Molecular confirmation of *Escherichia coli* isolates from *Bovine mastitis*

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Abstract
Escherichia coli is a major etiological agent of mastitis in cows leading to great economic losses in dairy production. The present study evaluates use of species specific Polymerase chain reaction (PCR) for molecular detection of E.coli associated with bovine mastitis. Milk samples from 25 bovine clinical mastitis cases presented to the University Veterinary hospitals were subjected to culture and isolation and the isolates were identified based on colony morphology, gram staining and biochemical tests.

Eighteen E. coli isolates obtained were further confirmed by species-specific PCR using primers targeting 16S r RNA gene which yielded amplicons of 232bp specific for E. coli from all samples. Polymerase chain reaction based detection method is less time consuming when compared with cultural isolation and identification. Rapid identification of pathogen will be helpful for initiation of prompt treatment which will aid in fast and successful cure from the condition.

Keywords: *Bovine mastitis, Escherichia coli, PCR.*

Introduction
Mastitis is reported to be one of the most costly diseases of dairy industry worldwide which results in reduction in the quality and quantity of milk. The main pathogens causing mastitis are bacteria which are classified as either contagious or environmental. The most common contagious bacteria associated with mastitis are *Staphylococcus aureus* and *Streptococcus agalactiae* whereas *Escherichia coli*, *Klebsiella pneumoniae* and *Streptococcus uberis* are the predominant environmental pathogens. Another important bacteria *Streptococcus dysgalactiae* is considered as both environmental as well as contagious pathogen[5].

Escherichia coli forms the major etiological agent of intramammary infections in cows leading to acute mastitis and causing great economic losses in dairy production worldwide[2]. Mastitis caused by *E. coli* usually occurs in sporadic form and may range from a subclinical infection to fatal systemic disease. The pathogenesis depends on immune response and genetic makeup of the host and virulence of the strain involved.

The important virulence factors associated with pathogenicity of *E. coli* include toxins, adhesins, invasins, capsule production, the ability to resist serum complement and iron scavenging and the isolates. The successful combinations of these virulence factors will be capable of causing clinical disease[8]. Mastitis due to *E. coli* is more common in cows around parturition and during early lactation and most often associated with local and sometimes severe systemic clinical symptoms. The severity of *E. coli* mastitis is mainly determined by cow factors than by pathogenicity of the organism and the environmental factors[3].

In *E. coli* mastitis, the host defence status is an important factor determining the outcome of the disease. Early and prompt diagnosis is more important in the management of mastitis. Conventional method of culture and identification of the pathogen is laborious and time consuming. Molecular assay such as polymerase chain reaction (PCR) is less time consuming and takes less than 24 hours to complete while identification of bacteria to the species levels by conventional microbiological and biochemical methods requires more than 72 hours[1]. The present study evaluates use of species specific Polymerase chain reaction for molecular detection of *E.coli* associated with bovine mastitis.

Material and Methods
Milk samples from the affected quarters were collected under aseptic conditions from 25 bovine clinical mastitis cases presented to the University Veterinary hospital during 2016-17. The samples were then subjected to culture and isolation and the isolates were identified based on colony morphology, gram staining and biochemical tests. Genomic DNA of the identified *E. coli* isolates was extracted using commercial DNA extraction kit following the manufacturer's instructions. The species-specific 16S r RNA gene of *E.coli* were amplified by PCR using the primers and protocol as in table 1 to 3.

Table 1
Species specific primers for *E.coli*

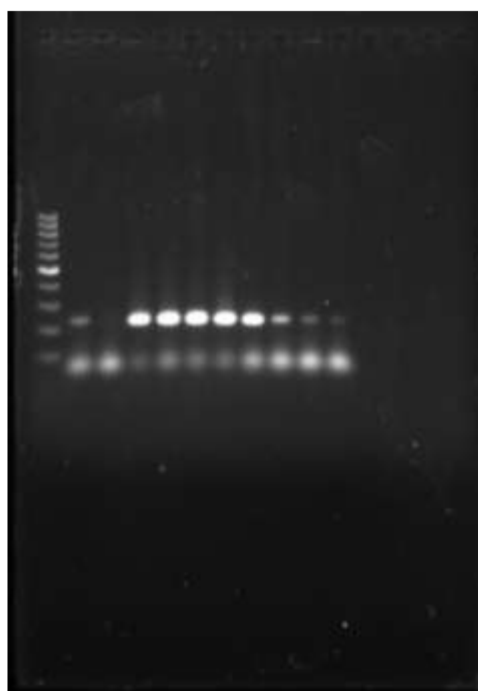
Primer	Sequence	Target Gene	Product Size
Eco 223	Forward: 5' ATC AAC CGA GAT TCC CCC AGT 3'	16S r RNA	232 bp
Eco 455	Reverse: 5' TCA CTA TCG GTC AGT CAG GAG 3'		

Table 2
PCR Reaction Mixture

Master Mix	12.5 µl
DNA Template	5.0µl
Forward Primer	1 µl
Reverse Primer	1 µl
Nuclease Free water	5.5 µl
Total	25µl

Table 3
PCR Conditions

Step	Temp and Time	Cycles
Initial Denaturation	94° C for 2 min	1
Denaturation	94° C for 45 sec	
Annealing	60° C for 1 min	35
Extension	72° C for 2 min	
	72° C for 10 min	1



1. Positive control
2. 2-Negative control
3. 3-10 samples positive for E.coli

Fig. 1: PCR products specific for *Escherichia coli*

The PCR products were analysed by agarose gel electrophoresis, ethidium bromide staining and UV transillumination and documented using gel documentation system.

Results and Discussion

Culture of milk samples from 25 animals with clinical mastitis yielded microbial colonies from 21 samples which were identified as *Escherichia coli* (18), *Staphylococcus aureus* (2) and *Streptococcus* spp. (1) based on colony characters, gram's staining and biochemical tests. The DNA extracted from *E.coli* isolates was subjected to PCR using species specific primers targeting 16S r RNA gene which yielded amplicons of 232 bp specific for *E.coli* (Fig. 1) from all the 18 samples.

Bacteriological culturing of the milk is the routine method used to identify the etiological agent in mastitis but in severe clinical cases, the results will be obtained late, most often by 48-72 hours to affect the specific treatment. Moreover, negative results with no growth of *E. coli* in culture are common because the cow's immune system has destroyed the bacteria by the time the milk sample is collected.

Polymerase chain reaction based detection method is more sensitive and less time consuming when compared with cultural isolation and identification. This method can also be used directly with mastitic milk which again will reduce the time required[1]. Earlier detection of pathogen will help in initiation of prompt treatment which will aid in fast and successful cure from the condition.

Escherichia coli is the most commonly isolated coliform species from intramammary infections and clinical mastitis. Prevalence of mastitis due to coliform infections was recorded widely throughout the world with varying rates. Results of the present study revealed higher prevalence of *E. coli* (85.74%) which is somewhat similar with Bradely[2] and Khaled et al.[7] Gangwal and Kashyap[5] and Hawari and Al-Dabbas[6] detected coliforms in the milk of 31.9% of the mastitic quarters in Jordan.

On the other side, El-Khodery and Osman[4] detected coliforms in 80.36% of the affected buffaloes. *Escherichia coli* was isolated from 21.28% quarter milk samples in Meerut, India by Verma et al.[10] Poor hygiene, husbandry and milking techniques were considered as the predisposing factors to environmental mastitis as well as milk contamination as stated by Maniruzzaman et al.[9] The dry period is the time for acquiring new subclinical infections and research indicates that 50% of the clinical coliform infections, occurring in the first 90 days of lactation, actually started in the dry period. The times of greatest risk for acquiring new infections during the dry period are two weeks after dry off and the prefresh/calving period.

Conclusion

Cows with severe cases of coliform mastitis should be treated with systemic and supportive therapies including fluids, anti-inflammatories and systemic antimicrobial therapy. Maintaining a clean, dry environment for cows is important to reduce exposure of the teat end to dirt and manure. This includes frequent scraping of alleys and holding pens and keeping stalls or areas where cows lie down clean and well-bedded. Pre- and post-milking teat disinfection and good milking practices are also important.

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