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RESEARCH ARTICLE

Molecular detection and antibiogram of *Escherichia coli O157* isolated from subclinical mastitis affected cows at Baghabari, Sirajganj

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ABSTRACT

Objectives: *E. coli* O157 is considered as one of the important pathogens causing subclinical mastitis in dairy cows. This study was undertaken to isolate *E. coli* O157 from the milk samples collected from subclinical mastitic cows using polymerase chain reaction (PCR) and investigate their antibiotic sensitivity patterns.
Materials and Methods: 50 California Mastitis Test (CMT) positive milk samples were collected from apparently healthy crossbreed dairy cows in Baghabari, Sirajganj. For the enrichment and isolation of the organism, nutrient broth, MacConkey agar, and EMB agar were used. Later on, observing the biochemical tests result, all the isolates of *E. coli* were confirmed by PCR using genus-specific 16SrRNA primers. PCR-positive samples were then screened for the presence of the *rfbO157* gene using gene-specific primers. The antibiotic sensitivity pattern of *E. coli* was assessed by the disk diffusion method against seven commonly used antibiotics.
Results: Altogether, 8 (16%) isolates of *E. coli* were obtained, among which 5 (10%) were *rfbO157* PCR positive. From the antibiotic sensitivity test, Gentamicin was the highest (75%) sensitive to the isolates, followed by Levofloxacin (62.5%), Cefixime (50%), Tetracycline (50%), and Ceftriaxone (25%). The highest resistance pattern was found against Ampicillin (100%) and Amoxycillin (87.5%).
Conclusion: Raw milk containing *E. coli* O157 does not only reflect the status of the dairy herd. Additionally, it poses a serious threat to human health if it is consumed raw or used to make any type of value-added food product.

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Introduction

Milk is regarded as a nutrient-dense food. Cow's whole milk typically includes about 3.5% milk fat. The knowledge regarding the health benefits of milk and milk products continues to develop. Milk has beneficial effects on inflammation, mild hypertension, and some types of cancer. However, it provides an ideal environment for the growth and spread of a range of bacteria that cause disease in humans [1].

Mastitis is defined by a rise in the number of somatic cells in the milk of the affected animal. Subclinical mastitis (SCM) is a severe problem for the dairy industry because no visible alterations in the udder or glandular tissues are observed, and the milk appears to be unaffected. However, if SCM continues to exist on the dairy farm, it will result in enormous losses. Due to SCM, milk production might be reduced by up to 80%. According to reports, Bangladesh's annual economic loss owing to lower milk production is projected to be roughly Taka 122.6 (USD 2.11) million [2]. A subclinically infected animal may serve as a source of infection for herd mates. If the sickness is allowed to persist for an extended period of time, it has a detrimental effect on the quality of milk [3]. Mastitis is more prevalent on farms with bigger herd sizes than on those with smaller herd sizes. Early identification of SCM in cows is critical for dairy animal survival and farmers' profitability. Numerous approaches for detecting SCM have been developed, taking into account physical and chemical changes in milk and the isolation of related organisms [4]. Among the assays, the

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California Mastitis Test (CMT), the White Side Test (WST), and the Surf Field Mastitis Test (SFMT) are arguably the most reliable for SCM screening.

Mastitis can be caused by a variety of microorganisms, including viruses, bacteria, mycoplasma, and yeast. In India, *Escherichia* (*E.*) *coli* is the most common etiological agent of SCM in cows. Clinical cases of mastitis in Bangladesh have been attributed to a variety of bacteria, including *E. coli*, *Staphylococci*, *Streptococci*, *Corynebacterium*, and *Bacillus* spp. At the Bangladesh Livestock Research Institute (BLRI) Regional Station in Sirajganj, Bangladesh, the frequency of SCM was 51%, with the majority of cows infected with a mix of *Staphylococcus* spp., *Streptococcus* spp., *E. coli*, and *Salmonella* spp.; however, some cows had a single bacterial infection [5]. *E. coli* is a Gram-negative rod-shaped bacterium belonging to the Enterobacteriaceae family frequently found in the lower intestine of warm-blooded species. It is transmitted to people primarily by ingesting infected foods such as raw or undercooked ground beef products, raw milk, and raw vegetables and sprouts that have been contaminated.

Over the last half-century, it has been increasingly clear that *E. coli* has a variety of distinct harmful strains. On the basis of their virulence genes, pathogenic *E. coli* strains are classified into pathotypes [6]. According to a previous study, the most prevalent *E. coli* serotypes recovered from mastitic milk are O55, O111, 0124, 0119, 0114, 026, O157, and O44 [7]. Generally, infections produced by this bacterium require antimicrobial therapy; however, antibiotic-resistant strains of bacteria cause more severe infections for a longer period of time than their antibiotic-susceptible counterparts. Numerous investigations have revealed an increase in antibiotic resistance in *E. coli* in recent years [8]. As a result, identifying the resistance pattern of this bacteria appears to be critical for lowering treatment costs.

Numerous investigations have been undertaken to determine the virulence factors of *E. coli* isolated from cows with clinical and SCM [9, 10]. Numerous studies have been conducted worldwide on milk and the bacteria found in tainted milk [11]. In Bangladesh, only a few studies on the isolation and molecular characterization of *E. coli* from raw cow and buffalo milk have been conducted [12-14]. Additionally, a few studies on the molecular identification of *E. coli* O157 from SCM have been conducted in Bangladesh [15]. In light of the preceding, the current investigation was designed to determine the prevalence, molecular identification, and antibacterial pattern of *E. coli* 0157 isolated from subclinical mastitic milk samples in Baghabari, Sirajganj district, Bangladesh.

Materials and Methods

Ethical statement

The research was conducted following established ethical norms and guidelines. Without injuring the cows, milk samples were obtained.

Sample collection

Three hundred milk samples were gathered for this investigation from the Baghabari neighborhood of Sirajganj. Prior to milk collection, the teat and tips were rinsed with clean water, antisepsis was performed with a swab soaked in 70% alcohol, and then milk samples were aseptically taken from the udder during the morning. All milk samples were collected in vials labeled with the cows' identifying numbers. CMT was used to detect SCM as instructed by the manufacturer (Cheil Bio Co. Ltd.). Positive samples were sent to the Department of Microbiology and Hygiene at Bangladesh Agricultural University, Mymensingh, using a cool box filled with ice.

Isolation and identification

The nutrient broth was employed as a primary enrichment medium for the organism. Bacteria were cultured and then streaked on Mac-Conkey agar (MC) (Himedia, India). On Mac-Conkey agar, the colony exhibited typical *E. coli* characteristics. It was then inoculated onto Eosin Methylene Blue agar (EMB) (Himedia, India), a selective media for *E. coli*. Each time, the temperature was maintained at 37°C for the duration of the incubation. The suspected colony was next stained with the Gram stain [16]. After performing a microscopic examination (100X) to confirm the *E. coli* isolates, a series of biochemical tests, including Catalase test, Indole test, sugar fermentation test (Dextrose, Sucrose, Lactose, Maltose, Mannitol), Voges-Proskauer test, and Methyl-Red test, were performed.

Table 1. Oligonucleotide primer used in this study.

Primer Name	Gene Targeted	Primer Sequence (5′-3′)	Amplicon size (bp)	Reference
EC16SrRNA-F	*16SrRNA*	GACCTCGGTTTAGTTCACAGA	585	[17]
EC16SrRNA-R		CACACGCTGACGCTGACCA		
rfbO157-F	*rfbO157*	AAGATTGCGCTGAAGCCTTTG	497	[15]
rfbO157-R		CATTGGCATCGTGTGGAC		

Extraction of genomic DNA

Each *E. coli* isolate's genomic DNA was isolated using the boiling technique. In brief, a single colony of each isolate was added to 200 µl of distilled water and then boiled for 10 min. Following boiling, the samples were immediately placed on ice for 10 min to induce a cold shock. Finally, 10 min of centrifugation at 10000 rpm were performed. The supernatant was collected and used as a DNA template for polymerase chain reaction (PCR) [18].

Molecular detection

To detect *E. coli*, a sequence of primers used in PCR assays at the molecular level. Table 1 lists the primers used in this study. The PCR mixture volume was 25 µl, which included 12.5 µl PCR master mixture (Promega, USA), 2 µl of each primer, 4 µl of template DNA, and 4.5 µl of nuclease-free deionized water [19]. The thermal profile utilized in this investigation followed the authors' recommendations.

Antibiogram

According to the author's description in his study [20], the disc diffusion method was employed to detect antimicrobial susceptibility assays. Seven regularly used antibacterial discs (Oxoid, UK) were utilized against the isolated *E. coli* while adhering to the Mcfarland turbidity standard. Amoxicillin (AMX, 30 µg), Ampicillin (AMP, 25 µg), Tetracycline (TE, 30 µg), Gentamicin (GEN, 10 µg), Ceftriaxone (CTR, 30 µg), Cefixime (CFM, 5 µg), and Levofloxacin (LE, 5 µg) were the antibiotics contained on the discs. The results were classified as susceptible, moderate, or resistant using CLSI-provided zone of diameter interpretative standards [21].

Results and Discussion

E. coli is the most prevalent Gram-negative bacteria that can cause SCM and is resistant to several antibiotics. Nevertheless, the presence of pathogenic *E. coli* in the environment is frequently overlooked. Numerous investigations have established the prevalence of *E. coli* in SCM cases in dairy farms throughout the world, most notably in developing nations such as Uruguay, Turkey, Brazil, Ethiopia, Mexico, and China [6, 22, 23]. A total of 300 cross dairy cows were selected from 60 farmers in 13 villages in Sirajganj district's Baghabari area. A total of 50 milk samples positive for CMT were collected and transferred aseptically in this study.

The media outlets employed in this study were chosen based on the previous researcher's expertise working in a variety of sectors related to the current study [11, 15, 24–27]. The colony features of *E. coli* observed in this investigation on EMB agar and MacConkey agar which were comparable to those reported previously [24, 25]. Gram stain revealed that the isolated bacteria were pink, tiny rod-shaped, and Gram-negative. Several authors concurred with these findings [15, 26]. Within 24 h of incubation, all isolates fermented dextrose, sucrose, fructose, maltose, and mannitol with acid and gas generation. As reported in several publications [5, 15], the results of *E. coli* isolates were positive. Additionally, the isolates demonstrated a positive response to the MR and Indole tests but a negative response to the V-P test.

The prevalence of *E. coli* in milk from cows with SCM was reported to be 16% (Table 2), which is almost identical to the 16.25% reported by Abdel-Rady and Sayed [28]. However, several studies indicated a lower incidence of *E. coli*, with Saidi et al. [29] reporting a prevalence of 7.5% and Mpatswenumugabo et al. [30], reporting a prevalence of 1.5%. This could be a result of differences in the environment and managerial practices. Haftu et al. [31] reported a prevalence of 27.3%, which is higher than the current study. In our study, out of eight *E. coli* isolates, five (62.5%) were positive for the *rfbO157* gene (Fig. 1). A comparable study showed a lower percentage of *rfbO157* gene-positive isolates at 11% by Garbaj et al. [31]. Sancak et al. [32] identified *E. coli* O157 in 11% and 6% of raw milk and herby cheese samples, respectively.

All eight *E. coli* isolates were tested against seven different antibiotics in this study. Gentamicin susceptibility was the greatest (75%), followed by Levofloxacin (62.5%), Cefixime (50%), Tetracycline (50%), and Ceftriaxone (25%) (Table 3, Fig. 2). The highest resistance level was seen against Ampicillin (100%) and Amoxycillin (87.5%). Haftu et al. [22], Thaker et al. [33], and Hinthong et al. [34] reported 100% resistance to ampicillin, which is consistent with the current findings. Rangel and Marin [35] demonstrated that Tetracycline was the most effective antibiotic. However, Tetracycline was found to be 50% sensitive in this study. Excessive antibiotic usage is a leading source of resistance to the organisms. Similar to the current research, Munsi et al. [36] determined that Ceftriaxone and Gentamicin were the most effective antibiotics.

Table 2. Prevalence of *E. coli* isolates.

Total samples	Culture positive *E. coli* samples	*16SrRNA* Positive Samples and prevalence (%)	*rfbO157* Positive	No. (%) of rfbO157 positive among isolates
50	8	8 (16%)	5 (10%)	5 (62.5%)

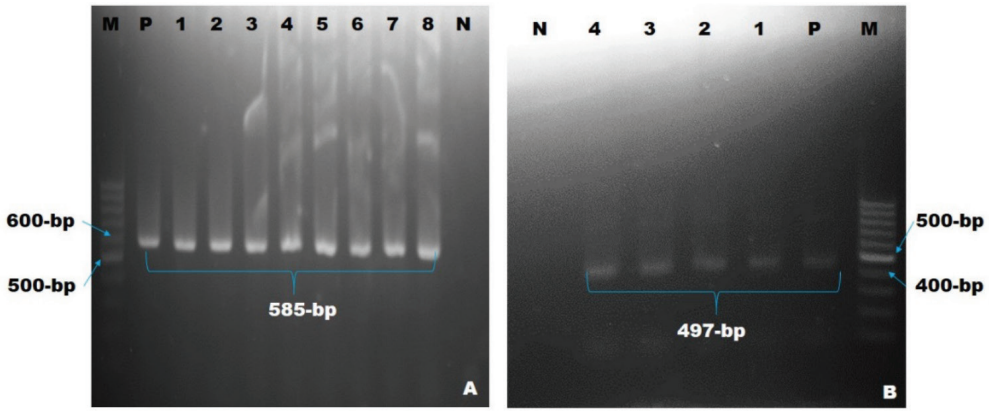

Figure 1. (A) PCR amplification of *16SrRNA* (585-bp) gene specific for *E. coli*, Lane 1-8 are test samples. **(B)** PCR amplification of *rfbO157* (497-bp) gene specific for *E. coli* O157, Lane 1-4 are test samples. In both case M= 100-bp DNA ladder, P= Positive control, N= Negative control.

Table 3. Antibiotic susceptibility pattern of isolated *E. coli.*

Antimicrobial agents	Resistant	Intermediate	Susceptible
Amoxycillin	7 (87.5)	1 (12.5)	0 (0.0)
Ampicillin	8 (100)	0 (0.0)	0 (0.0)
Tetracycline	1 (12.5)	3 (25.0)	4 (50.0)
Gentamicin	2 (25.0)	0 (0.0)	6(75.0)
Ceftriaxone	2 (25.0)	4 (37.5)	2 (25.0)
Cefixime	0 (0.0)	4 (50.0)	4 (50.0)
Levofloxacin	0 (0.0)	3 (37.5)	5 (62.5)

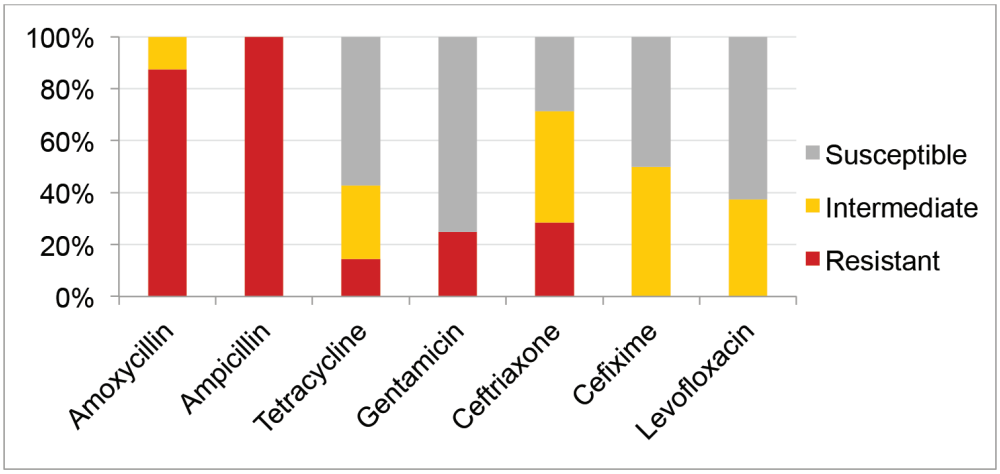

Figure 2. Antibiotic Susceptibility pattern of isolated *E. coli*.

Conclusion

Eight (16%) *E. coli* isolates were isolated from 50 samples, of which only five (10%) were *rfbO157* positive. Gentamicin was shown to be the most susceptible antibiotic (75%), followed by Levofloxacin (62.5%) and Cefixime (50%). The highest resistance levels were seen against Ampicillin (100%) and Amoxycillin (87.5%), respectively.

List of abbreviations

CMT, California Mastitis Test; SCM, Subclinical mastitis; WST, White Side Test; SFMT, Surf Field Mastitis Test; *E. coli*, *Escherichia coli*; EMB, Eosin Methylene Blue; PCR, Polymerase Chain Reaction; UV, Ultra-Violet.

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Conflict of interest

The authors declare that there is no conflict of interest toward the publication of this article.

Authors' contributions

Conceptualization, KHMNHN and MTR; methodology, SMF, AK and MMM; validation, KHMNHN and MTR; formal analysis, SMF and MMM; investigation, KHMNHN; resources, KHMNHN; data curation, AK and MAHS; writing—original draft preparation, SMF and MMM; writing—review and editing, MAHS and SD; visualization, KHMNHN; supervision, KHMNHN. All authors have read and agreed to the published version of the manuscript.

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