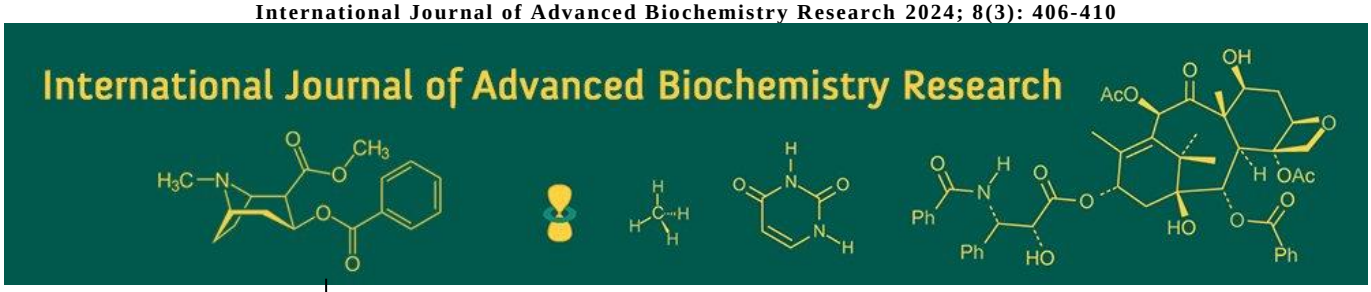

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The prevalence of *E. coli* in raw milk and their susceptibility to antibiotics

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Abstract
Escherichia coli, sometimes known as *E. coli*, is a beneficial member of the natural symbiotic bacterial flora found in the stomach. One of the main causes of bovine mastitis, which can range from a serious systemic illness to a subclinical mammary gland infection, is *E. coli*. Most commensals are benign, but some have developed virulence features that can spread through contaminated milk and infect otherwise healthy people. Antibiotic-resistant *E. coli* found in raw cow's milk may be the cause of resistance illnesses in people. The study looks into the antibiotic susceptibility of *E. coli* and how often it is in raw milk samples from dairy farms in Haryana.
For this cross-sectional investigation, 40 raw cow milk samples were gathered from different areas in Haryana. Following the presumed isolation of *E. coli* from raw milk, a two-stage NDRI assay was conducted to confirm the isolates of *E. coli* utilising quick procedures. The IMViC assays were used to biochemically characterise isolates of *E. coli* samples. Using the Kirby-Bauer method, antibiotic susceptibility was assessed for third- and fourth-generation cephalosporins.
The NDRI test verified that 15% of the 40 raw milk samples had *E. coli* germs in them. Every *E. coli* sample with phenotypic confirmation was susceptible to carbapenem and broad spectrum cephalosporin drugs.
15% of raw milk from Haryana's dairy had *E. coli*, although the bacteria did not show signs of drug resistance to cephalosporins.

Keywords: Bovine mastitis, milk, *E. coli*, antibiotic resistance

Introduction
Escherichia coli (*E. coli*) is a rod-shaped, facultatively anaerobic, Gram-negative bacteria *that* is classified as a member of the family Enterobacteriaceae within the Gammaproteo bacteria class (Aidara –Kane, 2012) [1]. Existing independently in nature it is also a part of normal bacterial flora in humans and animals. They benefit their hosts by producing vitamin K2 and prevent colonization of the intestine with pathogenic bacteria, having a symbiotic relationship (Cabrera *et al*., 2020) [2]. These bacteria live in the intestines of many animals and are usually transmitted to people when they eat foods contaminated with the bacteria (Clark *et al*., 1957) [3]. The majority of *E. coli* are harmless commensals of the mammalian gastrointestinal tract, yet some strains have deviated from this ancestral niche, adapting specific virulence traits that enable them to cause disease in otherwise healthy individuals (Duan *et al*., 2006) [4]. The resulting clinical syndromes may include gastroenteritis, urinary tract infection, septicemia, and meningitis.
Escherichia coli infection may itself be caused by at least six distinct *E. coli* pathotypes: enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), diffusely adherent (DAEC) and enteroaggregative *E. coli* (EAEC). These pathotypes exhibit distinct clinical, epidemiologic, and pathogenic profiles. Strains within each pathotype are characterized by shared virulence traits and can typically be further distinguished by O (lipopolysaccharide) and H (flagellar) antigens (Jafari e*t al*., 2012) [10]. Since its discovery, an increasing number of studies have implicated *E. coli* in causing persistent diarrhea, and traveler diarrhea among neonates and patients with weak immune systems. *E. coli* is the most frequent cause of community and hospital-acquired urinary tract infections (including infections of the kidney), bloodstream infection, meningitis and intra-abdominal infections such as peritonitis (Gebremedhinm *et al*., 2018) [7]. *E. coli* is also one of the major causative agents of bovine mastitis ranging from being a subclinical infection of

the mammary gland to a severe systemic disease (Suojala *et al*., 2013) [17].

Broad-spectrum antimicrobials are commonly used to treat *E. coli* mastitis (Erskine *et al*., 2003) [5]. Most of these antibiotics have been classified as "critically important" by the World Health Organization (WHO). The use of third and fourth-generation cephalosporins is of particular concern because they have been attributed to the development of antimicrobial resistance in certain bacteria (Erskine *et al*., 2003; Martinson and Walk, 2020) [5, 14]. Studies have been conducted in India in which antimicrobial-resistant *E. coli* were isolated from raw milk, mastitis milk, and unpasteurized milk (Ghatak *et al*., 2013; Kakkar *et al*., 2017) [8, 12]. It has been found that these bacterial species have characteristics of both multi-drug as well as carbapenem resistance. The major cause of the prevalence of ESBL and carbapenem-resistant bacteria is the indiscriminate use β-lactam antibiotics, horizontal transfer of genes by the mastitis milk isolate from human or environmental sources. Also among dairy animal's absence of antimicrobial treatment records, lack of written plans, and failure to complete an antimicrobial treatment course are the major factors that could lead to the inappropriate use of antimicrobials and the emergence of antibiotic-resistant bacteria (Sawant *et al*., 2007) [15]. Further European Food Safety Authority (EFSA) in 2013 stated that the β-lactam resistant bacteria are now seen as a new and potentially emerging problem in dairy animals because of the specific use of cephalosporins and β-lactam antibiotics for mastitis treatment (Kucukbasmaci et al., 2008) [13]. Isolates of *E. coli*, which are resistant to 3rd or 4th generation cephalosporin should be screened for ESBL and Carbapenem resistance. According to the National Action Plan (2017) on AMR, surveillance of AMR in humans, foods, animals and environment sectors focus on strengthening laboratories is one of the prioritized strategies for the containment of AMR in India. The present study investigated the Isolation and Identification of *E. coli* from Raw Milk Samples and the Antibiotic Susceptibility test for *E. coli* isolates.

Materials and Methods

Over the course of three months, the Department of Microbiology carried out this cross-sectional investigation. Forty raw cow milk samples were obtained from different regions in Haryana to be included in the study. Initially, 70% alcohol was used to sanitise the milking man's hands in preparation for collecting milk samples. Tap water was used to wash the cow's urine. The first few milk streams were thrown away, and the remaining streams were sampled right into the sample collection container. After being labelled, all of the sample bottles were quickly brought with ice packs to the laboratory.

Isolation and identification of *E. coli* in raw milk samples

Isolation of *E. coli* was performed as per the procedure laid down in the *IS 5887;* Methods for detection of bacteria responsible for food poisoning–isolation, identification and enumeration of *E. coli.* All milk samples were processed for the primary isolation of bacteria. Initially, 1 ml of milk samples was transferred into 10 ml of MacConkey broth tube in the bio-safety cabinet under aseptic conditions. Inoculated test tubes were incubated at 37 °C for 24 h. Positive isolates from enriched MacConkey broth tubes which showed a change in colour from purple to yellow were streaked on MacConkey agar and EMB agar plates and incubated at 37 °C for 24 hours**.** Gram staining was used to differentiate two large groups of bacteria based on their different cell wall constituents. It distinguishes between gram-positive and gram-negative groups. The Gram-negative short rods were presumptively considered as *E. coli* species and were subjected to biochemical tests.

Biochemical characterization of *E. coli*

The IMViC tests are a group of individual tests used in microbiology labs to identify an organism in the coliform group. The term "IMViC" is an acronym for each of these tests. "I" is for the indole test; "M" is for the methyl red test; "V" is for the Voges-Proskauer test, and "C" is for the citrate test.

Isolates having characteristic pink smooth colonies on MacConkey agar plates and greenish metallic sheen colonies on EMB agar plates were further evaluated for confirmation using the IMViC test.

Indole test

Prepared tryptone broth medium was inoculated with overnight grown culture and it was incubated at 37 °C for 24 h. Point five ml of Kovacs reagents was added, which was prepared by dissolving 10 g p-dimethyl-amino benzaldehyde in 150 ml amyl alcohol or iso-amyl alcohol and to which 50 ml of concentrated hydrochloric acid was slowly added. After adding the Kovacs reagent, the appearance of a distinct red color in the upper layer was an indication of the presence of indole.

Methyl red test

Prepared glucose peptone medium was inoculated with overnight grown culture and it was incubated at 37 °C for 48 h. After incubation, tubes were added with 2-3 drops of methyl red indicator solution into the medium. The appearance of red color was an indication of a positive reaction.

Voges-Proskauer reaction test

Prepared glucose peptone medium was inoculated with overnight grown culture and it was incubated at 37 °C for 48 h. After incubation, tubes were added with 0.6 ml of alpha-naphthol solution prepared as 5 percent solution in ethanol and 0.2 ml of 40 percent aqueous solution of potassium hydroxide. The positive reaction was indicated by the appearance of a rose-red color ring on the top of the tube was an indication of positive reaction.

Citrate utilization test

Prepared Simmons citrate agar slant was inoculated with overnight grown culture and slant tubes were incubated at 37 °C for 48 h. The change in color of the slant from green to blue indicated a positive reaction. The negative reaction was indicated by no color change.

Confirmation of *E. coli* isolates using rapid techniques by NDRI two-stage assay of Presumptive of *E. coli isolates*

Added 0.9 ml of sterile distilled water (pH 7.0) into the tube containing lyophilized

ECSM. Tubes were inoculated with 0.1 mL of milk and were incubated at 37 °C for 12±1.0 h. A change in color was observed from milky white to bluish-green.
Tube was centrifuged at 5,000 rpm for 10.0 min. The cell pellet was washed twice with 1.0 ml of Sodium phosphate buffer (pH- 6.8). After draining the supernatant, the sides of the tube were wiped with the sterile cotton swab to absorb the residual buffer without disturbing the pellet. Took stage-2 tube containing pre-lyophilized ESM and reconstituted with 200 µl of distilled water (pH 7.0). Transferred the contents of stage -2 tube to washed cell pellet and finally incubated at 37±1 °C for 3.00±0.15 h. Colour change was observed from yellow to blue which confirmed the presence of *E. coli.*

Antibiotic susceptibility test for *E. coli*

All the *E. coli* isolates obtained were subjected to an *in vitro* antibiotic susceptibility test (AST) as per the Kirby-Bauer method. This method is usually used for antimicrobial susceptibility testing and is recommended by the Clinical and Laboratory Standards Institute (CLSI). Mueller Hinton Agar Plates were prepared as per the manufacturer's instruction. A sterile non-toxic cotton swab on a wooden applicator was dipped into the standardized inoculum and was rotated. The soaked swab was firmly pressed against the upper inside wall of the tube to remove excess fluid. This inoculum was streaked the entire agar surface of the plate with the swab three times, turning the plate at a 60° angle between each streaking. Antimicrobial disks (Table 1) were placed on the surface of the prepared Muller Hinton agar plate which was inoculated with the test culture with the help of sterile forceps. Plates were transferred to the incubator and incubated at 35-37 °C for 16-18 hours.
The detailed AST for *E. coli* isolates was carried out using disk diffusion test by using antibiotics disk given in (Table 1) for detection of ESBL/Carbapenem-resistant *E. coli*. For preparation of inoculum isolates of *E. coli* were incubated in BHI broth at 37 °C for 24 h. overnight, grown cultures of *E. coli* were added into sterile saline until its turbidity reached equivalent to 0.5 McFarland standards (HI media Lab. Pvt. Ltd.).

Table 1: List of antibiotics disk used in antibiotic susceptible testing

Name of antibiotic	Symbol	Disk content (µg)
Cefotaxime	CTX	30
Cefepime	CPM	30
Ceftriaxone	CTR	30
Imipenem	IMP	10/10
Ertapenem	ERT	100
Meropenem	MRP	10
Cefoxitin	CX	30
Cefotaxime-clavulanic acid	CEC	10

Interpretation of result: Zones of inhibition were measured and compared with zone size interpretative table furnished by the CLSI (Clinical and Laboratory Standard Institute) and manufacturer and isolates were graded as sensitive (S), intermediate (I) and resistant (R).

Antibiotic Disc used were: Cefepime, Cefotaxime, Ceftriaxone, Cefoxitin, Cefotaxime-clavulanic acid, Imipenem, Meropenem, Ertapenem.

Table. 2 CLSI criteria for selection of antibiotics-resistant *E. coli*

S .No.	Antibiotic	ATCC (25922)	S (mm)	I (mm)	R (mm)
			Isolates		
1	Cefotaxime	30 – 36	26	23 – 25	22
2	Cefepime	31 -37	25	19 -24	8
3	Ceftriaxone	25 -32	21	18 – 20	17
4	Cefoxitin	26-30	>/= 22	19-22	</=21
5	Ertapenem	24 -31	22	19 – 21	18
6	Meropenem	28 – 35	23	20 – 22	19

Statistical Analysis

Data were analyzed using the statistical program SPSS for Windows, version 20 (SPSS Inc., Chicago, IL, USA). Being a cross-sectional study, the data is presented in the form of frequency tables. Figures are used to corroborate the findings observed during the microbial test.

Results

Forty raw cow milk samples were examined for the presence and isolation of *E. coli* variants in the current study. Based on the enrichment of milk samples in McDonald's broth, six samples of cow's milk were found to be positive for *E. coli* bacteria, or 15% of the samples overall. EMB or MacConkey plates were used for streaking in order to identify *E. coli* colonies inferentially. On EMB Agar, all six samples displayed a green metallic Sheen colour, while on MacConkey, they displayed a smooth pink colony. The positive strain of *Escherichia coli* was verified using the ATCC2599 strain. Gram staining was used to categorise the six *E. coli* isolates before microscopic inspection. Under a microscope, the *E. coli* was observed to have a rod-shaped pink colour and had lost its crystal violet colour.
On biochemical characterization by IMViC test, all 6 isolates of E coli were found to have the presence of indole using the indole test and was also positive on methyl red testing indicator. All samples of *E. coli* were observed to be negative on the Vogus Proskauer test and citrate utilization by the Simmons Citrate Agar slant test. The results were similar to isolate ATCC25922 (positive control) which was taken as positive controls (Table 3)

Table 3: Biochemical test for characterization of *E. coli* isolates using IMViC test.

Species	Indole Test	Methyl Red Test	Voges-Proskauer Test	Citrate Test
ATCC 25922	Positive	Positive	Negative	Negative
Escherichia coli Isolates	Positive	Positive	Negative	Negative

All the 6 cow milk samples which were biochemically identified were further confirmed for presence of *E. coli* using another rapid technique of NDRI 2 stage enzyme assay. The result showed that all six samples were also confirmed for the presence of *E. coli* strain. Similar to the conventional method.

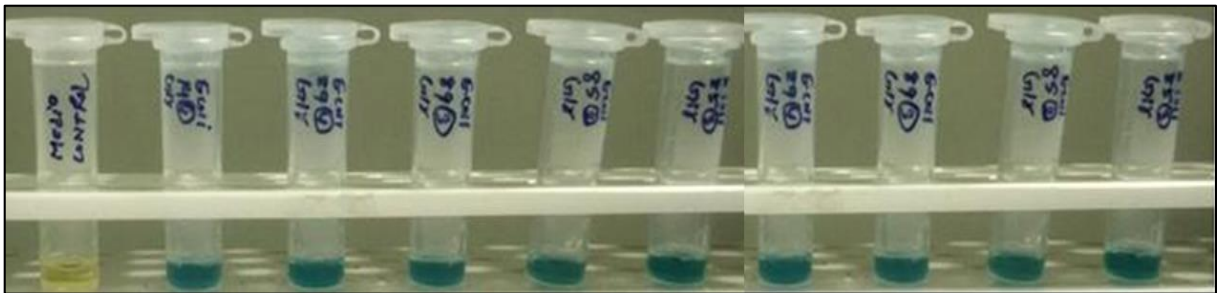

Fig 1: Enzyme Substrate assay for *E. coli*

Antibiotic resistant pattern of *E. coli* isolates (n=6) was determined by Kirby-baurer disc diffusion test. Based on the zone of inhibition due to antibiotic susceptibility pattern of resistance, *E. coli* isolates were classified as resistant, intermediate, and susceptible. The result observed that all the phenotypically confirmed *E. coli* (n=6) showed susceptibility to broad-spectrum Cephalosporin and Carbapenem antibiotics. (Table 4)

Table 4: *E. coli* isolates susceptibility to Broad spectrum Cephalosporin and Carbapenem antibiotics

Name of antibiotic	CTX	CPM	CTR	CX	CEC	ETP	MER	IPM
Sample No.5	S	S	S	S	S	S	S	S
Sample No.8	S	S	S	S	S	S	S	S
Sample No.11	S	S	S	S	S	S	S	S
Sample No.16	S	S	S	S	S	S	S	S
Sample No.32	S	S	I	S	S	S	S	S
Sample No.38	S	S	S	S	S	S	S	S

Discussion

The purpose of this study was to use IS method, or IS 5887, to isolate *E. coli* from raw milk samples. In all, forty samples were gathered from various locations around the Haryana district and subjected to the recommended protocol for the isolation of *E. coli*. Six (15%) of the samples tested positive for *E. coli*, with the isolates showing smooth pink colonies on MacConkey agar and a green metallic sheen on EMB agar. Under a microscope, the isolates were seen to be pink, rod-shaped bacteria, which are characteristic of Gram-negative rod-shaped bacteria. The isolates were further evaluated by the IMViC test, which demonstrated a positive response to the methyl red and indole tests, just like normal *E. coli*. The Antibiotic Susceptibility Test was conducted using a different antibiotic disc and the *E. coli* ATCC 25922 strain as a quality control culture. Many isolates were shown to be susceptible to cefepime (CPM), cefotaxime (CTX), cefotaxime-clavulanic acid (CEC), ceftriaxone (CTR), cefoxitin (CX), ertapenem (ETP), and impenem (IPM) based on their pattern of antibiotic resistance. Seiffert *et al.* (2013) and Harrington *et al.* (2006) [16, 9] conducted comparable studies in which they tested 380 raw milk samples for the presence and isolation of *Escherichia coli*. Results were favourable for 129 (33%) of the samples. It was found that cefoxitin, cefepime, streptomycin, and norfloxacin were effective against several isolates. In another study, out of 300 raw milk samples, 75 (or 25%) had positive *E. coli* tests. There have been reports of high susceptibility to imipenem, gentamicin, ertapenem, and kanamycine. Duan *et al.* (2006) [4] reported a 3.1% prevalence rate of ESBL producers among *E. coli* isolates from cattle (Suojala *et al.*, 2013; Fairbrother and Nadeau, 2006) [17, 6]. In a Turkish study, a 2.1% prevalence of ESBL among Enterobacteriaceae isolates was found in cattle (Suojala *et al.*, 2013; Jang *et al.*, 2017) [17, 11]. However, none of the *E. coli* isolates showed multidrug-resistant characteristics in our study. This may be due to less number of milk samples included in this study.

Conclusion

Every *E. coli* isolate shown susceptibility to cephalosporins of the third and fourth generations. The isolates of *E. coli* were also sensitive to cefoxitin and cefotaxime-clavulanic acid. Against carbapenem, imipenem, meropenem, and ertapenem, none of the isolates demonstrated susceptibility.

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