# Molecular detection of *Escherichia coli* from raw milk and milk powder of Camelus dromedarius

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## Abstract

The present study was proposed for molecular detection of *Escherichia coli* isolated from raw camel milk and camel milk powder samples by using traditional culture methods and then confirming it with molecular techniques. A total of 200 samples of raw camel milk (n=100) and camel milk powder (n=100) were aseptically collected from ICAR-National Research Centre on Camel, Jorbeer, Bikaner (Rajasthan). The samples were isolated on Mac Conkey agar and sub-cultured on Eosin Methylene blue agar to obtain pure isolated colonies. The isolated were further subjected to genotypic confirmation using species-specific primers targeting the *16srRNA* gene, *stx1* gene, and *stx2* gene through conventional polymerase chain reaction. The results revealed that 28% (28/100) of raw camel milk samples tested positive for *Escherichia coli*, while only 5% (5/100) of camel milk powder samples tested positive. All 33 isolates confirmed the presence of the *16s rRNA* gene, while the presence of *stx1* and *stx2* were not detected in *E. coli* isolates. On statistical analysis by Fischers test, it was found that there was significant difference in isolation of *E. coli* from raw camel milk odds ratio 7.32 (95% CI 2.62, 25.49). These findings suggested that there is a higher prevalence of *Escherichia coli* contamination in raw camel milk as compared to camel milk powder.

**Keywords**: Raw camel milk; *Escherichia coli;* camel milk powder; PCR

# Introduction

Foodborne diseases are a significant public health issue worldwide, with developing countries bearing most of the burden (Carbas *et al*. 2013). Milk and dairy products are consumed daily by billions of people because of their nutritional value (Ranjbar *et al*. 2018). Dromedary camels can produce significant amount of milk despite living in semiarid and arid climate zones. The milk from camel is valuable containing minerals like sodium potassium, copper, zinc, mercury and iron, rich in vitamin C and unsaturated fatty acid (Patel et al., 2016; Izadi, 2019). The raw milk can be microbially contaminated through multiple sources like udder, cleaning water utensils, dairymen and dust. Additionally, its nutritional value, which supports microbial growth varies depending on its storage (Saeed *et al*., 2022). *Escherichia coli* (*E. coli*) can be transmitted to raw milk and milk products by contamination during milking process along with poor hygienic practices (Garbaj *et al*. 2016). *Escherichia coli* is a facultative anaerobe and one of the normal inhabitants in the human and animal intestinal tracts. *E. coli* is categorised based on its virulence factors such as attaching and effacing *Escherichia coli* (AEEC), enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *Escherichia coli* (EPEC), enterohaemorrhagic *Escherichia coli* (EHEC), and Shiga toxin-producing *Escherichia coli* (STEC or VTEC) (Tassew, 2015; Saba *et al.,* 2015). Ruminants, especially bovines, are the major reservoir of STEC (Savoye *et al*., 2011). STEC strains are extremely infective to humans in low infectious doses, causing many food-borne diseases via the consumption of contaminated water or food (Dweik *et al*., 2012). These Shiga toxin-producing *Escherichia coli* contribute toward bloody gastrointestinal disorders including non-bloody and bloody diarrhoea, and haemolytic uremic syndrome in infected humans (Guth *et al*., 2012). Shiga toxin-producing *Escherichia coli* constitutes a subgroup of *Escherichia coli* capable of producing potent toxins known as Shiga 1 and Shiga 2 toxins (*stx1* and *stx2*). Foodborne diseases associated with STEC have been reported worldwide (Etcheverria and Padola, 2013). Food intoxication cannot be occurred in the absence of *stx*.

Traditional methods for detecting and identifying bacteria involve culturing, counting, and isolating specific colonies. Initially, bacterial subtyping was performed by examining their phenotypic characteristics, including serotyping, bio typing, and phage typing (Kretzer *et al*. 2007). In PCR, the *16srRNA* gene is an ideal amplification target because it is present in all bacteria and exhibits significant variation among different species and strains (Srinivasan *et al*. 2015). In the current study conventional PCR method was adopted for the identification and characterization of *E. coli* in raw dromedary camel milk and dromedary camel milk powder samples. By taking into consideration all these facts, the current study was planned with the objectives of molecular detection and characterization of *E. coli* isolated from raw camel milk and camel milk powder samples using *16srRNA* gene. The antimicrobial susceptibility patterns of isolated bacteria were also studied.

# Materials and Methods

**Sample collection**

A total of 200 samples of raw camel milk (n=100) and camel milk powder (n=100) samples were collected aseptically during September 2023 to January 2024 from the R & D cells of National Research Centre on Camel (NRCC), Jorbeer, Bikaner (Rajasthan). All the aseptic precautions were taken during sample collection to avoid external contamination. Milk samples were collected aseptically in sterile sampling bottles and milk powder samples were collected in sterilized plastic bags for the present investigation. On a daily basis, a total of 10 samples were collected and analysed. These included 5 samples of raw milk and 5 samples of milk powder. The milk powder samples were freeze dried. Samples were handled with sterile gloves, transported to the laboratory using ice packs, and stored in a chilled condition at 4 °C. Each sample was perfectly mixed before subjecting to bacteriological examination of *Escherichia coli*.

**Isolation and identification of *Escherichia coli***

Isolation and identification of *Escherichia coli* was accomplished within few hours. The samples of raw dromedary camel milk and milk powder were subjected to aerobic cultivation using standard methods as described earlier (Amita *et al*., 2024). Briefly collected 10 mL of each dromedary raw camel milk sample were added to 90 mL MacConkey broth and for dromedary camel milk powder, 10 gm samples were added to 90 mL MacConkey broth and homogenized. The homogenate was incubated overnight at 37 ℃ for enrichment of *Escherichia coli*. The sample was then streaked on MacConkey agar (MCA) and incubated for 24 hrs at 37 °C. After 24 hrs of incubation, lactose-fermented, pink-colored colonies were sub-cultured on Eosine Methylene Blue (EMB) agar plates for isolation of *Escherichia coli* and incubated for 24 hrs at 37 °C. Colonies showing greenish metallic sheen were regarded as *E. Coli* and were preserved for further bacterial identification of *Escherichia coli*. Simultaneously another single colony with similar characteristics was picked from agar plate and stained with Gram's stain. This presumptive *Escherichia coli* was inoculated onto nutrient slants and after incubation at 37 °C for 18-24 hrs, the slants were stored at 4 °C for further characterization. The recovered isolates were confirmed based on their morphological, cultural, and biochemical characteristics and further the isolates were confirmed by amplification of *E. coli* specific *16srRNA* gene.

**Isolation of DNA**

The genomic DNA isolation of *Escherichia coli* was undertaken using by Nucleo-pore gDNA fungal/bacterial mini kit (Genetix Biotech) by following the manufacturer's instructions supplied along with the kit. An overnight-incubated broth culture (1.5 mL) of the test organism was taken in a capped collection tube and processed as per the method described.

**Molecular identification of *E. coli* by conventional Polymerase chain reaction**

All the *E. coli* isolates were amplified using *16srRNA* gene of *E. coli* for confirmation (Table 1). The confirmed *E. coli* samples were then subjected to amplification of two virulence genes (*stx1 and stx2*) (Table 1).

PCR reaction (25 µl) consisted of primers (1µl each), DNA template (3µl), 2X Dream Taq Green Master Mix (12.5 µl) and nuclease free water (7.5µl) as mentioned in Table 02. The PCR was performed in Verti Thermal Cycler (Applied biosystem) with initial denaturation of 95℃ for 5 min, denaturation at 94 ℃ for 1 min, annealing at 50.2 ℃ for 1 min and primer extension at 72 ℃ for 1 min with 36 cycles. The final extension was done at 72 ℃ for 7 min.

Gel electrophoresis was used to visualize the target sequence amplicons. DNA samples were loaded into wells along with a DNA molecular weight marker (100 bp). Agarose gel (2%) along with ethidium bromide @0.5 µg/mL was used. Electrophoresis was then performed in 0.5 X TAE Buffer at 5V/cm for 60 minutes allowing for the separation and visualization of the amplified DNA fragments, which were compared to the DNA ladder for size estimation under gel documentation system.

# Results and Discussion

In the current study, a total of 33 samples (28 isolates from raw camel milk and 5 isolates from camel milk powder) were obtained of *E. coli*. All 33 isolates of *Escherichia coli* were selected for molecular confirmation by using species-specific primer targeting *16s rRNA, stx1 and stx2* genes. The samples were analysed in the laboratory using different cultural, biochemical and staining methods. The red/pink colonies grown on MacConkey agar (Fig. 1) were further streaked on EMB agar and *E. coli* metallic green colonies with a dark or purple centre were confirmed as *E. coli* strains (Fig. 2), small rod-shaped Gram-negative bacilli on Gram's staining (Fig.3).

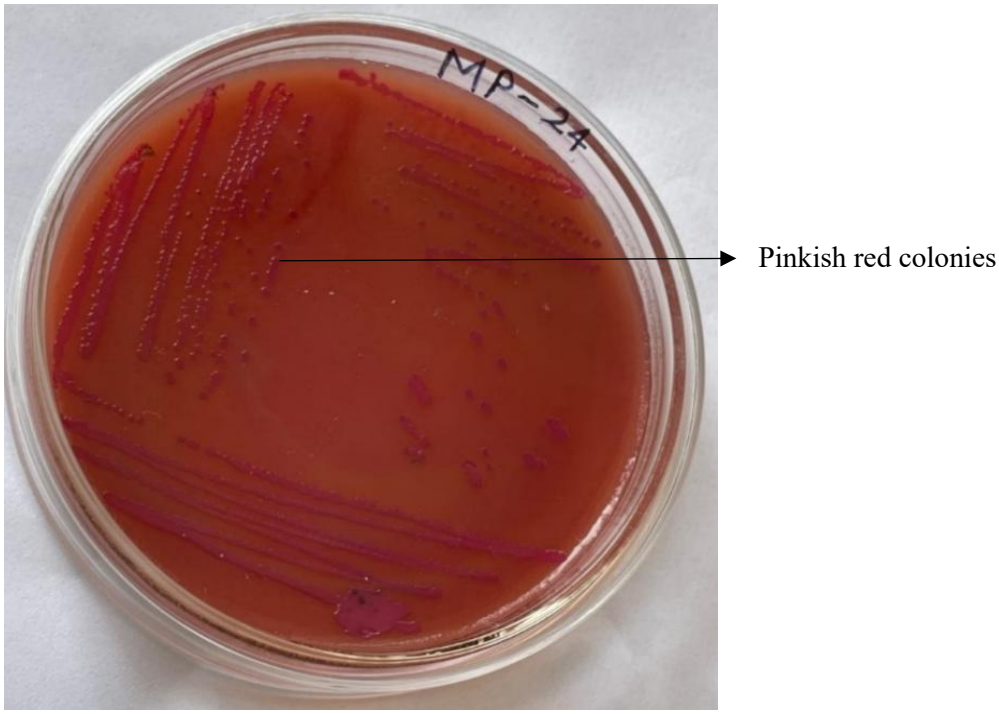


**Fig 1.** Isolation of *E. coli* on MacConkey Agar

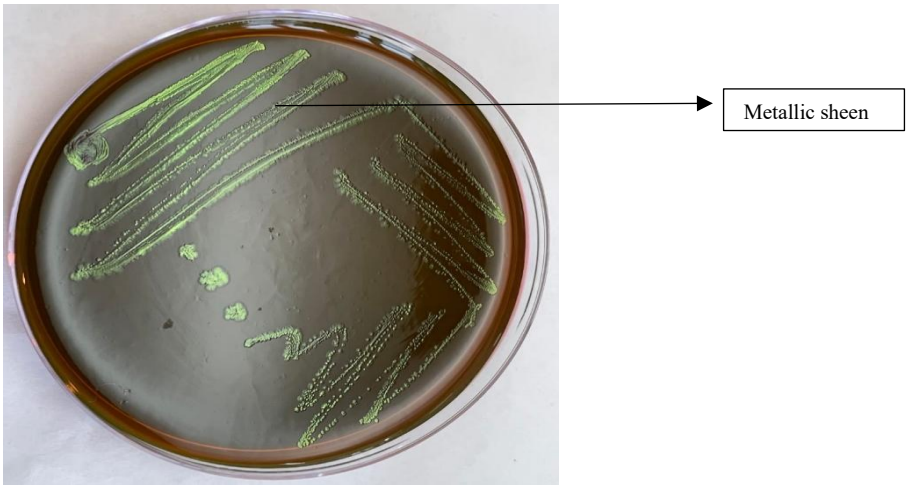


**Fig 2.** Isolation of *E. coli* on EMB Agar

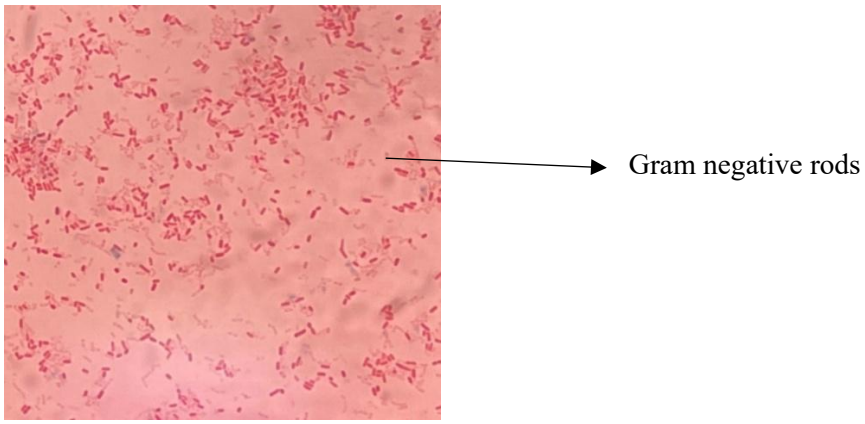


**Fig 3**. Gram staining of *E. coli*

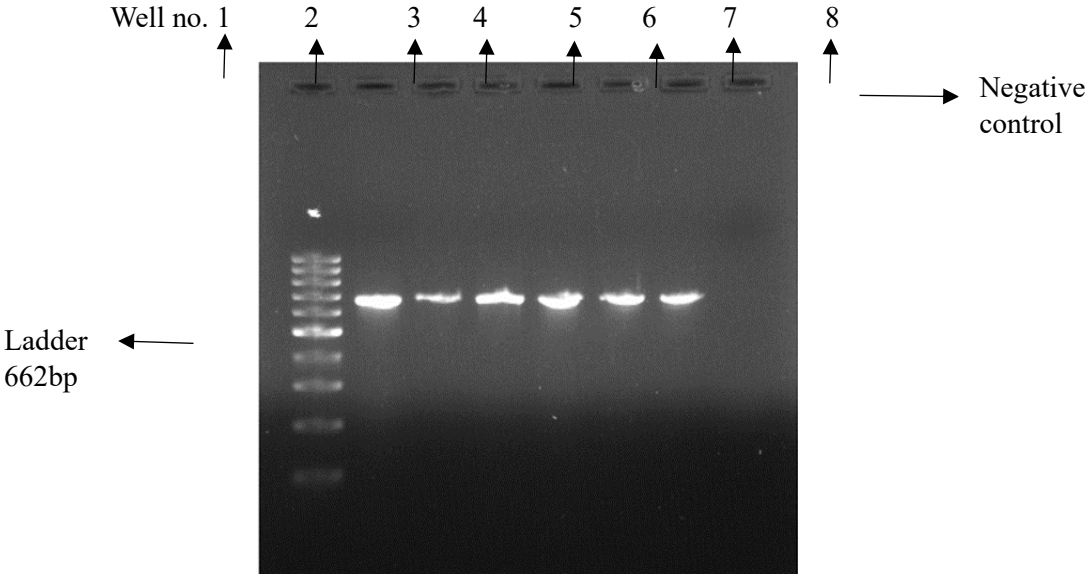


**Fig 4**. *16SrRNA* gene-based PCR for *E. coli*(662bp)

**Table 1**: Primers used for amplification of *Escherichia coli* isolates from raw dromedary camel milk and dromedary camel milk powder samples

| S. No. | Oligo Name | Sequence (5-3) | Size of Amplified Product (bp) | References |
|---|---|---|---|---|
| 1. | *16s rRNA* | F- 5'GCTTGACACTGAACATTGAG 3'<br>R- 5' GCACTTATCTCTTCCGCATT 3' | 662 bp | Khaled *et al*. (2010) |
| 2. | *stx1* | F- 5' ATAAATCGCCATTCGTTGACTAC 3'<br>R- 5' AGAACGCCCACTGAGATCATC 3' | 180 bp | Paton and Paton (1998) |

**Table 2**: Reaction mixture for PCR of *Escherichia coli* isolates

| Reagents | Volume (µl) |
|---|---|
| Dream Taq Green Master Mix, 2X | 12.5 |
| Primer-F(25pM/µl) | 1.0 |
| Primer-R(25pM/µl) | 1.0 |
| DNA template | 3.0 |
| Nuclease free water to make final volume | 7.5 |
| Total | 25 |

**Table 3**: Prevalence of *Escherichia coli* isolated from raw camel milk and camel milk powder samples:

| S. No. | Sample type | No. of samples | No. of samples positive for *E. coli* | Prevalence of *E. coli* (%) | 95% Confidence Interval | p- value | (Fisher' Exact Test) Odds ratio |
|---|---|---|---|---|---|---|---|
| 1. | Raw camel milk | 100 | 28 | 28 | 0.197, 0.3801 | 0.05 | 7.32 (95% CI 2.62, 25.49) |
| 2. | Camel milk powder | 100 | 5 | 5 | 0.0185, 0.118 | 0.28 | |

**Table 4**: Molecular detection *16S rRNA* and virulence genes for *Escherichia coli* isolates obtained from raw camel milk and camel milk powder samples

| Sample (Isolates) | Molecular detection | | |
|---|---|---|---|
| | *16S rRNA* | *stx1* | *stx2* |
| Raw camel milk (28) | + | - | - |
| Camel milk powder (5) | + | - | - |

The presence of *E. coli* was observed more in raw dromedary camel milk than in dromedary camel milk powder. On analysis by Fischer's Exact test, a significant difference was observed in prevalence of *E. coli* between raw dromedary camel milk and dromedary camel milk powder with odd's ratio 7.32 (95% CI- 2.62-25.49; $p \leq 0.05$), which indicates that there are 7.32 times higher chances of isolating *E. coli* from raw dromedary camel milk than dromedary camel milk powder (Table 3).

In our recent study, for raw dromedary camel milk (28/100) samples and dromedary camel milk powder (5/100) samples were contaminated with *E. coli* which is comparable to various other studies. Saeed *et al*. (2022) detected a prevalence of 8% (4/50) of *E. coli* from raw dromedary camel farm milk in Egypt. Another study conducted on milk samples collected from Bikaner, showed a prevalence of 14.5% (Verma *et al*., 2017). Highly variable results were shown by Elhaj *et al*. (2018) showed a prevalence of 25.99% from raw camel milk in middle region Saudi Arabia. The variation in isolation of *E. coli* from various regions can possibly be because of variation in the pre and post-production handling and management practices being followed at the farms, which predispose the milk to microbial contamination. All the 33 isolates obtained from raw dromedary camel milk (28) and dromedary camel milk powder (5) were found positive for the *16s rRNA* gene while the presence of *stx1* and *stx2* gene were not detected in any isolates. Similar studies on raw milk and milk products were conducted by the Islam *et al*. (2016), Parveen *et al*. (2021), Younis *et al*. (2021), Dowidar *et al*. (2023); Neamah *et al*. (2022); Ghali-Mohammed *et al*. (2023); Najm *et al.* (2022) and Elshiekh *et al*. (2019) for confirming the genotypes of *Escherichia coli* strains isolated from numerous cases.

Out of 33 *E. coli* isolates recovered from raw dromedary camel milk and dromedary camel milk powder, the presence of *stx1* and *stx2* were not detected (Table 4). PCR primers targeting the *stx1* and *stx2* genes of *Escherichia coli* amplified 180 bp and 255 bp fragments of DNA confirming the identity of *Escherichia coli* (Table 1). Similar studies on raw milk and milk products were also conducted by several researchers like Liu *et al*. (2021); Mohammadi *et al*. (2013); Sheikh *et al*. (2013); Vendramin *et al*. (2014) and Laila *et al*. (2017). The occurrence of *16s rRNA* and STEC genes in the examined samples emphasized the idea that raw camel milk may exhibit a potential health hazard to the consumers. To reduce the danger represented by this zoonotic agent to the

consumer health, it is important to minimize contamination of raw milk during milking procedures and regular surveillance of milk production is necessary. Powerful heat treatment for raw milk and milk products is required. Pasteurization of milk can effectively reduce the risk of foodborne illnesses associated with *Escherichia coli* and other pathogens present in raw milk. Informative educational programs should be conducted to publish awareness about STEC safety issues and hazards associated with raw milk consumption among consumers, dairy farmers and processors about the hazards associated with raw milk consumption.

**Conclusion**

The isolates and molecular detection of *Escherichia coli* was more in raw dromedary camel milk compared to milk powder. It highlights the difference in handling and management practices. Moreover, the detection of *Escherichia coli* in numerous samples is concerning, as it suggests poor quality emphasizing the need for appropriate measures to improve production and handling techniques, as well as to prevent contamination during processing.

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