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A Multiplex PCR Assay for Identification of Major Mastitis Causing Pathogens in Buffalo's Raw Milk and Evaluation of Their Sensitivity

Muhammad Safdar
Cholistan University of Veterinary and Animal Sciences

Yasmeen Junejo
Cholistan University of Veterinary and Animal Sciences

Mehmet Ozaslan
Gaziantep University

Abstract: Mastitis is a prevalent issue in dairy herds, particularly among buffaloes, leading to significant economic losses. This study developed a sensitive, specific, and cost-effective multiplex PCR assay for the identification of major pathogens responsible for mastitis in raw buffalo milk. A total of 200 milk samples were collected from various areas of District Multan and tested for the presence of pathogens. Additionally, the antibiotic susceptibility of drug-resistant bacteria was evaluated. The multiplex PCR assay demonstrated 100% specificity and high sensitivity (0.01ng/ul detection limit), identifying multiple pathogens simultaneously.

Keywords: Mastitis, Buffalo milk, Multiplex PCR, Pathogens, Antibiotic susceptibility, Sensitivity, Specificity.

Introduction

Mastitis is one of the most economically significant diseases affecting dairy herds worldwide, particularly in buffaloes, which are a major source of milk in many regions, including South Asia (Bari et al., 2022). It is characterized by the inflammation of the udder, mastitis not only affects milk production but also degrades milk quality, often resulting in contamination and rendering the milk unsuitable for consumption or further dairy processing (Reshi et al., 2015). The condition manifest in two forms: clinical and subclinical. Clinical mastitis shows obvious symptoms such as swelling, redness, and reduced milk yield, whereas subclinical mastitis, which often goes undetected, causes long-term damage to the udder and continuous bacterial shedding into the milk (Ruegg & Adkins, 2024).

Buffalo milk, known for its high nutritional content and versatility in producing dairy products like butter, yogurt, and cheese, is crucial to human diets, particularly in regions like Pakistan. However, its potential benefits are compromised when mastitis-causing pathogens contaminate the milk (Javeid et al., 2020; Saleem et al.). Several pathogens are implicated in mastitis, with the most common being *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Escherichia coli (Javeid et al., 2020; Saleem et al.)*. These pathogens vary in their persistence and impact on the milk supply, often causing severe economic losses to dairy farmers due to reduced milk yield, increased veterinary costs, and, in extreme cases, culling of affected animals (Parveen et al., 2021).

The traditional methods for detecting mastitis pathogens, such as bacterial culturing and biochemical tests, are time-consuming and lack the sensitivity needed for early detection, especially in cases of subclinical mastitis (Parveen et al., 2021; Reshi et al., 2015). In recent years, molecular techniques such as Polymerase Chain Reaction (PCR) have gained prominence due to their rapid, sensitive, and specific detection of pathogens. PCR-based methods can detect pathogens at low levels, even in asymptomatic cases, and are invaluable for early

intervention. However, conventional PCR typically targets a single pathogen per assay, making it less efficient for diagnosing multifactorial infections like mastitis, which often involves multiple pathogens (Javeid et al., 2020; Parveen et al., 2021; Saleem et al., 2019).

To address this limitation, multiplex PCR assays have been developed to detect several pathogens simultaneously in a single reaction. This study focuses on the development of a sensitive, specific, and cost-effective multiplex PCR assay for the identification of major mastitis-causing pathogens in raw buffalo milk (Safdar & Abasıyanık, 2013). Additionally, the study evaluates the antibiotic sensitivity of these pathogens to assess the presence of drug-resistant strains, which is critical for the management and treatment of mastitis (Awandkar et al., 2022). By integrating multiplex PCR into routine diagnostics, the detection of pathogens is significantly expedited, allowing for timely and more targeted treatment interventions. The information gained from this study will not only improve the understanding of pathogen prevalence in buffalo herds but also guide effective antibiotic use, which is crucial in the fight against antimicrobial resistance in dairy farming.

Material and Methods

Sample Collection

A total of 200 buffalo milk samples were collected from four regions in District Multan: Multan Saddar, Multan City, Jalalpur Pirwala, and Shujabad. The samples were collected from January 2022 to February 2024, stored at +4°C, and transported to the Genetic Lab for bacterial culturing and DNA extraction.

Bacterial Isolation and Identification

Milk samples were diluted and cultured on various agar media. The isolated bacteria were subjected to DNA extraction, and specific primers were designed for the identification of pathogenic bacteria via simplex and multiplex PCR assays.

Multiplex PCR Assay

The multiplex PCR assay was optimized to detect multiple pathogens in a single PCR tube. This assay was tested for both specificity and sensitivity, with various concentrations of bacterial DNA ranging from 50ng to 0.001ng.

Antibiotic Susceptibility Testing

Bacteria isolated from the samples were tested for antibiotic susceptibility using antibiotics such as Ciprofloxacin, Azithromycin, Gentamicin, Amoxicillin, Streptomycin, Erythromycin, and Norfloxacin. The effectiveness of these antibiotics was evaluated to determine resistance patterns.

Results

Specificity and Sensitivity of PCR

The multiplex PCR assay exhibited 100% specificity, accurately detecting all targeted pathogens, including *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus parauberis*, *Staphylococcus aureus*, and *Escherichia coli* (Figure is not available). Sensitivity tests showed that the assay could detect bacterial DNA concentrations as low as 0.01ng (Figure is not available).

Pathogen Prevalence

Out of the 200 samples, the following pathogens were detected: *S. uberis* (21%), *S. parauberis* (30%), *S. agalactiae* (19%), *S. dysgalactiae* (20%), *S. aureus* (32%), and *E. coli* (34%) (Figure 1).

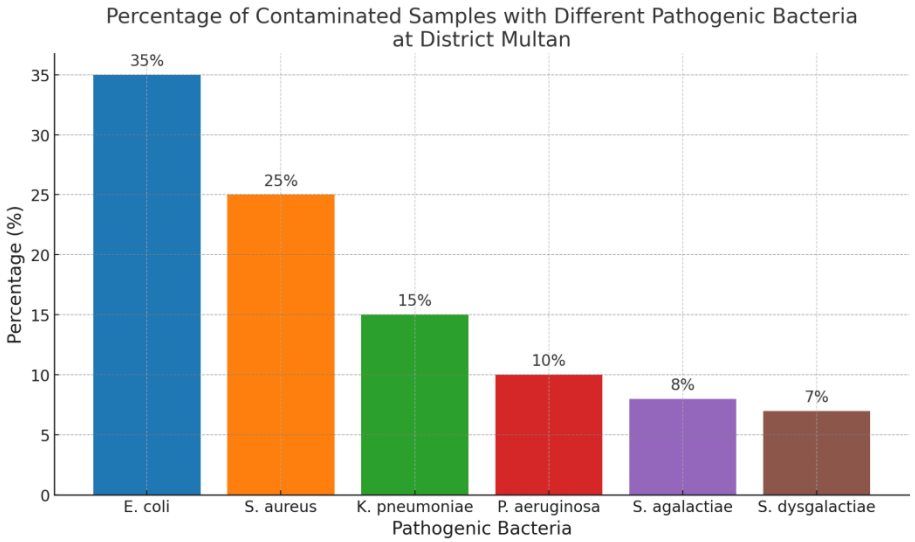

Figure 1. Percentage of contaminated samples with different pathogenic bacteria at district multan

Antibiotic Susceptibility

The antibiotic tests indicated varying levels of susceptibility among the pathogens. *E. coli*, for example, exhibited susceptibility to Ciprofloxacin and Azithromycin, while resistance was noted for Amoxicillin and Streptomycin (Figure 2).

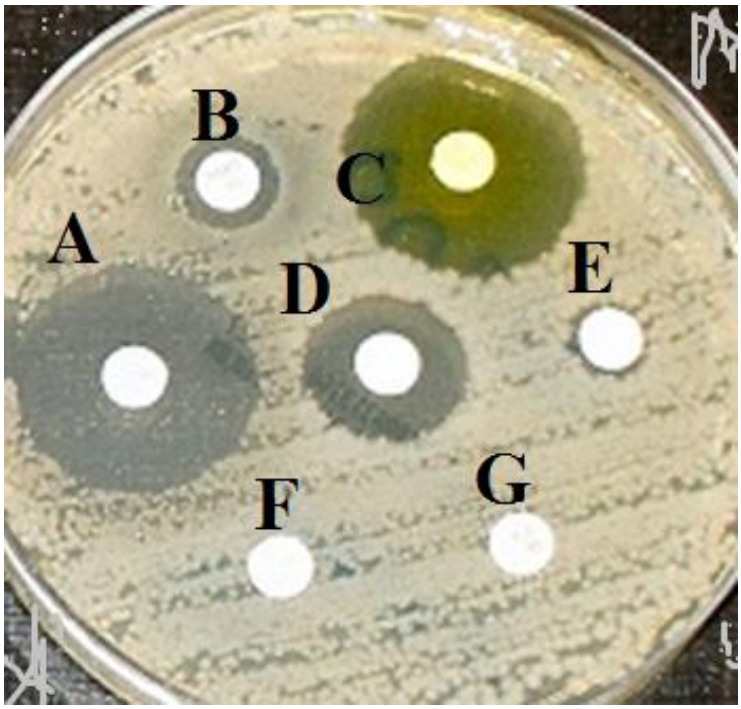

Figure 2. Antimicrobial tests on E. coli such as (A) Ciprofloxacin (B) Azithromycin (C) Gentamicin (D) Amoxicillin (E) Streptomycin (F) Erythromycin (G) Norfloxacin

Discussion

The development and validation of a multiplex PCR assay for the identification of major mastitis-causing pathogens in buffalo milk represents a significant advancement in the rapid diagnosis and management of this

economically critical disease (Parveen et al., 2021; Safdar & Abasıyanık, 2013). This study successfully demonstrated the multiplex PCR assay's high specificity and sensitivity, making it an invaluable tool for the simultaneous detection of multiple pathogens in a single reaction. The ability to identify pathogens such as Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, and Escherichia coli with 100% accuracy offers a clear advantage over traditional methods like bacterial culturing, which are laborious, time-consuming, and often less sensitive.

One of the key findings of this study is the assay's sensitivity, which can detect as little as 0.01ng/ul of bacterial DNA. This sensitivity is crucial for diagnosing subclinical mastitis, a form of the disease where infection persists without visible symptoms, leading to prolonged economic losses due to decreased milk production and quality (Safdar & Junejo, 2015; Safdar & Ozaslan, 2022; Safdar et al., 2023; Zaheer & Safdar, 2020). Early detection through multiplex PCR enables timely intervention, reducing the spread of infection within herds and minimizing the need for costly treatments or culling.

Furthermore, the study's results revealed a high prevalence of mastitis pathogens in buffalo herds in the Multan region, with E. coli and Staphylococcus aureus being the most frequently isolated bacteria (Zaheer & Safdar, 2020). These findings underscore the need for enhanced mastitis control programs in the region, including better hygiene practices, routine screening, and targeted antibiotic therapy. The study also evaluated the antibiotic susceptibility of the identified pathogens, revealing varying levels of resistance, particularly in E. coli isolates. This highlights the growing challenge of antimicrobial resistance (AMR) in dairy farming and the importance of responsible antibiotic use (Parveen et al., 2021; Zaheer & Safdar, 2020).

The development of a rapid, cost-effective diagnostic tool like multiplex PCR not only aids in better management of mastitis but also helps in minimizing the use of antibiotics, reducing the risk of AMR. As the assay is highly adaptable, it can be expanded to include additional pathogens or modified for use in other livestock species, making it a versatile tool for veterinary diagnostics.

Conclusion

The multiplex PCR assay developed in this study offers a rapid and reliable tool for the simultaneous detection of multiple mastitis-causing pathogens in buffalo milk. Its high specificity and sensitivity make it an ideal method for early diagnosis, which is crucial for effective management of mastitis in dairy herds. The study also highlights the importance of monitoring antibiotic resistance to improve treatment outcomes for mastitis-affected animals.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

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Author Information

Muhammad Safdar
Department of Breeding and Genetics, Cholistan University of Veterinary and Animal Sciences
Bahawalpur, Pakistan

Yasmeen Junejo
Department of Physiology and Biochemistry, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan
Contact e-mail: *yasmeen@cuvas.edu.pk*

Mehmet Ozaslan
Department of Biology,
Gaziantep University, Türkiye

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