MOLECULAR IDENTIFICATION AND ANTIBIOTIC RESISTANCE PROFILE OF *STAPHYLOCOCCUS AURUES* AND *ESCHERICHIA COLI* ISOLATED FROM MILK SAMPLES OF LARGE RUMINANTS

Aayesha Riaz, Arfan Yousaf, Evelyn Saba.

Faculty of Veterinary and Animal Sciences, PMAS-Arid Agriculture University Rawalpindi Pakistan

Introduction

In dairy cattle, mastitis (inflammation of udder) is caused by pathogenic bacteria living in the alveoli. Consequently, milk yield and quality are reduced due to the toxic substances produced by bacteria. As one of the leading diseases in the dairy industry, mastitis causes economic loss due to poor lactation, premature culling, wastage of milk and low milk yields due to overuse of antibiotics (Javed et al. 2022). *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* are the two most important organisms that contaminant the milk. These bacteria are also major causal organism of mastitis in cattle and buffalo.

Major human pathogen *S. aureus* causes a variety of clinical manifestations (Hasanvand et al. 2021). *S. aureus* is considered to be a cause of inflammation in animals and it is believed that this organism is responsible of 30-40% cases of mastitis. (Zigo et al. 2021).

One of the most common pathogens found in foods is *E. coli*. It is estimated that most *E. coli* are nonpathogenic, however a small number are highly pathogenic, causing hemolytic uremic syndrome, hemorrhagic colitis, and watery and bloody diarrhea in some cases.

Antibiotics are very essential for treatment of bacterial infections, both in human beings and animals. Antibiotic overuse has been linked to the development of antibiotic resistant bacteria, which ultimately renders the treatment of diseases in humans and animals impossible. Among the most prevalent bacteria that infect animals and are resistant to several drugs are *Klebsiella pneumoniae* and *Escherichia coli, Salmonella*, *Campylobacter*, *methicillin-resistant Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus,* and other bacteria identified in food animals can be harmful to the public's health. There have been very few reports on detection of pathogenic organisms using molecular methods being detected in cow and buffalo milk in Pakistan. Appropriate selection of antibiotic against the *Escherichia coli* and *Staphylococcus aureus* is crucial in mastitis treatment of cattle and buffalo. The objectives of the present study includes:

- The isolation of Escherichia coli and Staphylococcus aureus from the milk samples of buffalo and cow.
- Biochemical and Molecular characterization of S. aureus and E. coli from raw milk.
- Assessment of antimicrobial sensitivity profile of S. aureus and E. coli from raw milk.

Materials and Methods

Study location: Te study was conducted at Pir Mehr Ali Shah Arid Agriculture University Rawalpindi from October to December 2021.

Study population: The study population comprised apparently mastitic cattle and buffaloes. Samples were brought from different dairy farms of Rawalpindi.

Sample Collection: In this study mastitic milk samples were collected from dairy farms of Rawalpindi. In total, 30 samples (10 mL each) were tested. Twelve samples were collected twice from 12 different cattle's and six samples were collected from three different buffaloes at one month interval.

Isolation of *Staphylococcus aureus* and *Escherichia coli:* Pre-enriched milk sample was streaked on Nutrient and Mannitol salt agar for detection and isolation of *S. aureus* and was incubated at 37°C for 24 hours. Nutrient agar produces yellow-colored colonies while Mannitol salt agar is used to differentiate between (Yellow colonies) fermentation of mannitol and (Pink colonies) non-fermenting colonies which confirm the presence of *S. aureus*.

Milk samples of 200 µL quantity was aseptically streaked with MacConkey agar media and were incubated overnight at 37˚C. (If pink or bright red colonies will appear then these colonies will be used to culture in Eosin methylene blue agar (EMB) agar). *E. coli* were considered positive if the colonies of pink and greenish black with metallic shine appeared in EMB agar. To obtain pure colonies, positive colonies were sub cultured into EMB agar. The pure colonies were further subjected to biochemical analysis by using API Kit.

Molecular Identification by PCR: For investigation of bacteria, DNA extraction was performed. For PCR reaction 0.2µM of each primer, 0.1mM of each dNTP 0.15mM MgCl2, 1 unit of Taq DNA Polymerase , 2µl of DNA and made to final volume of 25µl with sterile nuclease free water.

Conditions for PCR reactions with *E. coli* include: Denaturation for 5 min at 96◦C, followed by 35 PCR cycles that consist of initial denaturation at 95◦C for 8 minutes followed by denaturation at 95◦C for 45seconds, annealing at 58◦C for 45 secs, extension at 72◦C for 45 sec and final extension at 72◦C for 8 minutes,.

PCR reaction conditions for *S. aureus* include: The initial denaturation takes place at 95°C for 5 minutes, the denaturation at 94°C for 45 seconds, the annealing at 60°C for 45 seconds, the amplification at 72°C for 30 seconds, and the final extension at 72°C for 10 seconds in a thermocycler for 30 cycles. (A 2% agarose gel at 90V was then used to resolve the PCR products.

Antibiotic sensitivity test

Antibiotic sensitivity test was performed in accordance with the guidelines established by the Standard Institute for Clinical Laboratory 2013 (Irshad et. al. 2020). The antibiotic discs impregnated Penicillin (5 mcg), Amoxicillin (5 mcg), Ciprofloxacin (5 mcg), Gentamycin (5 mcg), Erythromycin (5 mcg), Streptomycin (5 mcg) and Oxytetracycline (5 mcg), were used in a study.

Statistical analysis: Descriptive statistics (estimation of frequencies) were used to summarize the incidence of mastitis. The P value was statistically significant at ($P < 0.05$).

Results

Clinical examination of cattle and buffalo against mastitis from six dairy farms showed that, among 30 samples 18 samples were found positive for mastitis. The overall prevalence of mastitis based on Surf Field Mastitis Test (SFMT) and clinical examination was 60% (18/30) (Table 1). At farms level the highest prevalence rate was found between 12% to 24%. A significant difference ($P < 0.05$) was found between cattle and buffalo milk samples for mastitis.

Results

Table 1: Incidence of mastitis showed in different dairy farms.

Dairy farms	Animals tested	Positive animals	Percentage of positive samples
D1	5	2	12%
D2	5	3	18%
D3	5	2	12%
D4	5	4	24%
D5	5	4	24%
D6	5	3	18%
Total	30	18	60%

•All eighteen (mastitis positive) samples were cultured both on Nutrient agar, MacConkey agar, EMB agar.

• Out of total 18 samples *Staph aureus* and *E. coli* growth was found in 15 samples.

•*S. aureus* shows white/yellow color colonies on mannitol salt agar, while *E. coli* shows pink on MacConkey agar and green metallic sheen colonies on EMB.

•For identification of *E. coli* and *S. aureus* API E20 and API Staph Kit was used.

• The biochemical reaction showed that all, ***E. coli*** samples were oxidase negative, catalase positive and gram negative, VP negative and Indole positive. *E. coli* ferment all the basic sugars with the production of both acid and gas.

•The biochemical reaction for ***S. aureus*** isolates were catalase and coagulase positive and able to ferment mannitol salt agar with the production of yellow or white colored colonies. In Gram staining the organism revealed as gram positive, cocci shaped or arranged in clusters and purple-colored colonies.

Polymerase Chain Reaction (PCR)

An attempt was taken to amplify genes, which are typical of *E. coli* and *S. aureus*. For this purpose, 16S RNA gene of S. aureus and *E. coli* were selected for PCR amplification. Template DNA was prepared from the isolates, which were presumptively identified as *E. coli* and *S. aureus*, by their culture and biochemical tests. Template DNA was amplified to detect genes by using specific primer pairs (table 2).

Positive *E. coli* samples have shown 585bp and *S. aureus* have shown 409bp bands on agarose gel.

•16SrRNA gene confirmed the results of Biochemical analysis.

•Out of total samples 6 samples were found positive for *E. coli* and 4 samples were found positive for *S. aureus*.

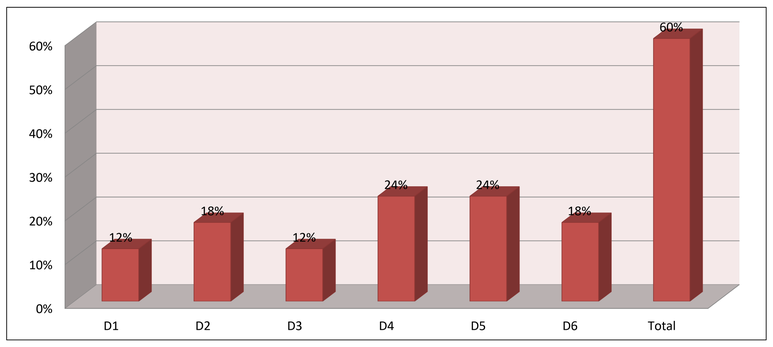

Fig 1: Percentage of mastitis incidence in different dairy farm

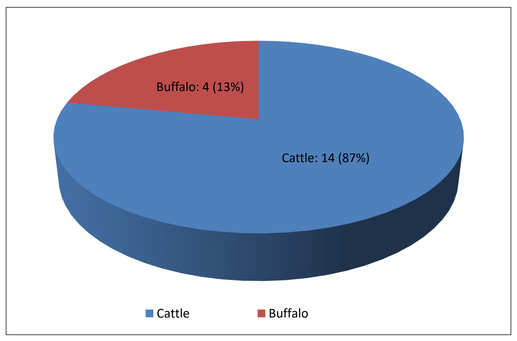

Fig 2: Percentage of positive mastitis animal species

Table 2. PCR conditions for different primers.

Target gene	Primer sequences (5'- 3')	PCR amplification (bp)	Denaturation	Annealing	Extention
ECO-1	GAC CTC GGT TTA GTT CAC AGA	550-bp	95◦C, 45s	58◦C, 45s	72◦C, 45s
ECO-2	CAC ACG GTC ACG CTG ACC A				
S. aureus F	GGA ATT CAA AGG AAT TGA CG GGG C	409-bp	94◦C, 45s	60◦C, 45s	72◦C, 10min
S. aureus R	CGG GAT CCC AGG CCC GGG AAC GTA TTC AC				

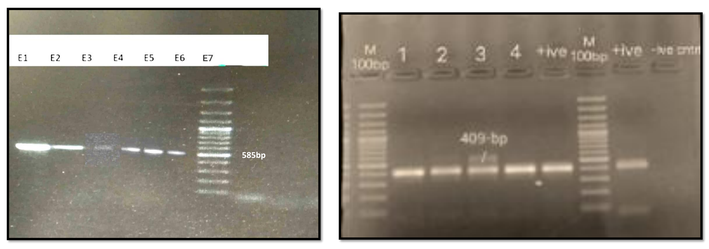

Fig 2 A: *E.Coli* B: *Staphylococcus aureus* Lane:M DNA ladder (100-bp).

Antibiotic Sensitivity Pattern

S. Aureus isolates showed high sensitivity against ciprofloxacin (100%) and Amoxicillin (75%) while gentamycin and streptomycin showed 25% sensitivity. Penicillin and Oxytetracycline are highly resistant against *S. aureus*.

E. coli showed high sensitivity against Ciprofloxacin and Amoxicillin (83.3%), followed by Erythromycin and Gentamycin (66.6%), Oxytetracycline showed sensitivity of (50%) and Penicillin is highly resistant against *E. coli.*

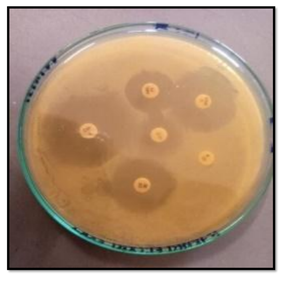

Fig 3 Testing the resistance of microbes in the lab. These petri dishes contain lawns of bacteria (creamy yellow) cultured from raw milk samples. The white discs each contain different antibiotics. Where clear zones appear around the discs, bacterial growth has been prevented by the antibiotics.

Conclusion

Out of 30 samples, 18 samples on the basis of surf field mastitis test (SFMT) and biochemical tests were found mastitis positive and overall prevalence rate was 60%. For further confirmation PCR based on 16SrRNA gene was used to confirm the presence of S. aureus and E. coli. Ten samples were confirmed as S. aureus and E. coli. Antibiotic test was performed to check resistance patterns and it was concluded that both pathogens showed high resistance against penicillin and were highly sensitive to Ciprofloxacin and Erythromycin. According to the results of this study, dairy animals in Rawalpindi are frequently infected with S. aureus and E. coli.