

MOLECULAR IDENTIFICATION OF PATHOGENS CAUSING CLINICAL AND SUBCLINICAL MASTITIS IN NILI-RAVI BUFFALOES

Yasir Ali, Akhtar Ali*, Ayesha Maqbool, Muhammad Asghar Qazi,
Tanveer Hussain and Asif Nadeem

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ABSTRACT

Mastitis is the leading cause of substantial economic losses due to decreased milk yield and treatment costs in buffaloes. It occurs in two forms; clinical symptoms and subclinical without symptoms but affects the milk yield caused by a wide range of pathogens including bacteria and fungi. Identification of causative agents is important for management and treatment. In this study milk samples from 30 buffaloes of *Nili-Ravi* breed were collected from different dairy farms and investigated for clinical and subclinical mastitis (CMT). Subclinical mastitis cases are followed by biochemical tests (catalase, oxidase, coagulase, etc.) to identify different microorganisms. DNA was extracted and the 16S rRNA (1500 bp) gene was amplified by PCR using universal primers. Amplicons were sequenced and analyzed for identification.

Out of 30 milk samples 13 (43.3%) were positive for clinical mastitis and 09 positive for subclinical mastitis (30%) while other 08 (26.7%) were negative samples having no growth. Out of 22 positive samples the most predominant bacteria were *E.coli* (31.8%) followed by *Staphylococcus* spp. (9.1%), *Pseudomonas aeruginosa* (9.1%), *Pseudomonas* spp. (9.1%), *Enterobacter* spp.

(9.1%), *Staphylococcus aureus* (4.5%), *Klebsiella pneumonia* (4.5%), *Klebsiella* spp. (4.5%), *Stenotrophomonas* sp. (4.5%), *Aeromonas* spp. (4.5%) and *Fungal* spp. (9.1%). Our findings highlight the significant prevalence of clinical (43.3%) and subclinical (30%) mastitis in buffaloes, with *E. coli* being the most common pathogen (31.8%) alongside a diverse range of bacterial and fungal species. The use of 16S rRNA sequencing provided precise pathogen identification, emphasizing the polymicrobial nature of mastitis. These findings underline the importance of regular monitoring and targeted management strategies to control mastitis and maintain dairy farm productivity.

Keywords: *Bubalus bubalis*, buffaloes, milk, clinical mastitis, sub-clinical mastitis, microorganism, 16S rRNA

INTRODUCTION

Mastitis is an inflammatory disease which affects the mammary gland. It is a common disease in dairy animals including cattle and buffaloes. It adversely affects the dairy animals leading to reduced milk yield and economic

losses to the dairy industry (Haxhiaj *et al.*, 2022). Mastitis is classified into clinical and subclinical forms based on duration, severity nature of the exudate, and primary cause (Ahmed *et al.*, 2016). Clinical mastitis is common with visible signs while subclinical has no signs but clots and high somatic cell count in milk and can be confirmed by California mastitis test, electrical conductivity of milk, etc. (Fagiolo and Lai, 2007). Mastitis is a bacterial infection in which a microorganism (pathogen) enters the mammary gland. These pathogens may be contagious, environmental, opportunist, and endogenous.

Buffaloes have high fat content in the milk and are valued in different regions of the world, particularly south Asia. *Nili-Ravi* buffalo is the most commonly domesticated buffalo in the Punjab region. They are prone to sub-clinical mastitis which remains underdiagnosis and poses challenges for the farmers and lead to significant underrepresentation (Stanek *et al.*, 2024). Subclinical mastitis, remains undiagnosed due to not visible sign and limited diagnostic facilities in the buffaloes rearing regions (Sharma *et al.*, 2011).

In Pakistan, 70 to 80% of mastitis is caused by *Staphylococcus aureus*, and *Streptococcus agalactiae* (Duarte *et al.*, 2016) while other organisms such as *E. coli*, *Klebsiella*, *Enterobacter*, *Streptococcus uberis*, *streptococcus faecalis*, *Corynebacterium bovis*, different species of *Staphylococcus* (other than *Staphylococcus aureus*) and *Mycobacterium bovis* etc. also causes mastitis (Hussein, 2008). Sources of mastitis include poor workers' hygiene, lack of proper management, and folded thumb milking can also damage teeth and chances of mastitis are also high in old animals because of fragile teat canals and weak immune. Pathogenesis of bacteria has three phases invasive phase, infection phase, and inflammation phase

(Khan and Khan, 2006).

Efforts to mitigate mastitis in buffaloes must account for their unique husbandary practices and management strategies. Challenges such as poor sanitation and inadequate nutrition exacerbate the prevalence and impact of the disease (Mota-Rojas *et al.*, 2024). Management strategies should include proper housing, sufficient nutrients and pathogen screening at subclinical stage using CMT or molecular detection. The 16S rRNA can detect mastitis microorganisms consistently with accuracy (Usui *et al.*, 2023), particularly in the buffaloes (Salman *et al.*, 2023). We performed the molecular screening of the clinical and subclinical mastitis from the buffalo milk samples.

MATERIALS AND METHODS

A total of 30 composite milk samples were collected during the period (November 2016 to September 2017) from different areas of Rawalpindi-Pakistan. Among these, 15 milk samples were taken from those buffaloes and have clear sign of mastitis and 15 milk samples from subclinical mastitis. For subclinical mastitis California Mastitis Test (CMT) was performed to determine somatic cell counts (SCC). These entire samples were collected aseptically by applying cotton swabs soaked in 70% alcohol. Milk samples of volume 5 ml were collected by discarding the first 2 to 3 streams of milk and brought to laboratory in the ice box.

Bacterial isolation and identification

Milk samples were plated on blood agar and MacConkey agar and incubated at 37°C for 24 h under aerobic conditions. Then bacteria were identified based on colony morphology, hemolysis,

microscopy, and other basic tests like Gram staining, coagulase, oxidase, catalase, indole, etc.

Genomic DNA extraction

Microbial DNA was extracted using a DNA extraction kit (Macherey-Nagel). In the first step, cells were harvested from a culture in a micro-centrifuge tube by centrifugation and then 100 µl elution buffer BE was added and cells were suspended. Then transfer to Nucleospin bead tube type B and add 40 µl buffer MG and 10 µl liquid proteinase K. Agitate for 4 to 12 minutes on a swing mill and add 600 µl buffer MG and mix and then centrifuge at 30 s at $11,000 \times g$. Transfer the supernatant to approx. 500 to 600 µl into Nucleospin Microbial DNA and wash with 500 µl buffer BW and 500 µl buffer B5 and then at last add 100 µl buffer BE.

Primer selection and optimization

Universal primer pair sequence was used: Forward 5'-AGAGTTTGATCCTGGCTCAG-3' and Reverse 5'-AAGGAGGTGATCCAGCCGCA-3' (Edwards *et al.*, 1989). This pair amplifies the 16S rRNA region of the bacterial genome. The PCR was carried out under the following conditions: DNA 2 µl, PCR buffer+MgCl₂ 2.5 µl, Forward primer 1.3 µl, Reverse primer 1.3 µl, dNTPs 0.5 µl, Taq polymerase 0.5 µl, PCR water 16.9 µl of final 25 µl volume. Touchdown PCR protocol was used to amplify with pre-heating (step 1) 95°C for 5 minutes followed by (step 2) 94°C for 30 seconds, (step 3) 57°C for 30 seconds, (step 4) 72°C for 1 minute, (step 5) Go to (2) 10 times, (step 6) 94°C for 30 seconds, (step 7) 52°C for 30 seconds, (step 8) 72°C for 1 minute, (step 9) Go to (6) 25 times, and final extension (step 10) 72°C for 10 minutes. Four µl of PCR products plus two µl loading dye were run using 1% agarose gel and visualized by UV in

Gel doc system and obtained 1500 bp amplicons. PCR products of all isolated bacterial strains were amplified for 16S rRNA gene. DNA ladder of 1 Kb size was run for confirmation of the amplicons.

Sequencing and phylogenetic analysis

The 22 amplicons were sent to (First BASE Laboratories Sdn Bhd (604944-X) Lot 7-1 to 7-3, Jalan SP 2/7, Taman Serdang Perdana, Seksyen 2, 43300 Seri Kembangan, Selangor, Malaysia) for Sanger sequencing after purification by PCR clean-up method. Sequences were then compared to the sequence present in the gene bank using the BLAST program at NCB and confirmed the microbial species. The phylogenetic tree was constructed using MEGA 7 software (Figure 3).

RESULTS AND DISCUSSIONS

Mastitis is an inflammatory disease of mammary gland in dairy animals. It has a great effect on milk yield and leads to economic losses and has clinical and subclinical types (Sharif *et al.*, 2009). We investigated 30 different milk samples of buffaloes for clinical and subclinical mastitis at random. Positive samples for clinical mastitis were 13 (86.7%) and 09 (60%) for sub-clinical mastitis, while 02 (13.3%) were negative for clinical and 06 (40%) were negative for sub-clinical mastitis as shown in Table 1. Other 08 (26.7%) were negative samples, having no growth.

The predominant bacteria were *E. coli* (31.8%) in the 22 samples, followed by *Staphylococcus* spp. (9.1%), *Pseudomonas aeruginosa* (9.1%), *Pseudomonas* spp. (9.1%), *Enterobacter* spp. (9.1%), *Staphylococcus aureus* (4.5%), *Klebsiella pneumonia* (4.5%), *Klebsiella* spp. (4.5%), *Stenotrophomonas* sp. (4.5%),

Aeromonas spp. (4.5%) and *Fungal* spp. (9.1%) as shown in Figure 1.

The 13 (43.3%) samples were positive for clinical mastitis and 09 were positive for subclinical mastitis (30%) while other 08 (26.7%) were negative samples having no growth. This shows that positive cases were high in clinical mastitis as compared to sub-clinical cases and *E. coli* was the most common organism isolated from both mastitis cases followed by *Staphylococcus* spp., *Pseudomonas* spp., *Klebsiella* spp., etc. These findings are similar to the other report in which 260 milk samples have subclinical, 140 have clinical and 100 were negative out of 500 milk samples (Enany *et al.*, 2012). The organisms isolated were *S. aureus*, *E. coli*, *P. aeruginosa*, *K. Pneumoniae*. In another report of 225 milk samples, 11.4% were clinical mastitis, 88.6% subclinical and 16.8% from these milk samples showed no growth. *Staphylococcus aureus* and *Escherichia coli* were the main isolated pathogens (Türkyilmaz *et al.*, 2010).

We have observed *E. coli* as the most prevalent organism in both clinical (30.8%) and subclinical (33.3%) mastitis. entry into the udder from mud, manure, washing the udder with contaminated water, inadequate drying and contaminated environment causing infection and low yield of milk. *E. coli* has been reported as a major source of infection at rate of 14.4% (Gao *et al.*, 2017). The prevalence of microorganisms causing mastitis varies from area to area and from species to species. It has been reported as 10.57% in dairy cattle (Navaneethan *et al.*, 2017), 3.6% in clinical and 5.8% in subclinical mastitis (Enany *et al.*, 2012), 7.51% (Ali *et al.*, 2011) and 11.1% (Srinivasan and Balachandran, 2013) in mastitis. *Staphylococcus aureus* is also the main pathogen that causes a great loss to the dairy industry (Karimuribo *et al.*,

2005). It causes a variety of infection in animal and human (Bartlett and Hulten, 2010). Previously, it has been reported that 70 to 80% of mastitis is caused by *Staphylococcus aureus*, and *Streptococcus agalactiae* in Pakistan (Duarte *et al.*, 2016). We found that *Staphylococcus aureus* incidence rate is 4.5% and another *Staphylococcus* spp. is 9.1%. Prevalence of which is also reported as 35% (236 out of 673) confirmed coagulase-positive *S. aureus* and 65% (437 out of 673) other *Staphylococci* (Hussain *et al.*, 2016). *Staphylococcus aureus* reported as predominant bacterial species in 214 isolates accounting for 49.53% of all the isolates followed by *S. hyicus*, *S. epidermidis*, *S. hominis*, *S. xylosus*, with a percentage of 8.88, 6.54, 1.40, 1.40 respectively (Ali *et al.*, 2008), similar to the 46.3% growth of *Staphylococcus species* (Jena *et al.*, 2015).

We found the growth ratio of *Pseudomonas aeruginosa* is 9.1%, and *Pseudomonas* spp. 9.1% (other than *P. aeruginosa*). It is also reported in studies of (Daly *et al.*, 1999); (Banerjee *et al.*, 2017) as major causing organism. It becomes a major problem in well-managed herds having a low prevalence of pathogens (*Staphylococci* or *Streptococci*). Prevalence rate can be decreased by testing water, shock-treated with chlorine and washing milking equipment regularly (Kirk and Mellenberger, 2006). We identified *Klebsiella pneumonia* at 4.5%, and *Klebsiella* spp. 4.5%, while *Klebsiella* spp., *K. pneumonia*, and *K. oxytoca* were reported as 34. 3%, 16.3%, and 8.1% respectively (Ahmed *et al.*, 2016). *Enterobacter* spp. overall growth is 9.1% which is 7.7% in clinical and 11.1% in subclinical is related to the result of 14.29% (Talukder *et al.*, 2013).

We used universal primers based on 16S rRNA gene sequence (Edwards *et al.*, 1989). The 16S rRNA sequence allows differentiation at the

Table 1. Percentage of isolated organisms from clinical and subclinical mastitis.

Bacterial Species	Clinical		Sub –clinical	
	N	%	N	%
<i>Escherichia coli</i>	04	30.8	3	33.3
<i>Pseudomonas aeruginosa</i>	02	15.4	-	-
<i>Pseudomonas</i> spp.	01	7.7	1	11.1
<i>Klebsiella pneumonia</i>	-	-	1	11.1
<i>Klebsiella</i> spp.	01	7.7	-	-
<i>Staphylococcus aureus</i>	01	7.7	-	-
<i>Staphylococcus</i> spp.	-	-	2	22.2
<i>Enterobacter</i> spp.	01	7.7	1	11.1
<i>Stenotrophomonas</i> sp.	-	-	1	11.1
Fungal spp.	02	15.4	-	-
<i>Aeromonas</i> spp.	01	7.7	-	-
Total	13	100	09	100

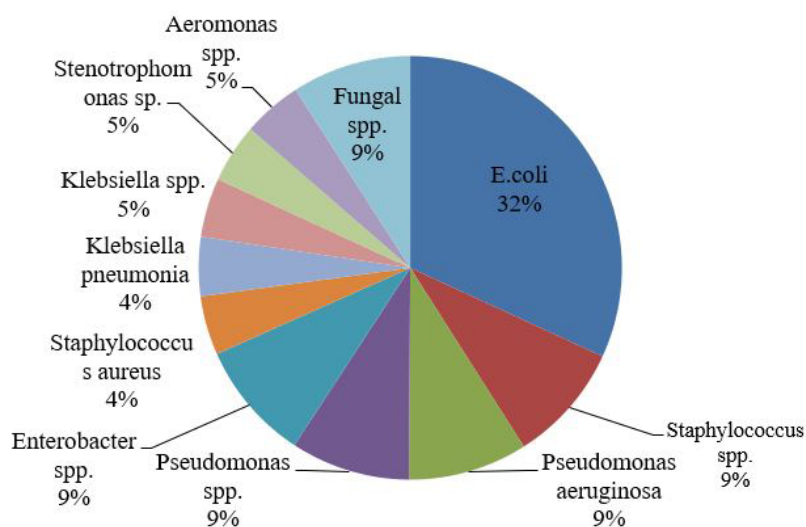


Figure 1. Pie chart of isolated microorganisms.

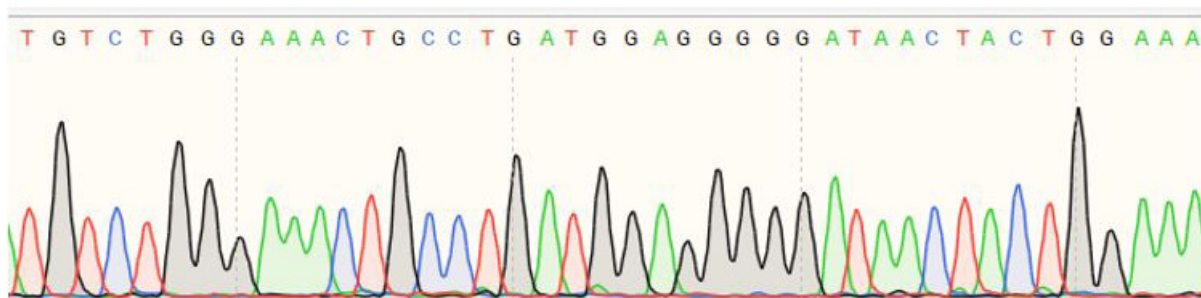


Figure 2. Chromatogram of sequence results.

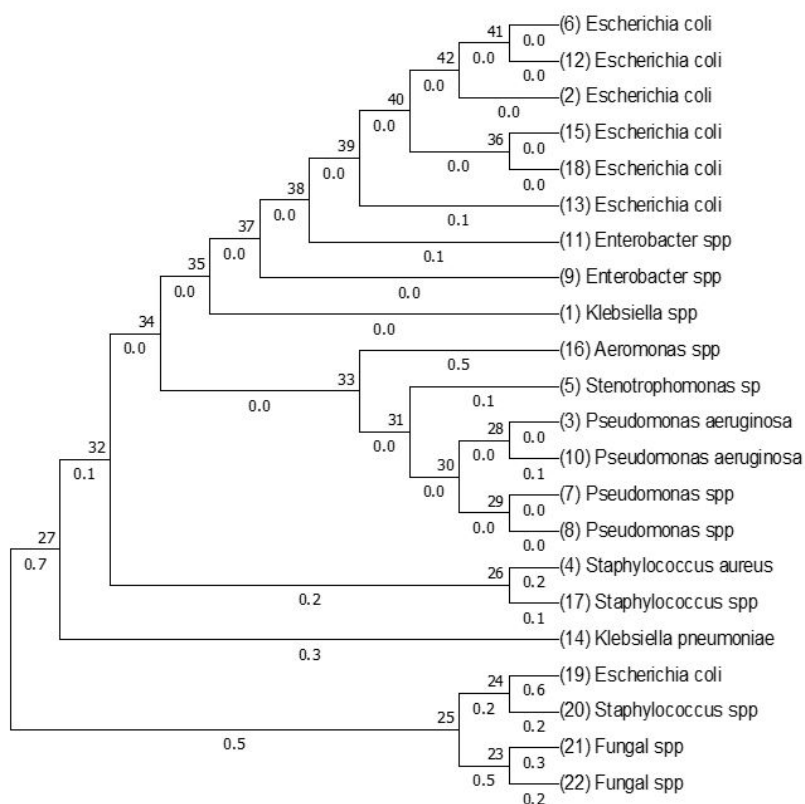


Figure 3. Phylogenetic tree based on 16sRNA gene sequences showing evolutionary relationship among microbial species using the Neighbor-Joining method in MEGA7 software. The sum of branch length for the optimal tree is 5.30380047. The taxa include *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Fungal spp.*, among others.

genus level across all major phyla of bacteria. It can also be useful to correlate with other species (Clarridge, 2004) as shown in Figure 3.

The study shows that subclinical mastitis is a hidden problem in buffaloes causing a reduction of milk and economic loss. It can be removed by good sanitary and hygienic measures. Molecular detection can improve the early detection of pathogens at the subclinical stage. Early detection provides an opportunity to mitigate and manage better treatment and prognosis. Therefore, clinical mastitis might be prevented if sub-clinical mastitis is treated timely.

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