and antibiotic sensitivity testing should be performed before prescribing antibiotics. In a study done by Chandrasekaran *et al*. (2013) [8]

on the treatment of resistant mastitis in dairy cows, 86.65% of isolated *E. coli* were found to be resistant, i.e., resistant to 1 or 2 antimicrobials, and only a few *E. coli* isolates (13.45%) were found to be multi-drug resistant, i.e., resistant to 3 or more antimicrobials. Antibiotics have been beneficial in enhancing growth, performance, and treating ailments in the dairy industry. However, treatment is often administered only after cows exhibit clinical signs without early screening tests or proper diagnosis, leading to haphazard antibiotic use. Subclinical mastitis is difficult to detect due to the absence of any visible indications and has major cost implications associated with decreased milk. In India, the annual economic loss to the dairy industry due to subclinical mastitis is estimated to be Rs. 43653 million. The incidence of coliform mastitis has increased since serious efforts have been made to eliminate *Staphylococcus aureus* and *Streptococcus sp.* mastitis (Stalberger, 1988). Recently, due to the unregulated use of various antimicrobial agents without AST, antibiotic resistance strains of pathogens have been developed, like MRSA (11.25% according to Joshi, 2012) and ESBL-producing *E. coli* (43% according to Sascha, 2012). And these strains are transmitted to humans through the ingestion of infected milk and have public health concerns.

**Objectives**

- To identify subclinical mastitis using an indirect test, i.e., the California Mastitis Test
- **Determine** the sensitivity of the California mastitis test
- **Isolate** and **phenotypically** identify coli from cattle with subclinical mastitis.
- Antibiotic sensitivity test to find out the choice of drugs against *E. coli*.

**Methods And Materials**

The research was carried out in Geetanagar, Rampur, and Shardanagar of Chitwan district. All these areas were in similar condition and contained significant cattle populations in both conventional and commercial farming systems. A cross-sectional study was conducted in the study area for the determination of the prevalence of subclinical mastitis and the antibiogram of *E. coli* in cow milk from September, 2017 to December 2017. The cattle milk under study was basically from conventional and commercial farms. A farm having more than 10 livestock units was defined as a commercial farm, and up to 10 large cattle units are considered conventional farms (DLS, 2013). 357 samples from animals of different farms were taken. Among 90 cattle, 50 cattle from commercial farm and remaining from individual farm were selected purposively. And all the microbiological lab works were done in National Cattle Research Program microbiology lab. After being swabbed with 70% ethyl alcohol, the teats were allowed to air dry. The initial milk streaks were thrown away. After that, milk was extracted aseptically into sterile vials marked fore, hind, right, and left. Within an hour, the samples were used in the lab. Utilizing a subclinical mastitis detector and comparing milk results with the subclinical mastitis reference range were required for subclinical mastitis detection. For the purpose of detecting subclinical mastitis, a California mastitis test was also carried out.

**Microbiological analysis of sample**

**Culture of milk sample:** The infected milk samples were streaked on nutrient agar and MacConkey agar, followed by overnight incubation at 37°C. Petri plates with no microbial growth after incubation were further incubated for 48 hours. Gram staining of colonies on nutrient agar was performed to identify gram-negative bacteria. The gram-negative bacteria exhibiting grayish-white colonies on nutrient agar and rose-pink colonies on MacConkey agar were subcultured on EMB agar at 37°C for 24 hours. Biochemical tests, including the indole test, the methyl red test, the Voges-Proskauer test, the citrate utilization test, and the oxidase test, were conducted using the gram-negative colonies from nutrient agar. The identification of *E. coli* was based on the following characteristics: large, smooth, opaque, or partially translucent moist greyish-white colonies on nutrient agar; rose-pink colonies on MacConkey agar; metallic sheen seen on EMB agar; positive indole and methyl red tests; negative Voges-Proskauer test; citrate test; and oxidase test.

**Antibiotic sensitivity test**

By Kirby-Bauer disc diffusion method using Mueller-Hinton agar plate following guidelines provided by the CLSI (2012). Briefly, 0.5 McFarland of bacterial suspension was inoculated on Muller Hilton Agar and following disk were placed: Gentamycin (10 mg), Ciprofloxacin (5 mg), Norfloxacin (10 mg), Tetracycline (30mg), Cefotoxime (30 mg) and Amoxyclav (30 mg)

**Data analysis**

- Data analysis was done using SPSS version 16.0.
- The association between different variables was analyzed using the Chi square test at a 5% level of significance.
- The MAR index was calculated from the AST data. The MAR index of an isolate is defined as a/b, where 'a' represents the number of antibiotics to which the isolate was resistant and 'b' represents the number of antibiotics to which the isolate was subjected (Jayaraman, Manoharan, Ilanchezian.

**Results**

**Prevalence of Subclinical Mastitis**

Early screening tests were done to identify subclinical mastitis, and out of 357 milk samples, 111 were identified as SCM. Among the 357 samples, 246 (68.90%) showed CMT negativity. 69 (19.32%) showed mild positive (+) and 42 (11.76%) showed strong positive (++). CMT in at least one quarter, but without clinical symptoms, was classified as SCM-positive. So the quarter-wise prevalence of SCM was found to be 31.09%. This has been illustrated in Figure 5.

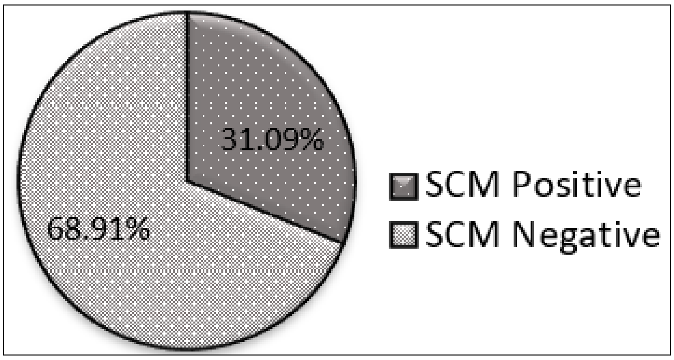


**Fig 1:** Prevalence of SCM in cow milk

### Quarter-wise prevalence of SCM

The prevalence of SCM in the left front, right front, left hind, and right hind was found to be 34.44%, 26.14%, 33.33%, and 30.34%, respectively. The highest prevalence was found in the left half. There was no significant difference in the quarter-wise prevalence of SCM (P< 0.05). This has been illustrated in Figure 2.

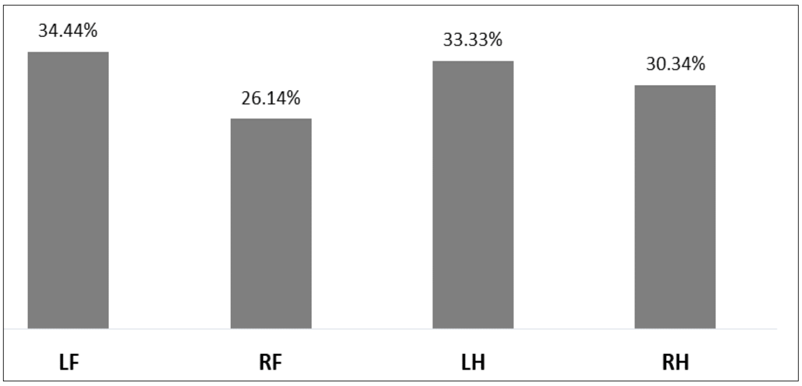


**Fig 2:** Quarter wise prevalence of SCM

### Farming system-wise prevalence of SCM

**Table 1:** Farming system-wise prevalence of SCM

| Farming system | SCM results | | OR(CI) | $\chi^2$ | P value | Result |
|---|---|---|---|---|---|---|
| | Positive n(%) | Negative n(%) | | | | |
| Commercial | 78(39%) | 122(61%) | 2.461 (1.53-3.96) | 14.073 | 0.000 | Highly significant |
| Conventional | 33(20.6%) | 127(79.4%) | | | | |

In my study prevalence of subclinical mastitis was high in commercial farming system which was found to be 39% than conventional (20.6%) which was highly significant ($p$<0.05).

**Sensitivity of CMT in response to bacterial growth:** Out of 111 positive milk samples, only 106 showed bacterial growth on bacteriological culture. The remaining five samples did not show any bacterial growth. Hence, the sensitivity of the CMT test in response to bacterial growth was found to be 95%.

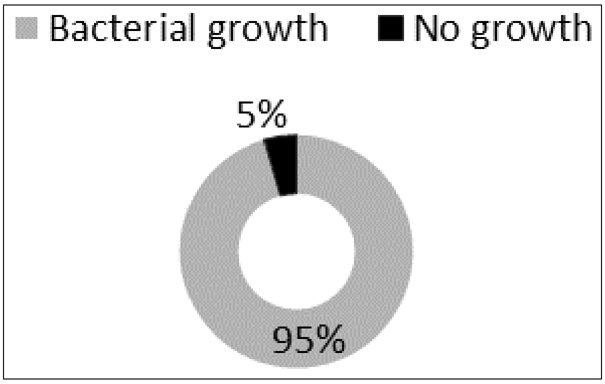


**Fig 3:** Sensitivity of CMT in response to bacterial growth

### Proportion of *E. coli* in cow milk with SCM

Out of 106 bacterial cultures, *E. coli* was isolated in 18 colonies through colony morphology and biochemical tests. Hence, the proportion of *E. coli* in cow milk with SCM was found to be 16.98%, which is given in the pie chart below.

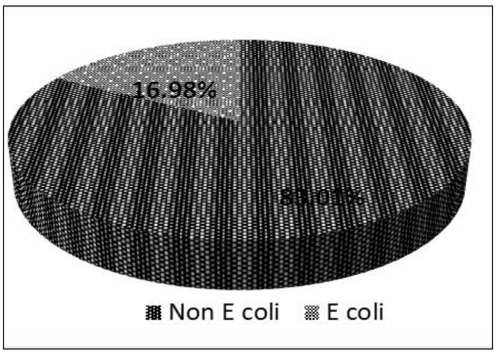


**Fig 4:** Pie chart showing proportion of *E. coli* in cow milk with SCM

**Antibiotic sensitivity results of isolates *E. coli:*** In my study *E coli* was more sensitive to Ciprofloxacin, Norfloxacin and Tetracycline with 100% sensitivity. Amoxyclav was found to be complete resistance. The graphical representation of sensitivity pattern of different antibiotics against *E. coli* isolates is given below.

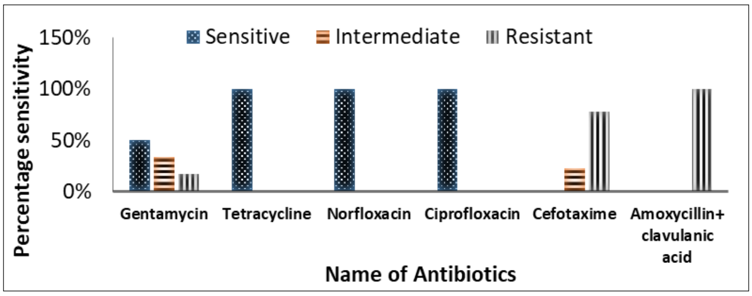


**Fig 5:** Bar graph showing AST results of isolates *E. coli*

**Multiple Antibiotic Resistant index of isolated *E coli***

In this study, 6 different antibiotics were used and MAR index was calculated for each *E coli* isolated. The graphical representation of MAR indices of individual bacterial isolates against 6 different antibiotics is shown in figure.

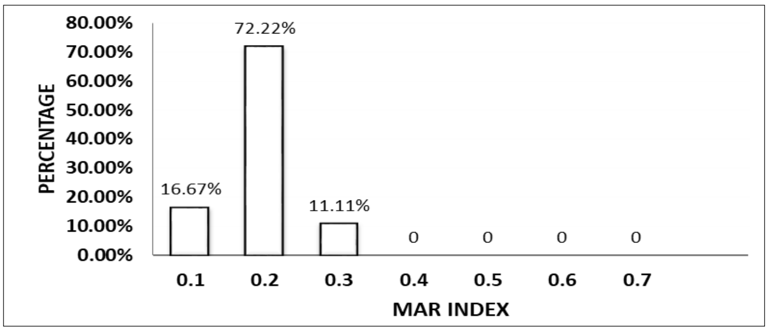


**Fig 6:** Bar graph showing MAR indexing of *E. coli* isolates on 6 different antibiotics

## Discussion

In a study of mastitis in Western Chitwan District, researchers using the CMT found the prevalence of SCM to be 30% in cows (Dhakal and Tiwari, 1993) [10], which was similar to the present research findings. Based on CMT, the overall prevalence of SCM was 33.33% (Sharma, 2015) [42] in Chitwan, another similar finding. In contrast to my findings, Shrestha and Bindari (2012) [45] found a 52% prevalence of SCM in Bhaktpur, Nepal, on the basis of CMT, which was higher than my findings and could be due to the different management practices used by those farmers. They also explained that their result of a higher prevalence was due to poor management practices. Poor hygiene and milking practices are reported to accelerate the disease (Pankey *et al*., 1984) [28]. Also, the findings , which were contradicted and lower than my findings, which are about 15.62%, The appropriate reason for such a difference might be due to the seasonality of the research, which was done on organized farms only. In my study, the prevalence of subclinical mastitis was higher in commercial farming systems than conventional ones, which

was statistically significant ($p$<0.05). This could be due to the high number of animals on commercial farms, and the chance of infection spreading from one animal to another is likely to be high. However, Rahman (2012) [31] in Bangladesh found no significant difference among farming systems (p>0.05). Quarter-wise, the occurrence of SCM was generally high in the left quarters. Statistically, there was no significant difference in the quarter-wise prevalence of SCM (p>0.05) which was similar to the findings of Shittu *et al*. (2012) [43] in Nigeria and Hashemi *et al*. (2011) [16] in Iran. Though an immediate explanation cannot be established for this observation, it is highly likely that in the process of milking, these particular quarters were milked first before the other quarters because most of the operators tend to be right-handed and sit first with the left animals (Shittu *et al*., 2012) [43]. Out of 111 positive milk samples, only 106 showed bacterial growth on bacteriological culture. My finding was similar to the findings of Saidi (2013) [35], who found 96% sensitivity of CMT in response to bacterial growth, and Teklesilasie (2014) [51], who found 97.6% sensitivity. The higher prevalence rate of *E. coli* in cow milk with SCM on the basis of bacteriological culture and biochemical properties found in the research of Hameed *et al*. (2008) [15], which was 16% in Pakistan, was similar to my findings. And also, the research done by Hashemi *et al*. (2011) [16] (13.64% in Iran) was in agreement with my findings. However, found a prevalence of 1.72% of *E. coli* in SCM, which was much lower than my findings despite the same procedure. The reason might be due to the different geographical location, climatic conditions during sampling, and hygiene practices of the study area. Also, Hamal (2015) found a 6.89% prevalence of *E. coli* in SCM in Chitwan district, which was lower than my findings, which might be due to different climatic conditions and a small sample size. The antibiotics Ciprofloxacin, Norfloxacin, and Tetracycline had a greater effect on *E. coli*. The widespread use of beta-lactam antibiotics to treat mastitis in cattle may be a contributing factor to resistance to cefotaxime and amoxyclav. Research done by Chandrasekaran *et al*., (2013) [8] found 86.65% isolated *E. coli* were found to be resistant i.e resistance to 1 or 2 of antimicrobials and few *E. coli* isolates (13.45%) were found to be multi-drug resistant i.e. resistance to 3 or more of antimicrobials which is similar to my findings. The reason might be due to the unregulated use of antibiotics or the prescription of drugs without AST that develop multiple antibiotic resistance strains.

**Conclusion and recommendations**

My research findings indicate a high prevalence of subclinical mastitis, highlighting its emergence as a significant managemental problem impacting animal health and causing economic losses. Coliform mastitis, caused by the environmental pathogen *E. coli*, is linked to poor hygiene and management practices. The study revealed a notable difference in infection rates among different farming systems, with commercial farming showing a higher prevalence. However, no significant variation was observed in quarter-wise occurrences of SCM. The sensitivity of 95% for CMT suggests its use as an early screening test for identifying SCM. The high proportion of *E. coli* in milk with subclinical mastitis underscores the emerging management challenges. The unregulated use of antibiotics without antibiotic susceptibility testing (AST) has led to an alarming increase in multiple antibiotic-resistant strains, posing a severe threat to veterinary and public health.

**Recommendations**

- Prior to writing an antibiotic prescription, an antibiotic sensitivity assay should be carried out.
- Emphasis on personal cleanliness and farm sanitation to lower the risk of coliform illness spreading.
- Regular use of early screening tests (CMT) should be done to identify the SCM so that proper prevention measures can be applied before it turns into a clinical infection.

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