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To Detect The Biofilm Producing *E. Coli* Isolates Of Bubaline Mastitis Milk Samples And Study Of Antibiotic Resistance Pattern Of *Escherichia Coli*.

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Abstract

Mastitis is the most common disease in dairy cows and buffaloes and has well-documented negative effects on animal welfare and dairy farm profitability. Antimicrobial treatment of infection can help to eliminate or reduce the occurrence of mastitis infection. The widespread use of antibiotics raised concerns about the emergence of antibiotic-resistant pathogens, prompting the dairy industry to reduce antibiotic use. As a result, alternative therapies for the prevention and treatment of bovine mastitis, particularly natural products derived from plants and animals, have been sought. *Escherichia coli* (*E. coli*) is a highly adaptable bacteria that may develop biofilms in certain situations. This study aims to explore the virulence character like biofilm production and resistance to antimicrobials of *E. coli* which are isolated from the bubaline mastitismilk samples.

16 milk samples from buffaloes with mastitis in coastal Andhra Pradesh were used in the current study. The original biochemical assays used to identify these isolates were the Indole test, Methyl red, Catalase, Spot Oxidase, Voges-Proskauer, Haemolytic activity on 5% Sheep Blood agar, and Biofilm Activity on Congo Red Agar. By using the Kirby-Bauer disc diffusion technique, the susceptibility of six antimicrobials to verified E.coli isolates was evaluated.

By using biochemical techniques, 5 samples from the 16 isolates were tentatively positive for E. Coli. The drug with the greatest level of resistance was penicillin (100 %). The findings indicate that the organisms are able to produce biofilm. They are rapidly

gaining genes that make them resistant to the standard antibiotics used to treat mastitis. In vitro study demonstrates the efficacy of penicillin against gram-positive mastitis pathogen is high.

Keywords

Antimicrobial resistance, Biofilm, Escherichia coli, Mastitis, Mammary gland, Milk samples.

1. Introduction

Bubaline mastitis is an inflammatory response of the mammary gland's udder tissue caused by physical trauma or microorganism infections. Due to lower milk output and poor milk quality, it is recognized as the most prevalent disease-causing economic loss in dairy industry. Bubaline mastitis, also known as "parenchymal inflammation of the mammary gland," is characterised by a variety of physical and chemical changes in milk as well as pathophysiologic changes. Logical modifications in udder glandular tissues (Roadsters et al., 2000). Mastitis is one of the most common and costly diseases affecting the dairy industry worldwide (Petrovski et al., 2006). Mastitis costs include decreased milk production, milk condemnation due to antibiotic residues, veterinary costs, culling of chronically infected cows, and the occasional death (Rahmeto Abebe, 2016).

Mastitis can also be clinical or subclinical in nature. Clinical mastitis is distinguished by its abrupt onset, changes in milk composition, milk appears watery with flakes and clots, and appearance, decreased milk production, the presence of the cardinal signs of inflammation in infected mammary quarters (red and swollen udders), and fever in dairy cows. It is easily visible and detectable. Sub-clinical mastitis, on the other hand, shows no visible signs on the udder or in the milk, but milk production decreases and somatic cell count rises (Khan MZ, Khan A, 2006).

Mastitis is a complex disease caused primarily by a variety of pathogens, with significant differences in infection patterns (clinical versus subclinical, acute vs. chronic) and no simple model that encompasses all possible facets of the disease (Fetrow et. Al., 2000). Mastitis is typically caused by bacterial pathogens that fall into two categories: contagious pathogens like Streptococcus agalactiae, Staphylococcus aureus, and Mycoplasma bovis, which reside predominantly in the udder and spread during milking, and environmental pathogens like Streptococcus species (Streptococcus uberis and Streptococcus dysgalactiae) and environmental coliforms (Radostits et al. 2000). The effects of antimicrobial treatment on the mammary microbiota have also been partially studied in humans and cattle (Hayashi, Mayu, et al,2023).

Escherichia coli is a terrifying pathogen, especially the "enterohemorrhagic *E. coli* strains that induce infection through milk and have a severe influence on human health (Hickey CD, 2015). *Escherichia coli*: The most common gram-negative pathogen is *E. coli*. It enters the udder via the teat, multiplies, and causes an inflammatory response in dairy cows. It can be found in the environment surrounding dairy cows, such as herd bedding, especially when wet conditions. Mastitis caused by *E. coli* is typically clinical and short-lived. Symptoms range from mild to severe, with only local signs (red and swollen udder) to severe with systemic signs (fever). Severe clinical mastitis induced by *E. coli* can cause irreparable tissue damage in the mammary gland, full cessation of milk output, and even death in a dairy cow. *E. coli* that causes mastitis can be resistant to practically all antimicrobial treatments (Eisenberger et al., 2018; Shafiq et al., 2021). Poverty, illiteracy, overpopulation, and starvation have all contributed to drug resistance. Based on these facts, India is often referred to as the "antimicrobial resistance capital of the world."(Sahoo, Sonali, et al.2023).

E. coli capable of forming biofilms in vitro, which is an important mechanism of protection and resistance to antimicrobials and plays an important role in microorganism virulence (Lucchesi, Paula MA, et al,2023). The adhesion of microorganisms to a surface, followed by cell division under favorable growth conditions (nutrients and temperature), results in surface colonization and is referred to as biofilm establishment. Lower levels of glucose could provide a stressful environment to the bacteria, resulting in increased production of biofilms during specific phases of the disease. *E. coli* breaks down the lactose in galactose and glucose in raw milk, and this latter is the main carbohydrateused by this bacterium (Andrews *et al*., 2008).

Biofilms protect microbial agents, phagocytosis, and sanitizers. The ability of bacteria to form biofilms complicates pathogen elimination, which can result in persistent infections. Resistance to antimicrobial agents is caused by the slowing of antibiotic diffusion through the biofilm matrix, an increased rate of mutation, the production of antibiotic-degrading enzymes, the presence of dormant bacterial cells with low metabolic activity, and increased doubling times in the inner layers of biofilms. There are several techniques for detecting biofilm development, however Congo Red Agar (CRA) and the Tube Method (TM) are often employed in clinical laboratories to determine bacteria's capacity to create biofilm. Because of their distinctiveness, low operational costs, and material availability (Hiby, Niels, 2011).

The present study was aimed for the detection and characterization of *E. coli* from raw milk samples from different dairy farms followed by further characterization and to know their antibiotic resistance patterns in vitro. And also know their virulence character like biofilm production.

2 Objectives

- To collect the samples from bubaline mastitis infected cattle (raw milk)
- To culture and process the collected milk samples.
- To isolate the *E. coli* colonies from the processed milk samples.
- Morphological characterization of *E. coli* samples.
- Biochemical characterization of *E. coli* isolates.
- To explore the antibiotic resistance of these provisionally confirmed *E. coli* isolates.
- To understand the virulence of provisionally confirmed *E. coli* isolates.
- Toxins
- Biofilm

3 Material and methods

3.1. Sample collection

A total of 16 fresh milk samples which found positive for clinical mastitis are collected in sterilized containers (bottles) and labeled based on temporary ID given to a cow from local dairy farms and from veterinary hospital from different places from coastal Andhra Pradesh, viz., Mangalgiri, Bhimavaram, Gudavali, Kunderu, Pedhavadlapudi. The collected samples were processed for the further isolation (Table 1).

S. No	Place of Sample Collection	Sample code
1.	Gudavalli	GDV-1
2.	Gudavali	GDV-2
3.	Gudavalli	GDV-3
4.	Gudavalli	GDV-4
5.	Mangalgiri	MG-1
6.	Mangalgiri	MG-2
7.	Kunderu	KN-1
8.	Kunderu	KN-2
9.	Pedhavadlapudi	PV-1
10.	Pedhavadlapudi	PV-2
11.	Pedhavadlapudi	PV-3
12.	Pedhavadlapudi	PV-4
13.	Bhimavaram	BHM-1
14.	Bhimavaram	BHM-2
15.	Bhimavaram	BHM-3
16.	Bhimavaram	BHM-4

Table 1: The given table represents the total number of isolates with the sample codes

3.2. ISOLATION AND IDENTIFICATION OF *E. COLI*

Enrichment and isolation of *E. coli* from the milk samples were performed under sterilized conditions. Firstly, the collected samples were inoculated in Nutrient Broth and Mueller Hington broth (MHB) at 37^0 C for 24hrs for enrichment and further the cultures are streaked on Eosin Methylene Blue (EMB) which is selective media for *E. coli.* The inoculated plates were incubated at 37^0C for 24 hrs.

3.3. MORPHOLOGICAL CHARACTERIZATION OF *E. COLI*

The suspected colonies were subjected to Gram's staining in order to understand the morphology of gram-negative rods.

3.4. BIOCHEMICAL CHARACTERIZATION OF *E. COLI*

The suspected isolates were identified using IMVIC tests which includes Indole test, Methyl red test, Voges- Proskauer and Citrate tests. The

E. coli isolates from the bovine mastitis samples were indole positive, methyl red positive, and where as Voges- Proskauer, citrate, oxidase wasnegative.

3.4.1. INDOLE TEST

A crimson or pink ring form at the tube's top as a result of the indole's reaction with the aldehyde in Kovac's reagent. To test the bacterium, a bacteria isolate is injected into peptone water, which contains tryptophan. Overnight at 37°C, the mixture is incubated. When a red or pink colored ring appears at the top of the liquid after a few drops of Kovac's reagent have been added, the reaction has been successful. A bacterium that produces indole is *E. coli*.

3.4.2. METHYL RED TEST

The test microorganism is introduced into glucose phosphate (MRVP) broth, which is made up of phosphate buffer and glucose, and cultured at 37°C for 48 hours. The tube is filled with three to five drops of MR reagent. When the bacteria have produced enough acid to counteract the phosphate buffer, red colour development is a positive reaction that takes place. The colour of Methyl Red-negative bacteria turns yellow. The bacteria *E. coli* is Methyl Red positive.

3.4.3. VOGES -PROSKAUER (VP) TEST

The test microorganism is placed in a tube of glucose phosphate (MRVP) broth to conduct the VP test, where it is cultured for 72 hours. Shake the test broth after adding 15 drops of alpha-naphthol. The broth should then contain five drops of potassium hydroxide (KOH) at a 40% concentration. The isolate is classified as VP negative after 1 hour of no colour change; leave the tube standing for 15 minutes to witness apositive red discoloration. VP is not expressed by *E. Coli*.

3.4.4. CITRATE TEST

Citrate utilization test the culture under test was streaked onto Simon's citrate slants and incubated at 37°C for 96 hrs. A positive reaction was indicated by appearance of blue colour and growth on the streak line *E. coli* is citrate negative.

3.4.5. CATALASE TEST

Two to three drops of 3% hydrogen peroxide was taken on glass slide, to test the catalase activity. The positive test indicates appearance of bubbles on H_2O_2 drops. *E. coli* is positive for catalase test.

3.4.6. SPOT OXIDASE TEST

The dye solution (N, N, N', N'-Tetramethyl-p-phenylenediamine) is poured onto blotting paper through a sterile filter. Using a sterile glass rod,2-3 colonies from an EMB plate were picked and gently touched onto blotting paper, and the development of any dark blue colour was observed (Carter and Cole, 1990). The Oxidase test results for *E. coli* are negative.

3.5. ANTIMICROBIAL SENSITIVITY TEST (ABST)

The antibiotics' minimum inhibitory concentration against *E. coli* species was determined using the Kirby- Bauer disc diffusion method. Mueller Hington broth samples are swabbed on Mueller Hington agar plates. The antimicrobial agents tested were Methicillin (10 mcg), Amoxyclav (30 mcg), Vancomycin (30 mcg), Erythromycin (15 mcg), Ciprofloxacin (5 mcg), Trimethoprim (10 mcg), Ceftriaxone (30 mcg),Ceftriaxone/Sulbactam (30/15 mcg), Streptomycin (25 mcg), Nitrofuranto (10 mcg). For 24 hours, plates were incubated at 37°C. The sensitivity patterns were read and classified as sensitive, intermediate, or resistant based on the diameter

of the inhibition zone (CLSI, 2021).

3.6. HAEMOLYSIS ACTIVITY

The hemolytic activity test conducted with 5% Sheep Blood Agar medium (Grema et al., 2015 and Quinn et al.2000).

3.7. Phenotypic detection of biofilm production

The production of biofilms by *E. coli* isolates was confirmed by culturing the organism on Congo red agar (CRA) medium. Culturing *E. coli* isolates in autoclaved CRA plates prepared by dissolving 37g brain heart infusion broth, 50 g sucrose, 10g agar, and 0.8g Congo red in 1 Ldistilled water was used to assess biofilm. Inoculated CRA plates were incubated for 24 hours at 37 0 c before being stored at room temperature for 48 hours. The appearance of black colonies with dry crystalline consistency indicated a positive outcome.

4. RESULT

4.1. ISOLATION

16 mastitis milk samples were collected and tested for *E. coli* presence. 31.25 % of the milk samples were tested positive for the presence of

E. coli provisionally.

4.2. MORPHOLOGICAL CHARACTERIZATION OF *E. COLI*

31.25 % of the milk samples were tested positive for the presence of *E. coli* provisionally, which were gram negative rod-shaped appearance (which were confirmed through the gram staining test) were picked up and maintained on Eosin Methylene Blue slants for further characterization by cultural and biochemical tests.

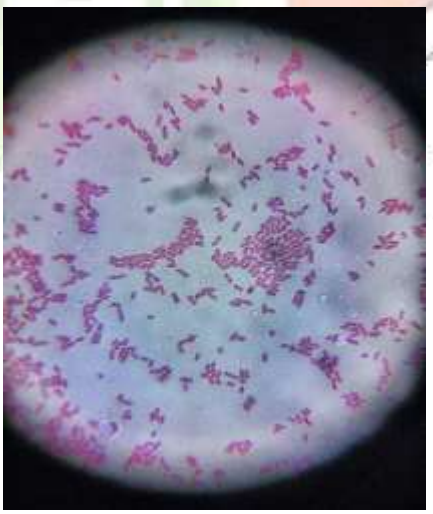
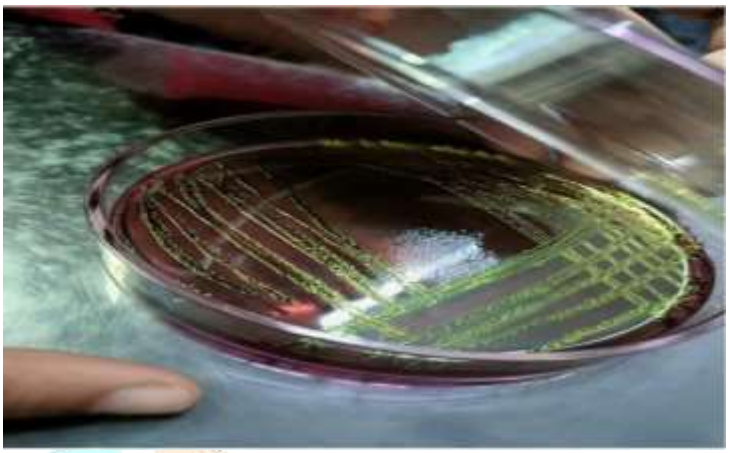


Figure 1: Image showing gram-negative rods by Phase Contrast Microscope

4.3: Cultural characterization of *E. coli*

Escherichia coli colonies were grown with a metallic sheen with a dark center on an EMB agar medium.

Figure 2: Eosin Methylene Blue Agar plate showing metallic sheen colonies.



BIOCHEMICAL CHARACTERIZATION

The provisionally confirmed *E. coli* isolates were catalase, indole, methyl red positive and Voges-Proskauer, citrate, oxidase negative.

HEMOLYSIS

The hemolytic activity of *E. coli* isolates proved that 40 % of isolates were α hemolytic and 20 % isolates were β hemolytic on 5% sheep bloodagar medium. The remaining 40 % isolates were non- hemolytic.

BIOFILM

60 % of provisionally conformed isolates were positive for biofilm production.

Figure 3: Black-colored colonies were observed on the Congo Red Agar Medium

ANTIMICROBIAL SUSCEPTIBILITY TESTING BY DISC DIFFUSION ASSAY

Antimicrobial Susceptibility Disc Diffusion Assay testing was carried out on the Muller Hington Agar plates for *E. coli* isolates. All 05 E. coli isolates were evaluated against 6 different antimicrobials. The drug with the greatest level of resistance was penicillin (100 %), Nitrofurantoin (80 %), Ciprofloxacin (40 %), Ceftriaxone+ Tazobactum (40 %), Erythromycin (20 %) followed by Gatifloxacin. From the study, the bacteria becoming more resistant to the antimicrobials commonly used to treat mastitis.

Table 2: The given table represents the antibiotic resistance pattern of provisionally confirmed

*E. coli i*solates

S. No.	Sample code	Erythromycin	Ciprofloxacin	Nitrofurantoin	Ceftriaxone/tazobactum	Penicillin	Gatifloxacin
1.	GDV-2	R	S	R	S	R	S
2.	MG-2	S	S	R	S	R	S
3.	PV-1	S	R	R	S	R	S
4.	BHM-3	S	S	R	R	R	S
5.	KN-1	S	R	S	R	R	S

DISCUSSION

E. coli is the most common pathogen causing environmental mastitis, and it is one of the most important coliforms that has received more attention due to its high prevalence in comparison to other mastitis pathogens.Control of udder health is important for the dairy production chain in light of food safety issues(Kovačević, Zorana, et al,2021). It can be found in dairy cow environments such as herd bedding, especially in wet conditions.(My, Tran Trung, et al.2023). Despite technological and medical breakthroughs, mastitis remains one of the most serious health issues in dairy cows and buffaloes. The most efficient ways of mastitis case reduction in dairy farms is pre-milking and post-milking teat disinfection

In the treatment of the disease, antibiotics are considered the first line of defence. However, the issue of antibiotic residue and antimicrobial resistance, as well as the impact of antibiotic abuse on public health, has led to many restrictions on uncontrolled antibiotic therapy in the dairy sector around the world (El-Sayed, A., Kamel, 2021). Increasing dairy production and animal welfare is a critical component in developing future policies aimed at ensuring food security, particularly in developing countries.(Neculai-Valeanu, 2022). which were isolated from animals with clinical mastitis characterized by mild, moderate, or severe symptoms and persistent infections, had the highest representation of virulence factors. Antimicrobial agents are extremely useful in developing treatments for bacterial infections. However, antibiotic-resistant bacteria pose a significant challenge to the veterinary and health professions, as well as dairy animal producers, because they have a negative impact on therapy. The extensive therapeutic use of antimicrobials has been linked to the development of resistance (Abo-Shama, 2014).

The present study aslo highlights the, pathogenicity of biofilm production and Antimicrobial resistance. E. coli exhibits the virulence characters(Orsi, Henrique, et al.2023).One property helping the bacteria to survive in the mammary gland and persist in dairy herds is a biofilm formation ability . The formation of biofilms is considered an important virulence factor of E.coli strains, as it facilitates their adhesion to biotic and abiotic surfacesThe ability of these pathogens to form biofilms adhering to the MG epithelium allows them to evade immune defences and cause recurrent or persistent infections (Zigo, František, et al,2022). It gains resistance towards the Antimicrobial drugs through changing its genes. This may lead towards the production of biofilm which is pathogenic to mankind and animals. Farm animals may transmit antibiotic-resistant bacteria to other animals, both wild and domestic. Humans are endangered by direct contact with such animals, as well as consumption of their meat or milk.Farm animals may transmit antibiotic-resistant bacteria to other animals, both wild and domestic.Humans are endangered by direct contact with such animals, as well as consumption of their meat or milk. Out of five isolates of *E. coli* three samples (60%) show the production of biofilm on congo red agar. It indicates that the chances of production of biofilm is high.

CONCLUSION

The present study using the clinical isolates from bubaline mastitis revealed the presence of virulence factors, by adopting resistance genes, biofilm formation and enterotoxins. This also causes the rural economic loses. Hence, with the help of this study we conclude that the environment of the dairy farms has to be hygiene and drugs must be given according to the bacterial resistance for the control of developing resistance to the present class of antibiotics and also to inhibit the production of biofilm in *E. coli* isolates.

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