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Research Article

**MICROBIOLOGICAL STUDIES OF BOVINE MASTITIS CAUSED BY
ESCHERICHIA COLI IN QUETTA, BALOCHISTAN**Shabir Ahmed^{1*}, Mumtaz Sanjrani¹, Zafar Ahmad², Asmatullah³, Deedar Ahmad khan⁴, Rezwan¹,
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Pakistan³ Department of Biotechnology, BUITEMS Quetta.⁴ Livestock and dairy development department Quetta, Balochistan.⁵ M.Phil Scholar Faculty of Pharmacy University of Balochistan Quetta⁶ Assistant Professor Department of Biology, Govt Degree College for Women, Chakwal**Abstract:**

Objective: The following study aims at determine the prevalence of bacterial mastitis in dairy cows in Quetta, to isolate and identify E.coli from dairy cows in Quetta, characterize biochemically and test for antibiotic sensitivity along with DNA extraction and confirmation of E.coli via PCR.

Methods and Materials: The milk samples were collected from different government and private dairy farms of Quetta city. The samples were streaked on MacConkey agar and kept in incubator at 37 °C for 24 hours. Antibiotic sensitivity test was performed by using disc diffusion Bauer technique and McFarland Turbidity Standard method 0.5 following CLSI protocols. The products were separated with 1% agarose gel electrophoresis and stained with ethidium bromide and images were documented during PCR.

Results: The overall prevalence of bovine mastitis among cows and buffalos was 38% with 18 % in government and 8% in private dairy farms. Antibiotics result showed that resistant to Vancomycin, Lincomycin, Carbenicillin, Kanamycin. The PCR amplification was positive for our isolation.

Conclusion: Present study concluded that E.coli is responsible for the high rate of mastitis among cows and buffalos in Quetta city. The E.coli found in the dairy farms of the Quetta was found to be resistant to the five antibiotics. This is an alarming state indicating the rising antibiotic resistance of E.coli towards the various antibiotic drug classes. The lack of awareness of the proper cleanliness and hygiene measures at dairy farms could be denoted as the reason of the higher prevalence of the mastitis in the dairy farms of Quetta.

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INTRODUCTION:

Mastitis is one of the common but significant diseases of dairy cows. It is important from economical view point. Costs due to mastitis include reduced milk production, condemnation of milk due to antibiotic residues, veterinary costs, culling of chronically infected cows and occasional deaths [1]. The mastitis marks in decreased milk production and has a long term treatment thus resulting in culling of animals before the completion of lactation period [2]. Inflammation of udder which is a physiological change leads to the decreased quality of milk. The worldwide annual losses caused by this disease estimates up to 35 billion USD[3]. According to a study the losses in this regard must be higher than estimated in Pakistan because the mastitis prevention practices like teat dipping and dry period antibiotic therapy is not much in practice [4]. Mastitis is caused by a wide spectrum of pathogens and, epidemiologically categorized in to contagious and environmental mastitis [5]. Contagious pathogens are those for which udders of infected cows serve as the major reservoir. They spread from cow to cow, primarily during milking, and tend to result in chronic sub-clinical infections with flare-ups of clinical episodes. Contagious pathogens include: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma spp.* and *Corynebacterium bovis* [6]. On the other hand, environmental mastitis can be defined broadly as those intra-mammary infections caused by pathogens whose primary reservoir is the environment in which the cow lives [7]. Environmental pathogens include *E. coli*, *Klebsiella spp.*, *Strept. dysgalactiae* and *Strept. uberis* and the majority of infections caused by these pathogens are clinical and of short duration [8].

Mastitis can also be classified as either clinical or sub-clinical. Clinical mastitis is characterized by sudden onset, alterations of milk composition and appearance, decreased milk production, and the presence of the cardinal signs of inflammation in infected mammary quarters [9]. It is readily apparent and easily detected. In contrast, no visible signs are seen either on the udder or in the milk in case of sub-clinical mastitis, but the milk production decreases and the somatic cell count increases[9]. It is more common and has serious impact in older lactating animals than in first lactation heifers [10]. Because of the lack of any overt manifestation, the diagnosis of sub-clinical mastitis is a challenge in dairy animal management and in veterinary practice [11].

In a number of surveys, mastitis has been recognized

is a key problem of livestock. There are different microorganisms such as *Staphylococcus aureus* and *Streptococcus agalactiae* and *Escherichia Coli* recorded to be the combine etiological agents of mastitis in buffaloes and cows in Pakistan [16]. *Escherichia coli* is an ecological pathogen and causes of clinical mastitis in early lactation period in high-producing cows [17]. Lipopolysaccharide (LPS) is the primary virulence reason in enteric bacteria[14], being accountable for most patho-physiological effect in *E. coli* mastitis[15]. The signs in acute mastitis caused by coliform are making by LPS and the release of inflammatory mediators[16]. There is no exact virulence determinant detected in *E. coli* isolate in mastitis.

Some available antibiotics are regularly used for the treatment of mastitis caused by *E. coli* [18], but the treatment results have been disruptive. Model studies of antimicrobial efficacy had been done to recommend a drug of choice for the treatment of induced *E. coli* intra mammary mastitis [19]. Some antimicrobial studies was reported with the inefficient results[20]. Some regions of world, now a day involved in using of Non-antimicrobial medicine for the treatment of mastitis, including glucocorticoids, nonsteroidal anti-inflammatory drugs (NSAIDs), regularly milking, liquid therapy and lactoferrin [19]. Antibiotics showed less efficient results due to high resistance rates between microorganisms. Furthermore, the *E. coli* strains are mostly resistant to some commonly available antibiotics [21]. Hygienic monitoring can be applied in direction for the management which reduced the incidence of *E. coli* mastitis. Whereas the efficacy of common practices is static and it is quietly different and changeable [22].

A focused study on contagious mastitis with emphasis on subclinical type is lacking in Quetta. It is therefore important to assess the effect of the *E.coli* in case of sub-clinical mastitis in Quetta are necessary to estimate and reduce loss attributed to the disease. Moreover, given the huge economic relevance due to lack of clinical visibility and subsequent effects, investigation of sub-clinical mastitis at herd-level is of paramount importance for designing feasible prevention and control strategy.

The following study aims at determining the prevalence of bacterial mastitis in dairy cows in Quetta, to isolate and identify *E.coli* from dairy cows in Quetta, characterize biochemically and test for antibiotic sensitivity along with DNA extraction and confirmation of *E.coli* via PCR.

MATERIAL AND METHODS

Collection of Milk Samples:

The samples were collected from different dairy farms of Quetta district. The samples were collected in pre-sterile and pre-labeled sample collecting containers. All the relevant information regarding history of animals, date of calving, milk production and appearance of clinical mastitis were collected on a pre-designed Performa and then the sample were shifted to laboratory within prescribed time and in favorable condition. To obtain refined results we divided the dairy farms into two categories i.e the dairy farms under the supervision of government and the dairy farms owned and managed privately.

Isolation and Identification

The samples were streaked on MacConkey agar and kept in incubator at 37°C for 24 hours. The colonies of *E.coli* were further confirmed with Eosine Methylene Blue agar, Brain heart infusion, gram staining, different biochemical tests (catalase, oxidase, indole, methyl red, vogues proskeur, urease test, citrate test, sugars fermentations test) and finally on PCR.

Antimicrobial Susceptibility Test:

Antibiotic sensitivity test was performed by using disc diffusion Bauer technique and McFarland Turbidity Standard method 0.5 following CLSI protocols. This test was done by using Mueller Hinton agar. The organism was accepted as sensitive and resistant by measuring the zone of inhibition.

Recognition of *E.coli* by PCR

A 25µl reaction volume was used for all PCRs, with mixtures that consists the following ingredients: 12.5µl of PCR Master Mix reagents, 9.5µl of grade water, 1µM KP-27F3 and KP-27B3 primers, and the same amount of DNA template was used. The PCR cycling parameters were: initial PCR activation, 94°C for 5min; amplification, 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; final extension, 72°C for 5min. The products were separated with 1% agarose gel electrophoresis and stained with ethidium bromide and images were documented.

RESULTS:

Result of Samples

In this study 100 milk samples were collected in which 38% were *E.coli* positive and 62% were negative as shown in Figure-1.

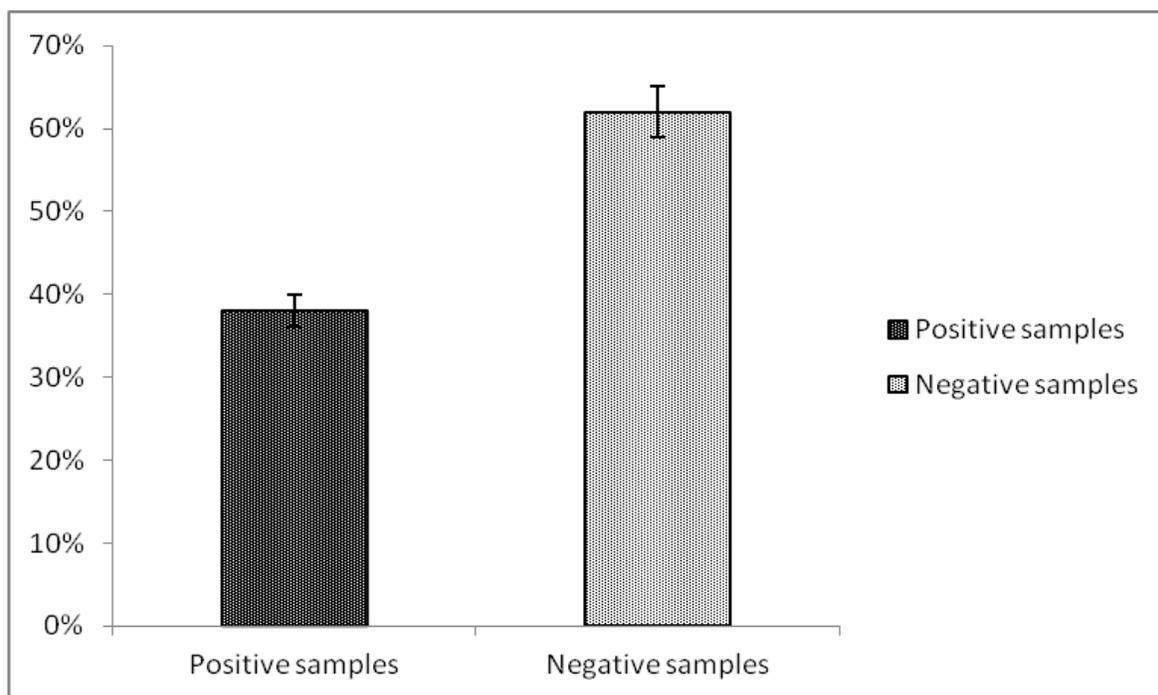


Fig 1: 38% were *E.coli* positive and 62% were negative

It was noted that among positive samples, 18% samples were from government dairy form Quetta, 12% samples were from private dairy form Jan Muhammad road and 8% samples were collected from private dairy form Raisani road as shown in figure 2.

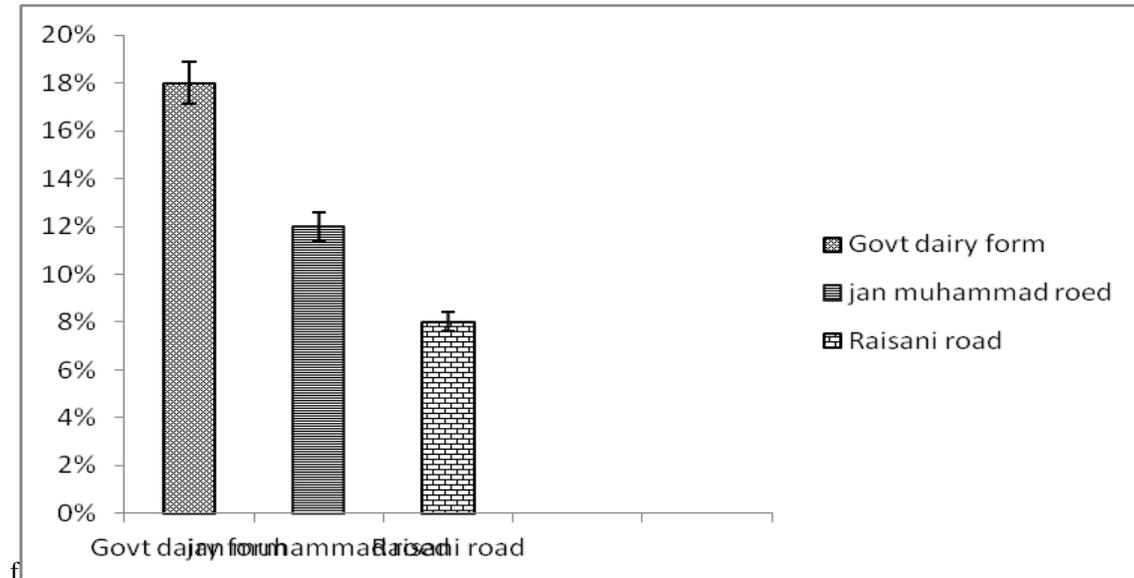


Fig 2: % samples were from government dairy form Quetta, 12% samples were from private dairy form Jan Muhammad road and 8% samples were collected from private dairy form Raisani road

While this study also indicated that cows were (10%) more affected as compared to buffaloes (28%) as shown in Figure-3

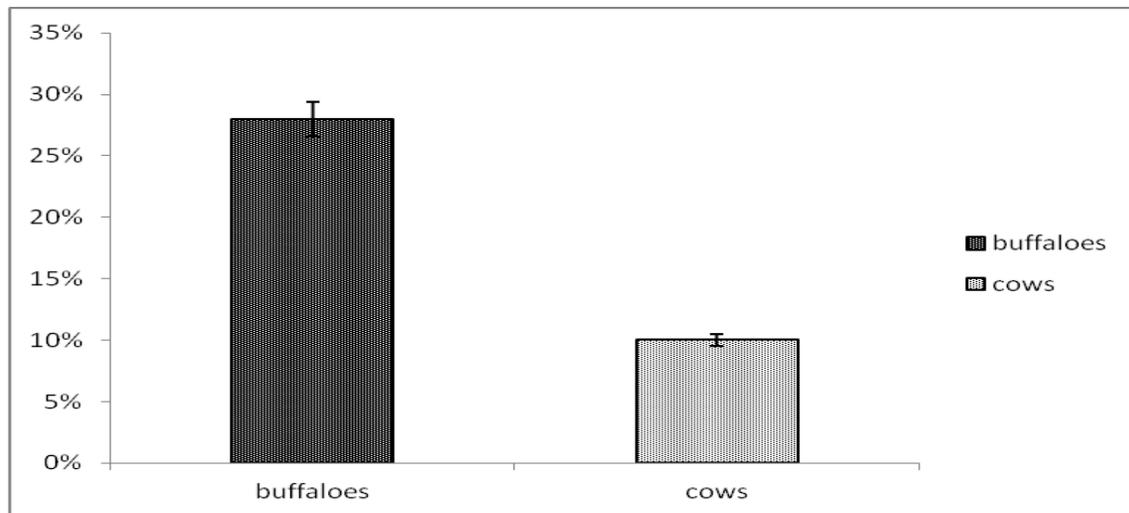


Fig 3: cows were (10%) more affected as compared to buffaloes (28%)

E.coli was confirmed through differential medium, gram staining and different biochemical tests that are shown below in Table-1.

Table1: Biochemical test properties

Biochemical test properties		<i>Ecoli</i>
Gram staining		-
Shape		Rod
Motility		+
Citrate test		-
Indole test		+
Methyl red test		+
Voges- Proskauer		-
Sugar fermentation tests	Glucose	+
	Lactose	+
	Sorbitol	+
	Mannitol	+
	Trehlose	+
	Dulicetol	+
Catalase test		+
Urea hydrolysis test		-

Antibiotic disc sensitivity test

Antibiotics result showed that *E coli* were sensitive to Ciprofloxacin (26mm), Tetracyclines (26mm), levofloxacin (22mm) Colistin sulphate (18mm), Cefixime (13mm) Azithromycine (12mm) while resistant to Vancomycin, Lincomycin, Carbenicillin, Kanamycin.

The zone of inhibition of organism against drug is given in Table-2.

Table-2 Antibiotic resistance and sensitivity test against *E.coli*.

Class	Antibiotics	<i>E coli</i>
1	Macrolides	Erthromycine
2	Pencillin	Carbencillin
3	Fluoroquinolone	levofloxacin
4	Macrolide	azithromycine
5	Tetracycline	Tetracycline
6	Aminoglycoside	Kanamycine
7	Polypeptide	Colistin.sulphate
8	Quinolones	Ciprofloxacin
9	Glycopeptides	Vancomycine
10	Lincosamides	Lincomycine
11	Cephalosporine	Cefixime

Confirmation of organism through PCR

Primers with the sequence of (5' CCGATACGCTGCCAATCAGT 3') and (5' ACGCAGACCGTAAGGGCCAGAT 3') were designed to allow PCR amplification of 884bp fragment of universal stress protein(usp gene). The PCR amplification was positive for our isolation as shown in Figure4.

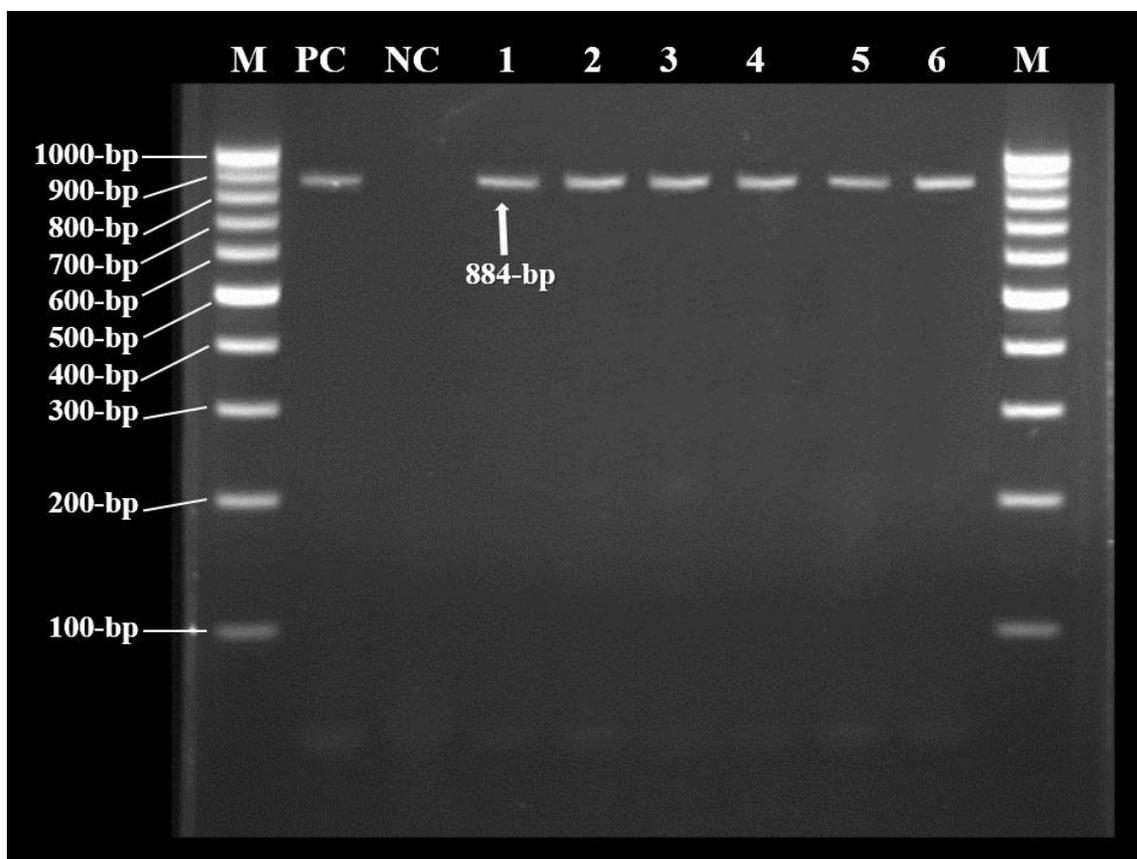


Fig 4: PCR for the detection of *E. coli*

PCR for the detection of *E. coli* showing a band size 884-bp. **Lane M:** 100-bp apart Molecular DNA Marker (Novagen, USA); **Lane PC:** Positive Control (884-bp); **Lane NC:** Negative Control; **Lane 1, 2, 3, 4, 5 and 6:** Positive for *e colish* showing a band size of 884-bp.

DISCUSSION:

This study has highlighted and brought the attention to the overall prevalence of bovine mastitis among cows and buffalos to be 38% in dairy farms of the Quetta city which is quite higher than a similar study conducted in Ethiopia which reported the prevalence of clinical mastitis to be 5.9%; which is inconsistent with 3.0% prevalence reported by Gizat *et al.* (2007) [23]. The variability in the prevalence of bovine mastitis is due to interaction of several factors mainly of management, environment and factors related to animal and causative organism. Mastitis-causing *E.*

coli are known to originate from the environment of the cow and their virulence probably reflects the situation of bacteria found in the intestines or feces of the cow [24]. The results from three different locations and two different dairy farms settings are however comparable which signifies that the prevalence of the bovine mastitis is quite higher due to *E.coli* in Quetta city. Our study also focused on the relative percentage of the infections in the cows and buffalos under similar dairy farm conditions. This comparison of prevalence of bovine mastitis has been missing in the previous studies which this study has tried to compensate. The relative comparison highlighted that there are more chances of buffalos to be infected with mastitis from *E.coli* species than the cows. The 28% of the infected samples were of the buffalos in comparison to the 10% of the infected samples belonged to cows. This has been depicted in Figure 3. *E. coli* which was the predominant isolate

in the current study could be associated with poor farm cleanness and stable areas. Although the literature on bovine *E. coli* mastitis is abundant, knowledge on its pathogenesis remains far from complete. Further studies should focus on host response and other cow factors related in this complex disease. One advantage of *E. coli* mastitis research is that good infection and inflammation challenge models are available; our cross-over study design diminishes the cow-to-cow variation. Field studies on spontaneous mastitis are also necessary to confirm findings of experimental research. For improved comparisons between different studies, the definitions of clinical *E. coli* mastitis characteristics (mild, moderate, severe, persistent) should be standardized. A deeper understanding of *E. coli* mastitis pathogenesis may help us to improve the immune defense of dairy cows against coliform mastitis and to develop more effective treatments. We did not study the effect of cows' age on host response, but evidence from earlier studies has shown that young cows are more resistant than older cows due to their more alert defence mechanisms [34,35]. This study focused on results of biological lab tests rather than the physical and clinical examination of the udders of cows and buffalos which distinguishes this study from other similar studies because of the definite certainty of the infected cattles rather than suspected appearances of the udders. For the purpose of the confirmation of the *E.coli* various techniques utilized included differential medium, gram staining and range of biochemical tests for the confirmation of *E.coli*. Biochemical tests utilized for this purpose have been shown in Table 1. The gram staining was found to be negative for the *E.coli* which is characteristic identification of the *E.coli*. The organism was identified to be of rod shape with motility. Citrate test indicated negative result. The other biochemical tests that indicated negative results include Voges-Proskauer, urea hydrolysis test, gelatin hydrolysis test, casein hydrolysis test. Whereas the indole test, methyl red test, sugar fermentation test and lysine decarboxylase test showed positive results. All these biochemical tests confirms the presence of the *E.coli* in the obtained samples.

Primers with the sequence of (5'CCGATACGCTGCCAATCAGT3') and (5'ACGCAGACCGTAAGGCCAGAT3') were designed to allow PCR amplification of 884 bp fragment. The PCR amplification was positive for our isolation as shown in Figure 4.

The sensitivity tests of *E.coli* were carried out in vitro. The *E.coli* were found to be sensitive towards

Ciprofloxacin (26mm), Tetracyclines (26mm), levofloxacin (22mm) Colistin sulphate (18mm), Cefixime (13mm) Azithromycine (12mm) while resistant to Vancomycin, Lincomycin, Carbenicillin, Kanamycin. Also this study identified the classes of antibiotics to which the *E.coli* have produced resistance in the dairy settings of Quetta.

CONCLUSION:

Present study concluded that *E.coli* is responsible for the high rate of mastitis among cows and buffalos in Quetta city beside other infective agents. The *E.coli* found in the dairy farms of the Quetta was found to be resistant to the five antibiotics out of the eleven antibiotics used in sensitivity tests. This is an alarming state indicating the rising antibiotic resistance of *E.coli* towards the various antibiotic drug classes. The buffalos are more prone to be infected and affected by mastitis resulting in economic dairy loss and increased cost of treatment and management of the cattle. Unhygienic environment at dairy farms pose a threat to other healthy cattle at the farm, if this issue is left unaddressed it may culminate into the economic loss to local dairy industry in long run. The lack of awareness of the proper cleanliness and hygiene measures at dairy farms could be denoted as the reason of the higher prevalence of the mastitis in the dairy farms of Quetta.

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