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Assessing the antimicrobial susceptibility of *Escherichia coli* isolated from raw and pasteurized bovine milk in Hisar, Haryana

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Abstract
The rise of multi-drug resistant *Escherichia coli* (*E. coli*) poses a significant and escalating concern for worldwide public health. This study aimed to examine the resistance to antibiotics in *E. coli* isolates derived from raw bovine milk and commercially pasteurized milk samples gathered from dairy farms situated in and around the Hisar district of Haryana. The susceptibility of the *E. coli* isolates to antimicrobials was assessed using the Kirby-Bauer disk diffusion method to ascertain their resistance patterns. The *E. coli* isolates from raw milk were highly resistant to penicillin (95.65%), erythromycin (95.65%), gentamicin (56.52%), streptomycin (56.52%), amikacin (39.13%), and aztreonam (39.13%). Whereas *E. coli* isolated from pasteurized milk were highly resistant to ceftazidime (100%), erythromycin (100%), penicillin (84.62%) and cefotaxime (76.92%). Nearly, all the *E. coli* isolates isolated from both raw (95.65%) and pasteurized milk (92.31%) belonged to multi drug resistant bacteria. In order to reduce contamination from foodborne pathogens, there's a necessity for innovative, effective, and feasible food safety measures and monitoring techniques specifically targeting multi-drug resistant pathogens.

Keywords: Antimicrobial susceptibility, *Escherichia coli*, raw, pasteurized bovine milk

1. Introduction
Due to the exponential growth in population, the total biomass of humans now surpasses that of animals raised for food globally. In response to this need, developing countries are shifting toward economically efficient and vertically structured intensive systems for livestock production (Van Boeckel *et al*., 2015) [15]. In these systems, the use of antimicrobials is crucial for maintaining animal health and improving productivity, leading to an escalation in antimicrobial consumption and the emergence of antimicrobial-resistant bacteria. The surge in antibiotic usage can be linked to several contributing factors, such as the utilization of clinical antibiotics in livestock feed, employing antibiotics to enhance animal growth, and the excessive use of antimicrobials in both human and animal domains (Walsh and Fanning, 2008) [16]. AMR presents a considerable worldwide risk to public and animal health, largely due to the improper selection and excessive utilization of antimicrobial substances in both human and animal (Ombarak *et al*., 2016) [9]. A significant concern in public health has been the emergence of foodborne bacteria that are resistant to multiple drugs. Multidrug resistance (MDR) refers to the ability to resist at least one antimicrobial agent across three or more categories of antibiotics (Magiorakos *et al*., 2012) [6]. Multidrug-resistant (MDR) *E. coli* stands out as a major obstacle in ensuring food safety (Rashid *et al*., 2013) [13]. The development of resistance to the primary antimicrobial treatments has been steadily increasing, posing a challenge for managing *E. coli* infections. Over time, there has been a rise in beta-lactam antimicrobial resistance within Enterobacteriaceae primarily due to the dissemination of extended spectrum beta lactamases (ESBLs) (Rasheed *et al*., 2014) [12]. This study aimed to establish the antimicrobial susceptibility profiles of *E. coli* strains isolated from raw and pasteurized samples gathered from dairy farms in and neighboring the Hisar district of Haryana.

2. Material and Methods

2.1 Collection of *E. coli* isolates

A total of 23 morphological distinct *E. coli* isolates were isolated from 30 bovine raw milk samples and 13 from 20 different brand pasteurized milk samples collected from dairy and local market in and around Hisar district of Haryana. The *E. coli* isolates were previously confirmed through both biochemical and molecular methods. (Jhandai *et al*., 2019) [4].

2.2 Antimicrobial susceptibility testing

The bacterial isolates were subjected to an *in vitro* antibiotic sensitivity test using the standard agar disk diffusion method as per the protocols recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012) [2]. This involved employing fifteen commercially available antimicrobial disks from eight distinct antibiotic classes obtained from HiMedia laboratories Limited, Mumbai. The isolates were assessed for their susceptibility to amoxicillin-clavulanic acid (AMC) (30 µg), cefoperazone (CPZ) (75 µg), penicillin (P) (10 µg), cefotaxime (CTX) (30 µg), cefpodoxime (CPD) (10 µg), ceftazidime (CAZ) (30 µg), chloramphenicol (C) (30 µg), ceftriaxone (CTR) (30 µg), amikacin (AK) (30 µg), gentamicin (GEN) (10 µg), erythromycin (E) (15 µg), streptomycin (S) (10 µg), tetracycline (TE) (30 µg), imipenem (IPM) (10 µg) and aztreonam (AT) (30 µg) by the disk diffusion assay in Mueller-Hinton agar. One individual bacterial colony sourced from a freshly cultivated pure sample was transferred into brain heart infusion broth (BHI) and then placed in an incubator at 37 °C for a duration of 6 hours. The resulting broth was adjusted to a McFarland 0.5 turbidity to attain the desired bacterial concentration. Muller Hinton Agar (MHA) plates, with a pH between 7.2 and 7.4, were then inoculated with this prepared sample using a sterile cotton swab. The swab was used to uniformly spread the inoculum across the surface of the media for even distribution. After the plates had dried, antibiotic disks were placed onto the inoculated plates using sterile forceps. The disks were carefully pressed onto the agar to ensure proper contact, followed by an incubation period at 37 °C for 24 hours. The next day, the width of the area where bacterial growth was inhibited around each disk was measured by positioning a clear ruler atop the plates. The outcomes were categorized as sensitive, intermediate, or resistant based on the standardized chart provided by CLSI in 2012 [2].

2.3 Statistical analysis

Statistical analysis for the study was conducted using STATA™ IC -15.0 software from StataCorp in College Station, TX.

3. Results and Discussion

All 36 E. coli isolates (23 from raw milk and 13 from pasteurized milk) underwent antimicrobial resistance testing against eight antibiotic classes and fifteen commercially available antibiotics. The outcomes of the isolates' antibiotic resistance tests are depicted in Table 1, along with Figures 1 and 2. A broad spectrum of antimicrobial drugs is presently employed globally for purposes such as promoting growth, preventing diseases, and for treatment of sick animal. This usage contributes to the emergence of multi-drug resistant foodborne pathogens (Jhandai *et al*., 2022) [4]. A significant concern in food safety and public health involves the rise of antibiotic-resistant bacterial pathogens in foodborne sources (Oniciuc *et al*., 2019) [10]. Numerous studies have highlighted that animal-based food products could serve as a crucial origin of multi-drug resistant (MDR) *E. coli* pathogens that affect humans (Rashid *et al*., 2013) [13].

Table 1: Antibiotic sensitivity pattern of *E. coli* samples collected from raw and pasteurized milk

Antibiotic	Raw milk (23 isolates)		Pasteurized milk (13 isolates)	
	Resistant isolate (%)	95% Confidence interval	Resistant isolate (%)	95% Confidence interval
AMC	4 (17.39)	4.95-38.78	0 (0)	0.0-24.70
P	22 (95.65)	78.05-99.89	11 (84.62)	54.55-98.08
CTX	7 (30.43)	13.21-52.92	10 (76.92)	46.19-94.96
CPD	6 (26.09)	10.23-48.41	1 (7.69)	0.19-36.02
CPZ	1 (4.35)	0.11-21.95	3 (23.08)	5.04-53.81
CAZ	6 (26.09)	10.23-48.41	13 (100)	75.29-100.0
CTR	4 (17.39)	4.95-38.78	3 (23.08)	5.04-53.81
AT	9 (39.13)	19.71-61.46	3 (23.08) b	5.04-53.81
IPM	7 (30.43)	13.21-52.92	3 (23.08)	5.04-53.81
TE	4 (17.39)	4.95-38.78	6 (46.15)	19.22-74.86
E	22 (95.65)	78.05-99.89	13 (100)	75.29-100.0
AK	9 (39.13)	19.71-61.46	3 (23.08)	5.04-53.81
GEN	13 (56.52)	34.49-76.81	4 (30.77)	9.09-61.42
S	13 (56.52)	34.49-76.81	3 (23.08)	5.04-53.81
C	1 (4.35)	0.11-21.95	0 (0)	0.0-24.70

Fig 1: Heat map displaying antibiotic susceptibility pattern of *E. coli* isolates from raw milk (n=23)

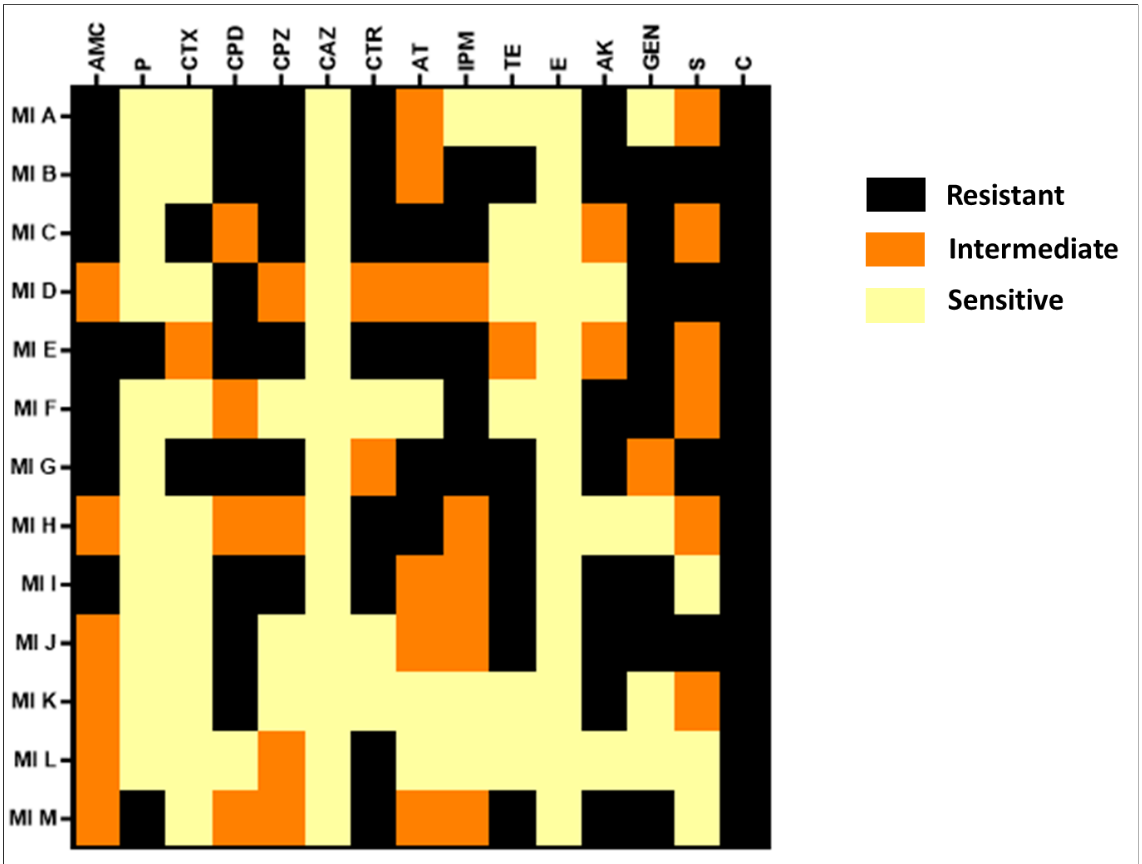

Fig 1: Heat map displaying antibiotic susceptibility pattern of *E. coli* isolates from pasteurized milk (n=13)

Penicillin (95.65%), erythromycin (95.65%), gentamicin (56.52%), streptomycin (56.52%), amikacin (39.13%), and aztreonam (39.13%) were found to be resistant in *E. coli* isolates from raw milk, Whereas *E. coli* isolated from pasteurized milk were highly resistant to ceftazidime (100%), erythromycin (100%), penicillin (84.62%) and cefotaxime (76.92%). However, the least resistance to antibiotics was noted for chloramphenicol (1; 4.35%), cefoperazone (1; 4.35%) for raw milk *E. coli* isolate and all isolate from pasteurized milk were sensitive for chloramphenicol and amoxicillin-clavulanic acid. Most of the isolates are resistant to penicillin and erythromycin, Variability has been observed regarding resistance to erythromycin and penicillin in various studies (Nazir, 2011 and Preethirani *et al*., 2015) [7, 11]. Nazir, 2011 [7] in Hisar, Haryana observed 32.8% and 3.1% resistance for ceftazidime and ceftriaxone in *E. coli* isolates obtained from pasteurized milk. Preethirani *et al*., 2015 [11] in Karnataka observed high resistance for cefotaxime (100%) and ceftriaxone (42%) in isolates from milk. The observed difference may be due to different hygienic measures. Third-generation cephalosporins exhibit broad range of action, especially against gram-negative organisms, making them potentially beneficial in addressing infections acquired within healthcare settings (Klein and Cunha, 1995) [5] and as observed in present study, the increased level of resistance against these antibiotics may restrict its use in human medicine in future. 23-30% *E. coli* in present study were resistant to imipenem. Rasheed *et al*., also analyzed isolates from milk samples and similar results were obtained, whereas, Badri *et al*., 2018 [1] have reported 19.90% isolates to be resistant to imipenem. Imipenem, belonging to the carbapenem class of drugs, is typically reserved for treating Gram-negative strains that are multidrug-resistant (Jhandai *et al*., 2022) [4]. In the present study, maximum sensitivity was observed against chloramphenicol among both type of samples. Chloramphenicol demonstrates efficacy against a broad spectrum of organisms, has excellent tissue penetration, and is a cost-effective medication. In numerous developing nations, chloramphenicol remains extensively employed in treating conditions like typhoid fever, anaerobic infections, bacterial meningitis in individuals with penicillin allergies, brain abscesses, and rickettsial infections (Nitzan *et al*., 2015) [8]. Jana and Mondal., 2013 [3] and Preethirani *et al*., 2015 [11] reported no resistance among *E. coli* isolates similar to present study, Whereas Rasheed *et al*., 2014 [12] and Skockova *et al*., 2015 [14] observed higher level of resistance. Variation may be due to difference in its usage in different part of the world. 22 out of 23 *E. coli* isolates from raw milk (95.65%: 95% CI- 78.05-95.65%) and 12 out of 13 *E. coli* isolates from pasteurized milk (92.31%: 63.97-99.81%) exhibited resistance to a minimum of three distinct categories of antimicrobial agents, indicating they were classified as MDR *E. coli* isolates.

The World Health Organization (WHO) assembled a list of crucial antimicrobials essential for human medical care, underscoring their priority or restricted usage for treating severe bacterial infections in humans. Some of these antimicrobials have been labeled as "Highest priority critically important" (WHO, 2016) [17]. Among these are cefotaxime, cefoperazone, ceftazidime, ceftriaxone and cefpodoxime (third-generation cephalosporins), along with erythromycin (macrolides), all identified in our study. Additionally, penicillins, imipenem, aminoglycosides, and aztreonam are classified as "Critically important" antimicrobials. The resistance detected in our study raises concern not just for their use in treating human infections but also for the potential transfer of resistance to humans, presenting a significant public health issue.

4. Conclusion
This study revealed a high resistance among *E. coli* isolates to penicillin, erythromycin, ceftazidime, and cefotaxime antibiotics. It was observed that over 90% of all *E. coli* isolates showed resistant to at least three classes of antimicrobial agents. We urgently need innovative and effective food safety monitoring systems, along with the genetic profiling of pathogens causing foodborne illnesses, particularly the multidrug-resistant strains, to manage and prevent contamination and diseases caused by foodborne pathogens.

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