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Molecular detection of *Escherichia coli* in caprine mastitis

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Abstract
Mastitis is one of the most expensive illnesses to deal within milch animals. Mastitis is divided into two types: subclinical and clinical. A total of 82 milk samples were collected from apparently healthy (52) and clinical mastitis (30) cases of goats in and around the Anand district. Total 76 bacterial isolates from mastitic milk were identified based on morphological, cultural, and biochemical characteristics. About 7.3% cases were found positive by cultural examination for *Escherichia coli* and molecular methods by PCR using species specific primer of *Escherichia coli.*

Keywords: *Escherichia coli*, coliform mastitis, PCR, clinical mastitis, subclinical mastitis

Introduction
Goat and Sheep are two major important livestock species in the Livestock Production System. A large part of the rural landless population and marginal farmers are dependent on them. Goat (*Capra hircus*) is a versatile animal and known as a poor man's cow in India. Goats are a major source of meat and dairy products (Haenlein, 2004) [12]. Goats adapt well to poor agricultural lands as well as arid areas. Goat milk is cheap, wholesome, easily digestible and nutritious (Panicker, 2015) [21]. After Dairy cattle, Sheep and Goat are the most important group of milk-producing animals in both temperate and tropical regions (Devendra and Coop, 1982) [9].

Inflammation of the mammary gland (udder) causing chemical and physical changes in milk produced by goats is called mastitis (Radostits *et al*., 2000) [23]. Mastitis causes economic losses in the forms of disposal of milk, treatment cost, reduced milk efficiency in following lactations. Mastitis is more frequent in goats due to intensive and semi-intensive management practices. It appears in two forms: sub-clinical and clinical, and may be acute or chronic.

Bacterial Etiological agents causing mastitis in goats are: *Staphylococcus aureus*, *coagulase-negative Staphylococci* (CNS), *Escherichia coli (E. Coli), Pseudomonas spp., Bacillus spp., Klebsiella pneumoniae, Streptococcus spp., Mycoplasma spp., Methicillin-resistant S. aureus (MRSA), Coliforms, Micrococci, Corynebacteria, Pasteurella* spp. and *Actinomyces spp.* have been reported by Kalogridou-Vassiliadou (1991) [14]; Deinhofer (1995) [8]; Mishra *et al.* (1996) [19]; Bergonier *et al.* (2003) [3]; da Silva *et al.* (2004) [6]; Amin *et al.* (2011) [1]; Marogna *et al.* (2012) [18]; Cortimiglia *et al.* (2015) [5]; Kumar *et al.* (2016) [16] and Gabli *et al.* (2019) [11].

E. coli causes coliform mastitis (Radostits *et al.*, 2000) [23]. Coliforms in milk could be caused by bacteria on teats, teat ends, teat canals, udder surfaces, mastitic udders, and contaminated water used to clean milking systems. Coliform bacteria spread from a contaminated region to the mammary gland, growing in the gland as they pass via the teat canal (Jayarao & Wolfgang, 2003) [13].

Material and Method
Cultural examination and morphological characteristics of bacterial isolates
A total of 82 milk samples were opened under aseptic conditions and streaked on 5 percent sheep blood agar (BA) using the sterile platinum loop for initial bacterial isolation, and the plates were incubated at 37 °C for 24-48 hours. The plates were checked for bacterial growth after incubation, and the morphological properties of bacterial colonies were reported. Each isolate's pure culture was determined based on morphological, cultural, and biochemical traits.

Each isolate's pure culture was then cultured on eosine methylene blue agar, where it will produce greenish metallic sheen. On Macconkey agar it will produced pink colour colonies, due to lactose fermenting property of *E. coli.*

Biochemical Characterization of Bacterial Isolates

1. Potassium Hydroxide (KOH) String test

On a clean microscopic slide, a loopful culture was taken from the blood agar and combined with an equivalent amount of 3 percent potassium hydroxide (KOH). The loop was lifted at many intervals after full mixing to test if the gel was developing. The *E. coli* will produce gel during the test.

2. Oxidase-Fermentation (O-F) test

The organisms were inoculated in two tubes of O-F basal medium by stabbing with straight wire. One tube was covered with a layer of soft paraffin. The tubes were incubated at 37 °C for up to 14 days.

3. Indole test

A few drops of xylene were added to the isolate's 2-day old culture in 2-3 ml of tryptone water (HiMedia) and thoroughly stirred to dissolve indole, followed by 0.2 ml of Kovac's reagent (HiMedia). The positive reaction was defined as the pink coloring of the xylene layer, whereas the negative reaction was defined as the yellow color.

4. Methyl-Red (MR) test

A 4-5-day growth of the isolate in 4-5 ml of glucose-phosphate peptone water (GPW) (HiMedia) was added five to six drops of MR reagent (HiMedia). The production of a pink or bright red hue was regarded as a positive reaction, while the production of yellow color was considered a negative reaction.

5. Voges-Proskauer (VP) test (Barritt's method)

To a 5-day old growth of the isolate in 5 ml GPW, 3 ml of 5% alpha-naphthol in absolute ethyl alcohol was added, followed by 1 ml of 40% (w/v) KOH. After 20 minutes, the development of a bright red hue indicated a positive reaction, whereas the yellow tint indicated a negative reaction.

6. Citrate utilization test

The culture was put on Slant of Simmon's citrate agar (HiMedia) and cultured at 37 °C for 7 days. A good reaction was defined as the development of an intense blue hue on the medium, whereas an inability to utilize citrate was shown by no change in the medium's color.

7. Urease test

Slants of urea agar (HiMedia) were inoculated, incubated at 37 °C for 3 days, and monitored. The development of a pink hue in the slant reaction indicated a positive reaction, but the inability to hydrolyze urea was indicated by no change in the color of the medium.

PCR-Based Identification of *E. coli*

DNA Extraction from the colony

With slight adjustments, the colony template DNA was produced according to Antony *et al*. (2006) [2]. The bacterial colonies were picked up from the blood agar plate or the brain heart infusion agar plate and suspended in 100 µL milli Q water. For 15 minutes, those samples were boiled. Centrifugation was employed to remove the cell debris, and 5 µl of the supernatant was employed as a template DNA.

Quantitation and Quality assessment of DNA

Using the standard that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml, DNA was measured using an ND-1000 spectrophotometer (Nanodrop Technologies Inc., USA).

Concentration of DNA (µg per ml) = Optical Density at 260 × Dilution factor × 50

Where 50 is the concentration of DNA at one O.D.

To determine sample concentration and purity, the UV absorbance was measured at 260 and 280 nm wavelengths. The O.D. ratio of 260:280 was used to determine the purity of DNA. DNA with a purity ratio of 1.8-2.0 was deemed to be of good quality.

PCR Primers

Primers specifically used for *E. coli* was reported by Riffon *et al*. (2001) [24].

Table 1: Details of primers used for amplification of target genes for identification of *E. coli*

Primer Sequence (5'-3')		Size of amplified products (bp)
Eco 223	ATCAACCGAGATTCCCCCAGT	232
Eco 455	TCACTATCGGTCAGTCAGGAG	

Table 2: Steps and conditions of thermal cycling for primers in PCR

Primers (forward and Reverse)	Cycling Conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
Eco 223 Eco 455	95 °C 5 min	94 °C 45 sec	64 °C 45 sec	72 °C 90 sec	72 °C 10 min
Repeated for 35 cycles					

Result

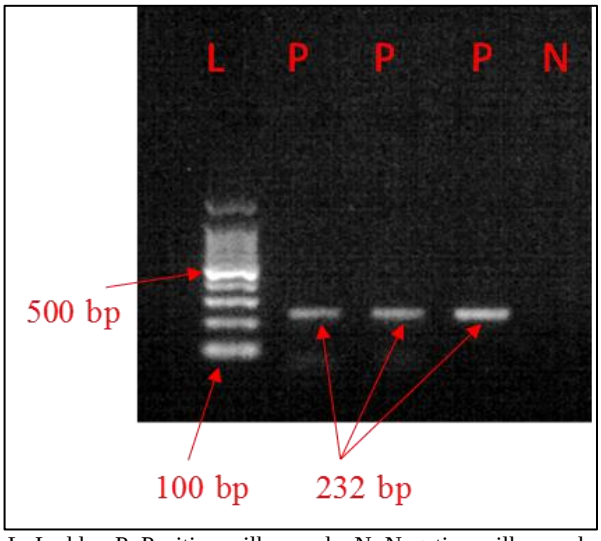
Based on the above morphological, cultural, and biochemical features, 7.3% (6/82) of *E. coli* isolates (two isolates from clinical mastitis and four isolates from sub clinical mastitis) could be identified in the current investigation.

E. coli isolates have greyish color colonies on blood agar, are Gram negative bacilli, positive KOH test (Fig:1), catalase positive, oxidase negative, pink color colony on MacConkey agar (Fig;2), the greenish metallic sheen on EMB agar and IMViC pattern ++ - -. The TSI reaction was seen in all isolates as yellow slant and yellow butt, without gas generation.

The *E. coli* specific DNA sequence coding for the 23S rRNA was amplified using PCR. All six isolates were found positive by PCR, yielded 232bp size on agarose gel electrophoresis (Fig; 3).

Fig 1: Positive KOH test for *E. coli*

Fig 2: Lactose fermenting pink colonies of *E. coli* on MacConkey agar



L: Ladder, P: Positive milk sample, N: Negative milk sample

Fig 3: Agarose gel electrophoresis of amplified PCR product of *E. coli* of 232 bp

Discussion

The *E. coli* were present in 7.3% cases of clinical as well as of subclinical mastitis in goats. Various researchers such as Deinhofer (1995) [8] 1.7%, Mishra *et al.* (1996) [19] 18.2%, Moshi *et al.* (1998) [20] 26.69%, Kostelič *et al.* (2009) [15] 2%, Amin *et al.* (2011) [1] 6.67%, Patel (2012) [22] 5.97%, Ferdous *et al.* (2018) [10] 22.5%, 6.9% and Mahlangu *et al.* (2018) [17] 5.9% were isolated the *E. coli* from mastitis.

All six *E. coli* strains identified by cultural and biochemical methods were examined for PCR-based identification using DNA sequence coding for the 23S rRNA gene and all were confirmed to be positive, generating a 232 bp amplification result. PCR-based *E. coli* identification has already been carried out by several researchers, including Riffon *et al.* (2001) [24], Bhanderi (2008) [4], Patel (2012) [22] and Vatalia *et al.* (2020) [25].

E. coli is the major organism in coliform mastitis in goats, it is mostly responsible for sub clinical mastitis rather than the clinical mastitis. It will produce inflammatory changes in udder and producing the flakes in milk. It affects the productivity of animal, in term of milk production. If problem persist for long time, it will cause the serious complication like gangrenous mastitis and leads to permanent production losses.

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