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Prevalence and Risk Factor Analysis of Escherichia Coli–Associated Subclinical Mastitis in Cholistani Cattle

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Abstract

Subclinical mastitis is a major constraint to dairy productivity, particularly in indigenous cattle managed under extensive and semi-intensive systems. The present study investigated the prevalence of Escherichia coli–associated subclinical mastitis, evaluated associated risk factors, and determined the antimicrobial susceptibility patterns of E. coli isolates in Cholistani cattle from Rahim Yar Khan, Punjab, Pakistan. A total of 385 milk samples were collected from lactating cows during 2023–2024 and screened for subclinical mastitis using the Surf Field Mastitis Test. Samples positive for subclinical mastitis were subjected to bacteriological culture on selective media for isolation and identification of E. coli using morphological and biochemical characteristics. Risk factors related to animal, management, and hygiene practices were analysed using univariate and multivariate logistic regression models. Antimicrobial susceptibility of confirmed E. coli isolates was assessed using the disc diffusion method following CLSI guidelines. The overall prevalence of subclinical mastitis was 48.5%, while E. coli was isolated from 20.3% of subclinical mastitis cases. Significant associations were observed between subclinical mastitis and poor milking hygiene, inadequate milker care, high milk yield, body condition, and grazing type. Fluoroquinolones, including enrofloxacin, ciprofloxacin, and norfloxacin, exhibited the highest in-vitro efficacy against E. coli isolates, whereas notable resistance was observed against commonly used antibiotics such as amoxicillin. The findings highlight the epidemiological importance of E. coli in subclinical mastitis and emphasise the need for improved management practices and judicious antimicrobial use to mitigate economic losses and antimicrobial resistance in dairy systems.

Keywords: Cholistani cattle, *E. coli*, Prevalence, Subclinical Mastitis, Susceptibility

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Introduction

Mastitis is the inflammation of the parenchyma of the mammary glands. It is characterised by physical and chemical changes in milk and pathological changes in the glandular tissues of the udder. It is manifested by alterations in the physical and chemical composition of milk (Bhakat *et al*., 2020). Pakistan is an agricultural country with two key sectors: crops and livestock. Together, they account 45% of the labour force and contribute 21% to the GDP. Livestock alone accounts for 12% of the GDP, while the entire agricultural sector contributes 56%. Both sectors have a significant positive impact on reducing poverty and improving the economy (Shahzad *et al*., 2022; Rehman *et al*., 2017).

In Pakistan, approximately 35 million people are engaged in livestock farming (Qayyum *et al*., 2018; Rehman *et al*., 2017). Cattle are the main source of milk globally. The demand for milk in Pakistan has risen to 2.2% in the past few years and 3% increase worldwide (Jalil *et al*.,2022; FOA. 2019). Pakistan is the world's third-largest milk producer, with an annual production of 57 million tons (Khalid *et al*.,2024; Sattar, 2022). Mainly, 80% milk produced in Pakistan comes from small-scale dairy farming in rural areas, about 15% is produced in peri-urban areas, and the remaining 5% in urban areas (Khalid *et al*.,2024; Sattar, 2020). The livestock in Pakistan face different diseases routinely, but mastitis is a significant obstacle in developing the dairy industry (Khan *et al*., 2015; Karahan *et al*., 2011).

Mastitis is the most expensive disease for dairy animals. It reduces milk production and milk quality, leading to milk rejection by consumers or processors. Additionally, indirect losses are an increase in expenses. Mastitis is ranked as the second most significant disease affecting dairy animals after Foot-and-Mouth Disease (FMD) (Azam *et al*., 2020). It results in culling, decreased animal value, diagnostic expenditures, as well as veterinary and medication costs (Azam M *et al*., 2020). Mastitis is typically present in two forms: clinical, which is visible and subclinical, which is invisible.

In subclinical mastitis, there is an abrupt increase in the somatic cell count (SCC) exceeding 200,00/mL and lower milk production (Cheng *et al*., 2020; Abebe *et al*., 2016). Mastitis is now considered the most common disease affecting the dairy industry, with nearly two-thirds of its economic losses caused by subclinical mastitis, abbreviated as SCM (Iqbal et al., 2020; Khan et al., 2022). For screening of subclinical mastitis, easily available tests in the field conditions are the Surf field mastitis test (SFMT), California mastitis test (CMT) and White side test. (Alam *et al*., 2021). In countries like Pakistan, cases of mastitis are increasing due to poor disease prevention and reporting systems (Jabbar *et al*., 2020).

In Pakistan prevalence rate of mastitis is about 93% in cows and 48% in buffaloes (Amber *et al*., 2018). Different pathogens can cause mastitis that induce various immune responses in the udder, and it required specific response for each type of pathogen to provide protection (Ali *et al*., 2022). *E. coli* is a prevalent etiological agent of bovine mastitis, which usually leads to clinical and acute mastitis (Mahmood and Alam, 2019). However, the severity of symptoms can vary greatly, ranging from mild to severe. Sometimes, the infection can persist for a long time (Blum *et al*., 2014).

E. coli is the primary causative agent in over 80% of coliform mastitis cases (Abegewi *et al*., 2022). *E. coli* are Gram-negative, rod-shaped, motile bacteria

belonging to the family Enterobacteriaceae (Zaatout *et al*., 2022; Shahrani *et al*., 2014). Various risk factors, including housing conditions, nutritional practices, and milking techniques, have significant effects on the type and severity of the pathogens that can infiltrate the mammary gland (Sah *et al*., 2020). The common reservoirs of *E. coli* are the bedding material, water, flies, the stable and the bovine gastrointestinal tract (Klaas *et al*., 2018). Milk could be contaminated by Shiga toxin-producing *E. coli* (STEC) strains, which pose a significant risk due to their high zoonotic potential (Abegewi *et al*., 2022; Raza et al., 2023).

The chance of mastitis increases with the increase in temperature and humidity index (Salman *et al*.,2024; Bokharaeian *et al*., 2023). Antimicrobial resistance (AMR) has become a major public health concern worldwide because of the heavy use of antibiotics in both humans and livestock (Tepeli *et al*., 2023; Higham *et al*., 2018). Subclinical mastitis causes a dramatic decrease in milk production and quality; it is 15–40 times more common than clinical mastitis (Goulart *et al*., 2022).

Objectives

Focusing on the importance of *E. coli* subclinical mastitis in cattle, the objectives of the research were (1) the prevalence of *E. coli* in subclinical mastitis of Cholistani cattle, (2) the association of different assumed risk factors related to subclinical mastitis in Cholistani cattle, and (3) susceptibility of different antibiotics against *E. coli* isolates of subclinical mastitis.

Materials and Methods

The primary goal of this research was to study the prevalence of subclinical mastitis caused by *E. coli* in cattle, associated risk factors, along with its *in-vitro* antibiotic susceptibility profile in Cholistani cattle in District RAHIM YAR KHAN, Punjab, Pakistan.

Ethical consideration

The purposes of the study were explained to the dairy farmers, and they agreed to take part in the study. By signing a consent form from the dairy farmers, the animals were included in the research.

Study Area

The study was conducted in Rahim Yar khan one of the districts of cholistan region. A surface area of about 11,880 km², and lies between 27°40' and 29°16' N and latitudes and 60°45' and 70°01' E longitudes above sea level. The major factor in selecting the region was to address the basic and major causes of the local Cholistani cattle.

Study design and population

Cholistani Cattle herds were randomly selected based on how easy they were to access and whether the farmers were willing to participate by signing the consent forms. Milk samples and risk factors data were collected from all milking cows in each herd, except for those that had received antibiotics in the past fifty days.

The study focused on lactating cows used for dairy production, including cows raised for both milk and beef. The cows were kept under three different systems.

Intensive system: Cows are usually kept in a specific area and handled carefully.

Semi-intensive system: Cows graze on pasture but receive extra feeding, especially during milking days, and are penned at night.

Extensive system: Cows graze in the day but are kept in enclosures at night, which is the most prevalent feeding system in Rahim Yar Khan.

Sample Collection

A total of 385 milk samples (n=385 Cholistani Cattle) were collected during 2023–2024. The samples were collected

from various herds in the area surrounding the district of Rahim Yar Khan. The sample size was determined by assuming a 50% prevalence of *E. coli* at 95% confidence interval, following the recommendations of Thrusfield (2007).

Sampling was done in the early hours of the morning before the cows were milked. The hands were washed and disinfected, then put on a pair of gloves before collecting milk from each cow. The udder was thoroughly washed with clean water and dried with towels. After that, the teats were disinfected with 70% ethanol and allowed to dry, with a piece of cotton wool for each teat. The first few streams were discarded, and about 15 ml of milk was collected directly from the teats into a pre-labelled, sterile plastic Falcon tube with screw caps (Khan et al., 2022). The samples were then transported in a cool box at 4°C to the Medicine Laboratory in the department of clinical medicine and surgery, The Islamia University of Bahawalpur.

During sample collection, data regarding different risks associated with the occurrence of subclinical mastitis were recorded on a data capture form. The questionnaire collected information about each cow, including its age, breed, parity, stage of lactation, and any previous history of mastitis. The details about the herd size, the type of management system used, milking methods, and No. Of Milking, Hygienic condition during milking, and the type of floor in their living area were also collected.

Isolation and identification of coliforms

The Samples that were positive for subclinical mastitis were further cultured for *E. coli* on a selective medium, MacConkey agar culture media (OXOID, UK) and incubated at 37˚C for 24–48h. *E. coli* was confirmed by morphological appearance, lactose fermentation and Gram staining as performed by (Qayyum *et al.*, 2016).

Antibiotic Susceptibility

A panel of 9 antibiotic discs were used including Septran 25ug (Trimethoprim1.25ug + sulfamrthoxazole 23.75ug Bioanalyse®), Tylosine (30ug Bioanalyse®), Oxytetracycline (30ug OXOID®), Enrofloxacin (5ug OXOID®), Norfloxacin (10ug OXOID®), Gentamycin (10ug OXOID®), Ciprofloxacin (5ug Bioanalyse®), Lincomycin (10ug OXOID®), Amoxicillin (10ug OXOID®), Augmentin 30ug (Amoxicillin 20ug + clavulanic acid 10ug Bioanalyse®). The activated growth of *E. coli* (1 × 10^8 CFU/ml) was swabbed on Mueller-Hinton agar (OXOID, UK). The antibiotic discs were applied gently to the Mueller-Hinton agar (OXOID, UK). Incubation of the petri plates was done at 37°C for 24-48 hours in the incubator. Zones of inhibition were evaluated using Vernier calipers then compared with criteria established by the Clinical and Laboratory Standards Institute (CLSI, 2020) to determine whether *E. coli* is resistant or sensitive.

Statistical analysis

Univariate analysis was done to find out the association of all determinants in subclinical mastitis of Cholistani cow recorded on the data capture form. Selected determinants or the variables having p value <0.05 were further analysed by a multivariate logistic regression model. The statistical analysis was done using the software "SPSS version 27".

Results

Prevalence of Subclinical Mastitis

The total number (n=385) of milk samples of Cholistani cattle was collected from Rahim Yar Khan, one of the districts included in cholistan region. These were screened by SFMT. The prevalence of subclinical mastitis in RAHIM YAR KHAN

was found to be 48.5% (187/385), as shown in Figure 1.

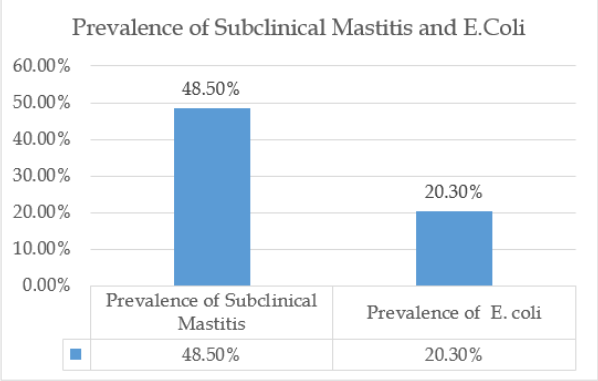

Figure 1: Prevalence of Subclinical mastitis and *E. coli*

Prevalence of *E. coli* in Subclinical Mastitis

The sample that was found positive for subclinical mastitis was further cultured for *E. coli* on MacConkey agar, and *E. coli* was confirmed by morphological appearance, Gram staining and biochemical test (Lactose fermentation). The prevalence of *E. coli* by the culture method was found to be 20.3 (38/187), as shown in Figure 2.

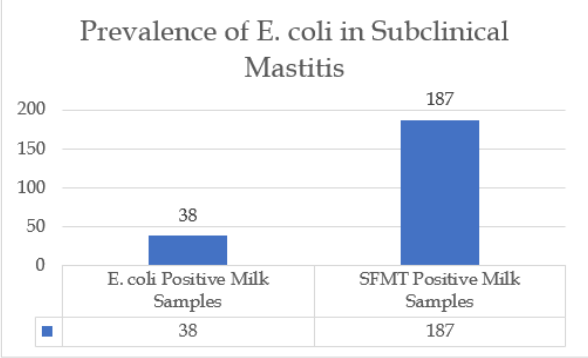

Figure 2: Prevalence of *E. coli* in Subclinical mastitis

Risk Factors association

The subclinical mastitis in Cholistani cattle was statistically associated with several risk factors identified by logistic regression analyses. Initially, all assumed risk factors (11) were analysed by univariate analysis, including Physiological Status, Milking Frequency, Milker Care, Hygiene during Milking, Milk Yield, Use of Teat Dips, Presence of Ticks, Body Health, Feed and Water, Grazing Type and Veterinary Services.

The univariate analysis identifies several key risk factors for subclinical mastitis. Significant associations were observed with poor milking care, lack of hygiene during milking, and high milk yield, all of which were associated with a higher occurrence of subclinical mastitis ($p < 0.001$). The use of teat dips and mixed grazing systems was also a significant risk factor. These findings suggest that improved hygiene, better milking care, and consistent use of teat dips could potentially reduce the risk of subclinical mastitis in this population.

Those variables that were found statistically significant in univariable analysis were further analysed by multivariable logistic regression (Table 1). The multivariate logistic regression model was used to assess the combined impact of these variables while controlling for potential confounders. The final model included key factors that were found to significantly influence the risk of subclinical mastitis. Notably, poor milker care during milking (OR = 0.141, $p < 0.001$) was associated with a lower likelihood of subclinical mastitis, while poor hygiene during milking (OR = 19.446, $p < 0.001$) dramatically increased the risk. High milk yield (OR = 7.962, $p = 0.002$) and the presence of thin body condition (OR = 6.679, $p = 0.014$) were also significant predictors of subclinical mastitis. Mixed grazing types (OR = 28.000, $p < 0.001$) further heightened the risk, indicating a substantial interaction between grazing practices and mastitis occurrence. In conclusion, this analysis highlights the critical role of proper milker care, hygiene practices, and management strategies in mitigating the risk of subclinical mastitis in Cholistani cattle. Interventions focusing on

improving milking hygiene, ensuring consistent teat dip use, and optimising grazing systems could potentially reduce the incidence of this condition in Cholistani herds.

The multivariate logistic regression identified several significant risk factors associated with the likelihood of a positive test result. Notably, poor hygiene during milking was associated with a substantial increase in risk (OR = 19.446, $p < 0.001$), while poor milker care significantly decreased the likelihood of a positive outcome (OR = 0.141, $p < 0.001$). High milk yield and certain body health statuses also showed significant associations. However, some variables, such as the feeding system and tick presence, did not show a significant impact.

Table 1: Univariate Analysis Table

Variables	Variable Levels	Positive (+ve)	Negative	p-value
Physiological Status	Lactating	350	34	<0.001
	Dry	34	0	
Milking Frequency	Once	337	47	0.032
	Twice	47	0	
Milker Care	Poor	226	158	<0.001
	Good	158	68	
Hygiene during Milking	No	228	156	<0.001
	Yes	156	72	
Milk Yield	High	52	332	0.008
	Low	328	4	
Use of Teat Dips	No	356	28	<0.001
	Yes	27	1	
Presence of Ticks	Yes	47	337	0.015
	No	333	4	
Body Health	Normal	349	35	0.017
	Thin	29	6	
Feed and Water	Well fed	374	10	0.042
	Under fed	10	0	
Grazing Type	Mixed	126	258	<0.001
	Separate	258	0	
Veterinary Services	Veterinary Officer	182	202	<0.001
	Veterinary Assistant	161	23	
	Self	40	0	

Table 2: Multivariable Association of Selected Determinants

Variables	Response	Odd Ratio (Exp(B))	p-value
Physiological Status	Lactating	6.513	0.009
Milking Frequency	Once	2.504	0.210
Milker Care	Poor	0.141	0.000
Hygiene during Milking	No	19.446	0.000
Milk Yield	High	7.962	0.002
Teat Dip Use	No	-	1.000
Tick Presence	Yes	0.757	0.614
Body Health	Thin	6.679	0.014
Feed and Water	Underfed	4374882069.721	0.999
Feeding System	Grazing + Stall Feeding	0.000	1.000
Grazing Type	Mixed	28.000	0.000
Disease Management	Self	53	0.999

Antibiotic Susceptibility Profile of *E. coli*

A panel of 9 antibiotic discs were used and tested antibiotics is as follows; Trimethoprim + sulfamrthoxazole (1.25ug,23.75ug) 60% (29/38), Tylosine (30ug) 50% (19/38), Oxytetracycline (30ug) 52.63% (20/38), Enrofloxacin (5ug) 100% (38/38), Norfloxacin (10ug) 100% (38/38), Gentamycin (10ug) 71.05% (27/38),

Ciprofloxacin (5ug) 100% (38/38), Lincomycin (10ug) 60.52% (23/38), Amoxicillin (10ug) 31.57% (12/38) and Augmentin Amoxicillin + clavulanic acid (20ug, 10ug) 78.94% (30/38) as shown in Figure 3.

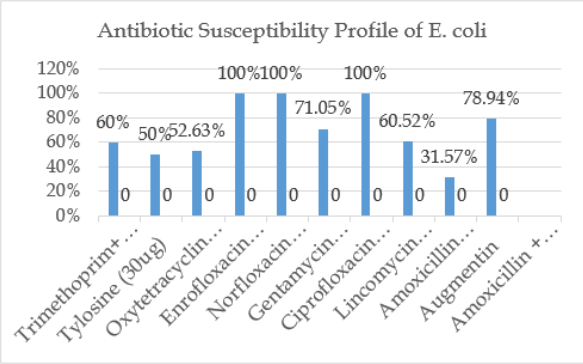

Figure 3: Antibiotic Susceptibility Profile of *E. Coli.*

Discussion

E. coli is a prevalent etiological agent of bovine mastitis, which usually leads to clinical and acute mastitis (Cheng and Han, 2020). However, the severity of symptoms can vary greatly, ranging from mild to severe. Sometimes, the infection can persist for a long time (Blum *et al.*, 2014). *E. coli* is the primary causative agent in over 80% of coliform mastitis cases (Abegewi *et al.*, 2022). *E. coli* are Gram-negative, motile, rod-shaped bacteria belonging to the family *Enterobacteriaceae* (Patout *et al.*, 2022; Sharani *et al.*, 2014). Various risk factors, including housing conditions, nutritional practices, and milking techniques, have significant effects on the type and severity of the pathogens that can infiltrate the mammary glands (Keshav *et al.*, 2020). The common reservoirs of *E. coli* are the bedding materials, water, insects, the feeding area and the gastrointestinal tract of cattle (Klaas *et al.*, 2018).

The current study helps in finding the prevalence of subclinical mastitis in Rahim Yar Khan, one of the districts included in the Cholistan region of Punjab, Pakistan and also helps in identifying the risk factors significantly associated with the occurrence of coliform mastitis. The study also evaluated the sensitivity of different antibiotics against isolates of *E. coli* from subclinical mastitis. In this study, 385 milk samples were screened by SFMT. The prevalence of subclinical mastitis in RAHIM YAR KHAN was found to be 48.5% (187/385). The findings of the present study are almost similar to those of Jalil *et al.* (2022) and Akhtar *et al.* (2012), who reported the prevalence of SCM as 45%, 51.8% and 53%, respectively. The higher prevalence of 67.3% subclinical mastitis as compared to the present study was reported by Khan *et al.* (2019). Tariq *et al.* (2021) and Qayyum *et al.* (2016) showed 42% and 21.96% prevalence in their studies, which is less than the present study. Research conducted by Sadaf *et al.* (2016) in Northern areas of Pakistan reported much less prevalence of subclinical mastitis 28.6% compared to the current research. There are multiple reasons behind the variation in prevalence of subclinical mastitis in different studies, such as inadequate milking hygiene and improper milking procedures, which increase the risk of bacterial infections causing subclinical mastitis. Unclean milking equipment can also introduce pathogens into the udder, leading to higher rates of infection. High-stress environments, such as overcrowding, increase the risk of subclinical mastitis (Arikan *et al.*, 2024).

The samples that were positive for subclinical mastitis (187/385) were further processed for *E. coli.* It was found that the prevalence of *E. coli* from subclinical mastitis was 20.3% (38/187). Ullah *et al.* (2024) and Sadaf *et al.* (2016) have reported 18.5% and 17.54% prevalence of *E. coli, which* is almost similar to this study.

Higher prevalence of *E. coli*, 21.9% and 40.1%. Was reported by Abegewi *et al.* (2022) and Tariq *et al.* (2021). The reasons behind this variation of *E. coli* prevalence are many, as it is an environmental pathogen; its presence in the udder leads to mastitis. This variation is also influenced by various aspects of farm conditions and treatment practices for *E. coli* mastitis; another major reason is increased resistance against antibiotics (Jalil *et al.*, 2022). The different assumed risk factors associated with the occurrence of subclinical mastitis were evaluated. The risk factors, such as milk care and hygienic practices in milking, yield of milk, using teat dips, tick presence, mixed grazing and services by denarians were key factors and associated with the subclinical mastitis Tariq *et al.*, 2021; Altaf *et al.*, 2020). Aqib *et al.* (2017) revealed that farm workers involved in the milking process, poor hygiene conditions, tick presence and lack of teat dips were associated with subclinical mastitis. In conclusion, this analysis highlights the critical role of proper milker care, hygiene practices, and management strategies in mitigating the risk of subclinical mastitis in Cholistani cattle. Interventions focusing on improving milking hygiene, ensuring consistent teat dip use, and optimising grazing systems could potentially reduce the incidence of subclinical mastitis in Cholistani herds.

The susceptibility of different antibiotics against *E. coli* was evaluated. It was found that Enrofloxacin, Ciprofloxacin and Norfloxacin were found 100% sensitive against *E. coli* isolates of subclinical mastitis from dairy cows.

The current study showed that Gentamycin is 71.05% sensitive against *E. coli*, which is in line with the Sohidullah *et al* (2024) and Aslam *et al* (2021), who reported that Gentamicin is 75.6% and 73.4% sensitive, respectively. Ahmad *et al.* (2021) reported100% and 85% sensitivity of Gentamicin, which is much higher than the present study, which may be due to less use of antibiotics and effective management of animals. Ullah *et al.* (2024), Abegewi *et al.* (2022) and Hasan *et al.* (2016) revealed sensitivity of gentamicin 38%, 48.6% and 52.6%, respectively, which is much less than that of this study. The current study showed that Amoxicillin is 31.57% sensitive against *E. coli,* which is closely in line with Abegewi *et al.* (2022) that it was 29.7% sensitive. Ullah *et al.* (2024) and Al Emon *et al.* (2024) revealed 00% sensitivity, which shows Amoxicillin is 100% resistant to *E. coli.* Sohidullah *et al.* (2024) and Hasan *et al.* (2016) revealed that amoxicillin sensitivity was 62.1% and 47.3%, respectively, which is slightly higher than this study and also indicates that amoxicillin was still sensitive to *E. coli.* The reason behind this difference may be due to the treatment of mastitis and other diseases, and no rational use of antibiotics at the farm level, contributing toward development of antibiotic resistance. The current study showed that Enrofloxacin is 100% sensitive against *E. coli,* which is in line with the findings of Sohidullah *et al.* (2024). Ullah *et al.* (2024) and Aslam *et al.* (2021) in which they revealed 77% and 53.4% sensitivity of Enrofloxacin. These variations in Enrofloxacin sensitivity may be due to different areas of study, development of antibiotic resistance and different farm practices. Ciprofloxacin was fount 100% sensitive, which aligns with Al Emon *et al.* (2024), in which they revealed that Ciprofloxacin is 100% sensitive. The reason behind this is that ciprofloxacin resistance is not as developed as compared to other antibiotics that are used in treating subclinical mastitis.

The present study shows that Norfloxacin is 100% sensitive against *E. coli*,_which is similar to Abegewi *et al.* (2022), in which they showed 89.2% sensitivity. Ullah *et al.* (2024) and Aslam *et al.* (2021) revealed 77% and 56.7% sensitivity of Norfloxacin against *E. coli*, which is slightly lower than that of this study. Ullah *et al.* (2024) showed 62% sensitivity of Amoxicillin/clavulanic acid in their study, which is slightly lower than this study, which shows 78.94% sensitivity. Aslam *et al.* (2021) revealed 30% sensitivity, which is much lower than that of this research. The reason behind this variation could be the higher antibiotic resistance.

Conclusion

The study concluded that *E. coli* is one of the major causative agents of subclinical mastitis. The risk factors, such as physiological status, milker care, hygiene during milking, milk yield, and grazing type, were significantly associated with the likelihood of subclinical mastitis. Specifically, cattle with poor milker care during milking, inadequate hygiene practices, or managed under mixed grazing systems were at a significantly higher risk of developing subclinical mastitis. The susceptibility of different antibiotic against *E. coli* isolates showed that Trimethoprim + sulfamrthoxazole (1.25ug,23.75ug) 60% (29/38), Tylosine (30ug) 50% (19/38), Oxytetracycline (30ug) 52.63% (20/38), Enrofloxacin (5ug) 100% (38/38), Norfloxacin (10ug) 100% (38/38), Gentamycin (10ug) 71.05% (27/38), Ciprofloxacin (5ug) 100% (38/38), Lincomycin (10ug) 60.52% (23/38), Amoxicillin (10ug) 31.57% (12/38) and Augmentin Amoxicillin + clavulanic acid (20ug, 10ug) 78.94% (30/38).

Recommendation

Coliform (*E. coli)* subclinical mastitis is a very important management problem in dairy farming. There is a large space for researchers to study coliform mastitis. Research should focus on improving early detection methods through rapid diagnostic tools to minimise severe cases. The study recommended that future studies may be conducted on *E. coli* mastitis, particularly to identify its resistant isolates, and new treatment strategies may be developed for the treatment of resistant isolates of *E. coli.*

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