www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(12): 492-498

www.thepharmajournal.com
Received: 26-10-2023
Accepted: 29-11-2023

K Pavani
Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, SVVU, Tirupati, Andhra Pradesh, India

PV Siddartha
Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, SVVU, Tirupati, Andhra Pradesh, India

Prashanth S Bagalakote
Department of Veterinary Public Health and Epidemiology, Veterinary College Shivamogga, Karnataka, India

C Siva Swetha
Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, SVVU, Tirupati, Andhra Pradesh, India

A Jagadeesh Babu
Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science, SVVU, Gannavaram, Andhra Pradesh, India

Corresponding Author:
K Pavani
Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, SVVU, Tirupati, Andhra Pradesh, India

Study on isolation, molecular detection of virulence genes and evaluation of ESBL producing ability of *Escherichia coli* isolated from raw milk

K Pavani, PV Siddartha, Prashanth S Bagalakote, C Siva Swetha and A Jagadeesh Babu

Abstract
Aim: The following study was aimed to detect *Escherichia coli* in raw milk samples, determine the virulence gene profile by PCR and to evaluate the ESBL producing ability of the isolates phenotypically.
Materials and Methods: 125 raw milk samples were collected from nearby local farms and milk merchants of Chandragiri mandal, Tirupati district, Andhra Pradesh. In order to isolate and identify *E. coli*, the collected samples were processed and put through standard procedures. Later by using PCR, all of these suspected *E. coli* isolates were verified for species-specific characterisation targeting *usp*A gene. Further, these isolates were evaluated for the presence of virulence genes *viz.*, *stx*1 and *stx*2 by PCR. Using the double disk diffusion method, the ability of these *E. coli* isolates to produce Extended Spectrum Beta-lactamase (ESBL) was determined. And also risk factors were evaluated by an observational survey with semi structured questionnaire that was constructed using Epi Info™ software.
Results: Out of 48 presumptive *E. coli* isolates, only 35 isolates were confirmed by PCR targeting *usp*A gene with an amplicon size of 884bp. Among these, 14 (40%) isolates harboured *stx*1 gene, 8 (22.85%) isolates positive for *stx*2 gene, while 4 (11.43%) isolates harboured both *stx*1 and *stx*2 genes. Further, out of 35 isolates only 9 (25.7%) isolates were positive for ESBL production and shown varied sensitivity response to different antibiotics.
Among ESBL antibiotics studied, Aztreonam (68.5%) had the highest sensitivity, followed by Cefotaxime (48.5%), Ceftazidime + Clavulanic acid (45.7%) and the least sensitivity was shown by Cefoxitin (8.5%).
Conclusions: The findings of this study suggest that hygienic, sanitary, and biosafety precautions should be taken during the milk-production process to prevent the spread of pathogens, as raw milk may serve as a source of virulent and antibiotic-resistant *E. coli*.

Keywords: *Escherichia coli*, milk, PCR, ESBL, resistance

Introduction
Worldwide, food-borne infections are the main cause of public health issues. As per FAO, India is one of the world's top producers of milk, with production rising by 59% over the past three decades from 530 million tonnes in 1988 to 843 million tonnes in 2018. Globally, milk is the dairy product that people consume the most which is a good source of essential nutrients including calcium, proteins and vitamin-D. In addition to its nutritional value and health benefits, raw milk's high-water activity and complex biochemical composition serves as an ideal environment for the growth and multiplication of a wide variety of microorganisms (Ashenafi M and Beyene F *et al.*, 1994) [1]. Microbial contamination of milk is due to entry of bacteria from farm environment by practising unhygienic milking practices. The other contaminating sources responsible for milk contamination are milch animal's skin and udder; water used for cleaning of animal and the milking pails, soil of milking environment, milker's hygiene and milking equipment (Oliver *et al.*, 2005) [20]. The most prevalent coliform bacteria that is typically found in the microflora of mammal's lower gastrointestinal tracts is *Escherichia coli*. Of all the microorganisms, *E. coli* is common contaminant and is a reliable indicator of faecal pollution in unsanitary milking environments (Diliello *et al.*, 1982) [7].
The presence of bacteria in raw milk can increase the risk of consumption of harmful toxins and spreading of food-borne pathogens constituting a public health hazard. *Escherichia coli* harbours six *usp* (Universal stress protein) genes- A, C, D, E, F, and G. The *usp*A gene is the most crucial of these for *E. coli* survival during cellular development, adhesion, and motility (Nachin *et al.*, 2005) [17].

Among the *E. coli* serotypes, *Escherichia coli* infections that produce Shiga toxin (STEC) pose a serious threat to public health since foodborne spread of these bacteria results in over one million illnesses annually worldwide (Devleesschauwer *et al.*, 2019) [8]. The two phage-encoded cytotoxins known as Shiga toxins, *stx*1 and *stx*2, are generally responsible for the pathogenicity of STEC which causes various complications like diarrhoea, haemolytic uremic syndrome (HUS) and haemorrhagic colitis (HC) (Paton *et al.*, 1998; Brett *et al.*, 2003) [22, 5].

Antibiotic-resistant bacteria are caused by the enzyme - Extended Spectrum Beta-lactamases (ESBL). They degrade and destroy the antibiotic, rendering it ineffective against an infection. One of the most prevalent ESBL-producing bacteria is *E. coli*, and its prevalence is also increasing globally by inappropriate administration of antibiotics. The ESBL-producing *E. coli* strains isolated from raw milk samples are potentially hazardous as they can harm consumers and calves and contribute to the transmission of these pathogens which are resistant to antibiotics (Batabyal *et al.*, 2018) [3]. Clavulanic acid inhibits the activity of extended-spectrum β-lactamases (ESBLs), a rapidly developing class of β-lactamases that can hydrolyse aztreonam and third-generation cephalosporins alike. (Paterson *et al.*, 2005) [21].

Thereby, the objective of the current study was to determine the occurrence of *E. coli* in raw milk as well as the virulence genes (*stx*1 and *stx*2) and their ESBL production.

Materials and Methods

Sample Collection: 125 raw milk samples were collected from nearby local farms and milk merchants of 5 villages (Tondavada, Mallaih palli, Dornakambala, Chandragiri and Mittapalem) of Chandragiri mandal located near Tirupati city, Andhra Pradesh (Fig. 1). The samples were collected in sterilized sampling bottles during the milking hours, early in the morning which were then transported to the laboratory in an ice box within 2 hours for further microbiological testing.

***Escherichia coli* Isolation and identification:** Each collected milk sample was inoculated into Nutrient broth (1ml of milk in 9ml broth) and subjected to overnight incubation at 37 °C. After incubation a loop of inoculum was streaked on MacConkey agar and Eosin Methylene Blue (EMB) agar plates and incubated at 37 °C for 24 hours. Later, the inoculated plates were observed for pink coloured colonies on MCA and greenish metallic sheen colonies on EMB agar. Further for confirmation, the specified colonies on EMB and MCA agar plates were subjected to grams staining and biochemical tests *viz.*, IMVC tests (Indole production, Methyl red, Voges Proskauer, Citrate test), Catalase test, Urease production and Nitrate reduction tests (Edwards *et al.* (1972) [9].

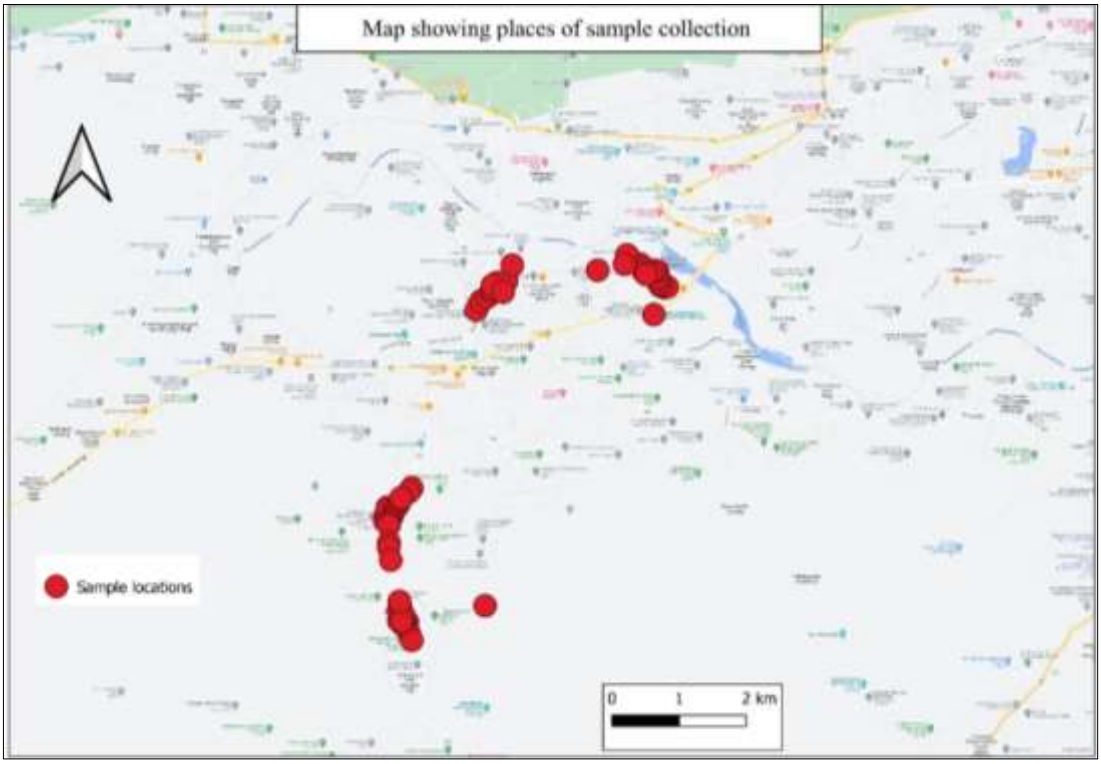

Fig 1: Map showing places of sample collection in Tirupati

DNA Isolation

Extraction of DNA was done by Boiling and Snap chilling method as per standard protocol (Virpari *et al.*, 2013) [29]. Initially all the culturally and biochemically confirmed *E. coli* isolates were cultured overnight in Nutrient broth at 37 °C. A volume of 1.5 ml of enriched broth culture that had been incubated for 18 hours was centrifuged for 10 minutes at 10,000 rpm, and the pellet was then reconstituted in 100 µl of nuclease-free water. Subsequently the mixture was subjected to heat lysis in boiling water bath at 100 °C for about 10 minutes after which it underwent rapid chilling in an ice box at -20 °C and centrifuged at 10,000 rpm for 5 minutes. Finally, the supernatant was stored at -20 °C and utilized as a DNA template for the PCR test. Using Nanodrop, the concentration of DNA was measured (Thermo Scientific, USA).

Polymerase Chain Reaction (PCR)

All the extracted DNA samples were put through PCR to target the *usp*A gene for species-specific characterization

followed by detection of virulence genes *viz.*, *stx*1 and *stx*2 with specific primers. Table-1 lists the primer sequences that were used in the current investigation. Using 12.5 µl of master mix, 4 µl of DNA template, 1 µl of each primer, and 6.5 µl of nuclease-free water, a 25 µl PCR reaction was conducted. As indicated in Table 2, the PCR experiment was carried out in a thermal cycler with a heated cover under standardized cycling settings.

The PCR products that were amplified were put through to 1.5% agarose gel electrophoresis with a 100 bp DNA ladder in a horizontal electrophoresis apparatus at 5V/cm, and ethidium bromide at 0.5 µg/ml. Using the Alpha innotech gel documentation system, the gel was examined under UV trans illumination in order to observe the bands.

Table 1: Specifics about the primers that are used for detection of *usp*A, *stx1 and stx2*

Primers	Primer sequence (5' to 3')	Amplicon size	Reference
*usp*A-F	CCGATACGCTGCCAATCAGT	884 bp	Chen and Griffiths (1998) [6]
*usp*A-R	ACGCAGACCGTAAGGGCCAGAT		
*stx*1-F	ATAAATCGCCATTCGTTGACTAC	180 bp	Paton and paton (1998) [22]
*stx*1-R	AGAACGCCCACTGAGATCATC		
*stx*2-F	GGCACTGTCTGAAACTGCTCC	254 bp	Paton and paton (1998) [22]
*stx*2-R	TCGCCAGTTATCTGACATTCTG		

Table 2: Standardized thermal cycling conditions for PCR assay

Steps	Standardized	conditions	No. of cycles
	***usp*A**	***stx*1 and *stx*2**	
Initial denaturation	94 °C for 5 min	95 °C for 5 min	1
Denaturation	94 °C for 1 min	94 °C for 45 sec	
Annealing	55 °C for 1 min	59 °C for 45 sec	30
Extension	72 °C for 5 min	72 °C for 1 min	
Final extension	72 °C for 5 min	72 °C for 6 min	1
Hold/standby	4 °C for ∞	12 °C for ∞	

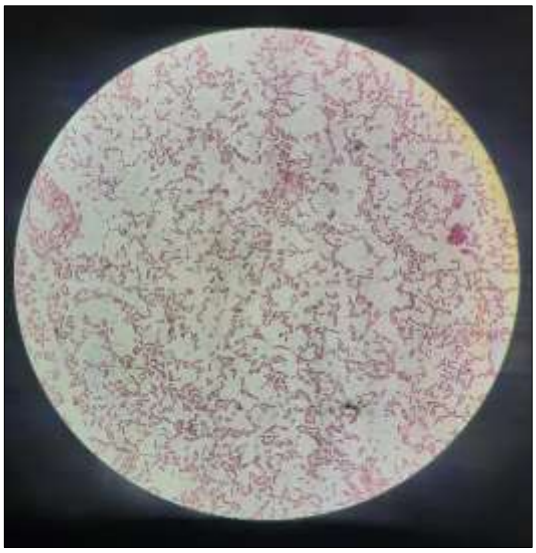

Fig 2: Gram staining showing pink coccobacillary rods of *E. coli*

Antimicrobial sensitivity test and ESBL production

The Kirby-Bauer method was used to measure the antimicrobial sensitivity of each confirmed *E. coli* isolate against the selected antibiotic discs. Furthermore, using the double disk diffusion method, all of the isolates were examined for the production of Extended Spectrum Beta-lactamases (ESBLs), and the results were interpreted in accordance with the guidelines provided by CSLI (2021).

Briefly, fresh overnight cultures of *E. coli* were inoculated into sterile PBS solution and matched with 0.5 McFarland's turbidity standard. After spreading a sterile swab-based bacterial culture evenly over Mueller-Hinton agar plates, antibiotic discs were placed as per standard (Rodrigues *et al*., 2004) [24] and the plates were incubated for 24 hours at 37 °C.

Risk factor analysis

Questionnaires related to milking management, animal and disease management were prepared using Epi Info™ software. An observational survey was done at farm level.

Results

***E. coli* prevalence rate:** On testing 125 milk samples by cultural and biochemical tests, a total of 48 isolates given a characteristic result for *E. coli* giving an overall prevalence of 38.4% (Table-3).

Molecular identification of *E. coli*'s *usp*A and *stx* genes

35 of the 48 *E. coli* isolates tested positive for the species-specific PCR targeting *usp*A gene, resulting in a prevalence of 28% (Fig. 3). The 35 *E. coli* isolates that underwent PCR confirmation were tested for the presence of the pathogenic genes *stx*1 and *stx*2, which are generated by *E. coli* that produces Shiga toxin (STEC) (Fig. 4). Of them, 14 (40%) isolates harboured *stx*1 gene, 8 (22.85%) isolates positive for *stx*2 gene while 4 (11.43%) isolates carried both *stx*1 and *stx*2 genes (Table-3).

Table 3: *E. coli* prevalence in raw milk samples as determined by PCR and cultured techniques

Total number of samples collected and tested	No. of samples positive by cultural methods	No. of samples positive by PCR (*usp*A)	Prevalence of *E. coli* that produces Shiga toxin		
			stx1	***stx2***	***stx1* & *stx2***
125	48 (38.4%)	35 (28%)	14 (40%)	8 (22.85%)	4 (11.43%)

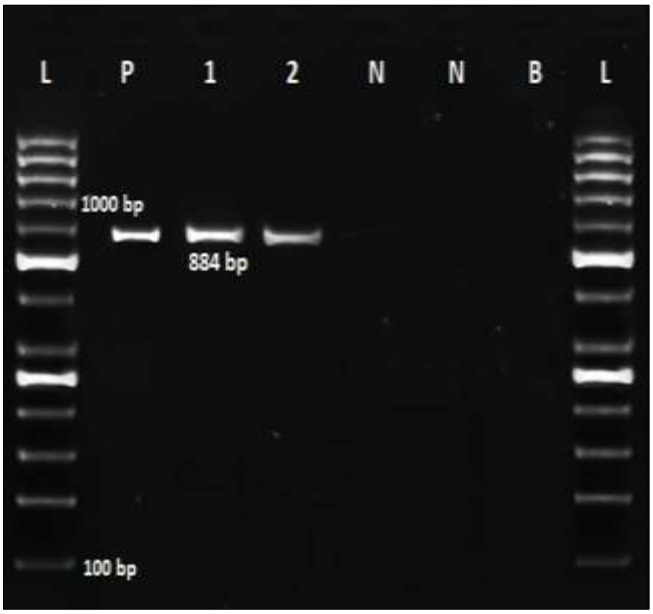

Fig 3: Agarose gel displaying the 884 bp *E. coli usp*A gene product amplified by PCR. P - Positive control, N - Negative control, L - DNA Ladder, Lane 1 to 2 - Positive samples, B – Blank

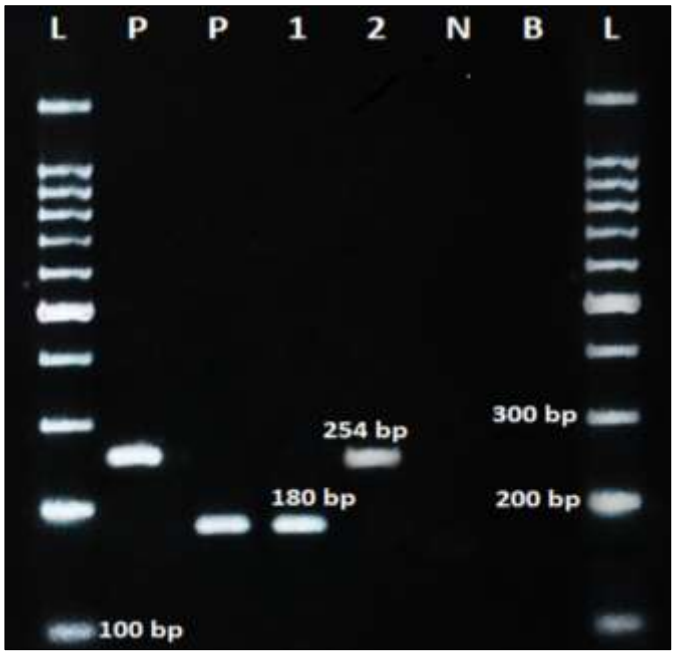

Fig 4: Agarose gel demonstrating the *stx*1 and *stx*2 genes of *E. coli* amplified by PCR, P - Positive control, N - Negative control, L - DNA Ladder, Lane 1 - Amplified DNA of *stx*1 gene (180 bp), Lane 2 - Amplified DNA of *stx*2 gene (254 bp), B – Blank

Antibiotic sensitivity pattern and ESBL production

The double disk diffusion method was used to perform phenotypic screening for ESBL production on all 35 PCR-positive isolates. Nine (25.7%) of the 35 isolates had positive ESBL production results. When the zone inhibition of cefotaxime plus clavulanic acid or ceftazidime plus clavulanic acid was ≥5 mm greater than their respective single discs, the test was considered positive (CLSI 2021).

Highest sensitivity was observed against Aztreonam (68.5%), Cefotaxime (48.5%) and Ceftazidime + Clavulanic acid (45.7%) (Fig. 5). Cefoxitin (71.5%) showed the greatest resistance, followed by Ceftazidime (60%) (Table-4).

Table 4: Patterns of antibiotic susceptibility in *E. coli* isolates from raw milk

S. No.	Antimicrobial agents	Sensitive	Intermediate	Resistant
1.	Imipenem (IPM), 10µg	11 (31.5%)	14 (40%)	10 (28.5%)
2.	Cefotaxime (CTX), 30µg	17 (48.5%)	11 (31.5%)	7 (20%)
3.	Cefoxitin (CX), 30µg	3 (8.5%)	7 (20%)	25 (71.5%)
4.	Ceftriaxone (CTR), 30µg	11 (31.5%)	14 (40%)	10 (28.5%)
5.	Ceftazidime (CAZ), 30µg	7 (20%)	7 (20%)	21 (60%)
6.	Ceftazidime + Clavulanic acid (CAC), 30/10µg	16 (45.7%)	10 (28.6%)	9 (25.7%)
7.	Aztreonam (AT), 30µg	24 (68.5%)	7 (20%)	4 (11.5%)

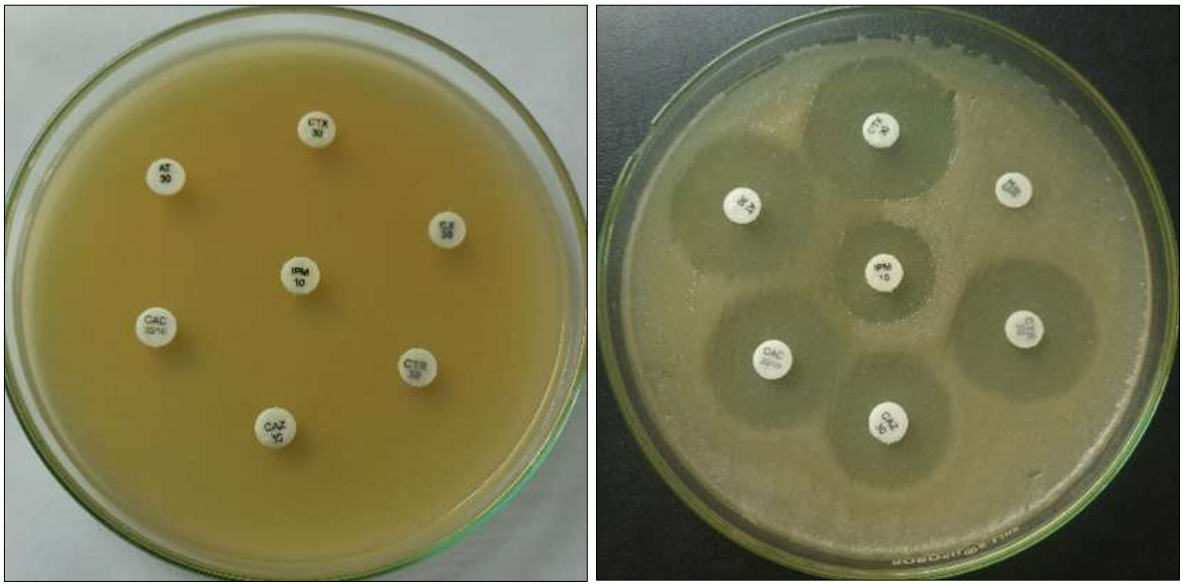

Fig 5: Antibiotic sensitivity patterns of the *E. coli* isolate

Risk factor analysis: Following the results of completed questionnaire and observational survey conducted the risk factor analysis was shown in the bar graphs (Fig. 6); (Fig. 8) and the herd size was shown in the pie chart (Fig. 7). Before milking, the majority of farm members (100%) had the practice of cleaning their animals' udder and teats using stored water (74%), among them 80% of households clean their barn on daily basis. Most (96%) of the respondents practice milking twice per day. More than half of the respondents (66%) housed their animals in soil type of bedding conditions. Henceforth, the factors such as bedding conditions, thorough washing of udder and teats before milking, teat dipping practice, barn cleaning frequency and source of water used are majorly seemed to contribute for microbial contamination in milk.

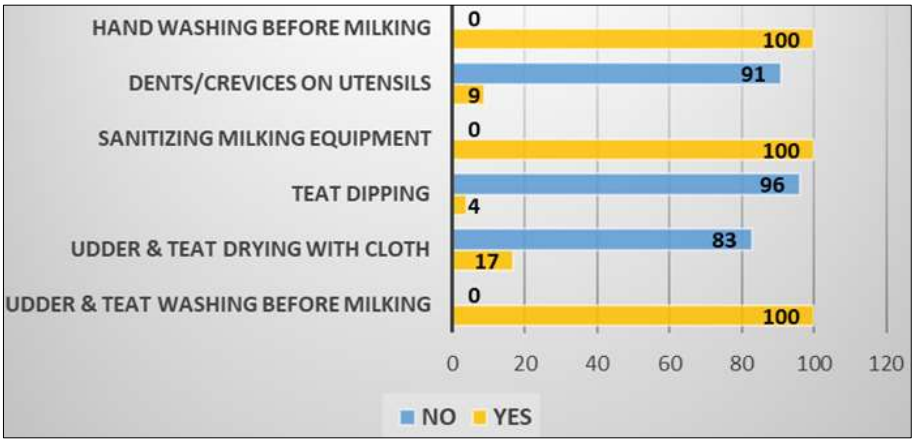

Fig 6: Analysis of risk factor from milking management

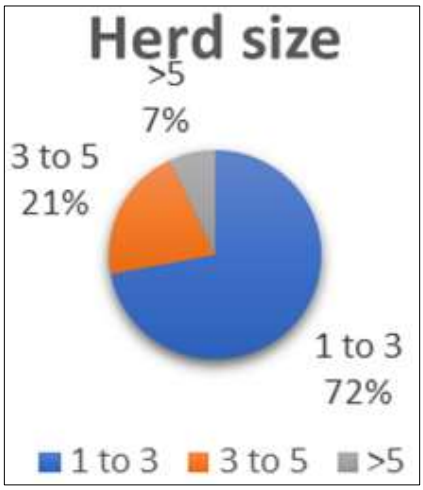

Fig 7: Pie chart representing Herd size

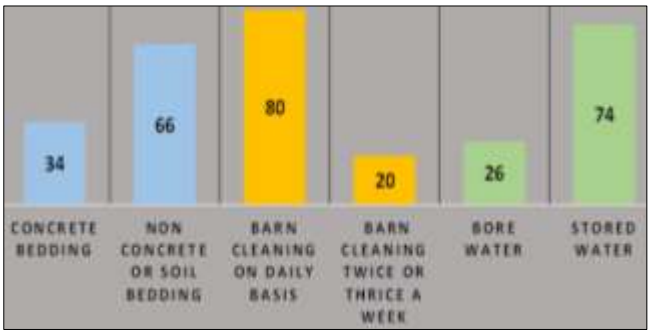

Fig 8: Risk factor analysis from farm environment management

Discussion

E. coli belonging to *Enterobacteriaceae* family are facultative anaerobic, non-sporulating, and gram-negative bacteria. The species is classified in to different pathotypes *viz.* Entero-

invasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), Diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), Enterohemorrhagic *E. coli* (EHEC), Shiga toxin producing *E. coli* (STEC) and Attaching and Effacing *E. coli* (AEEC) (Bavaro *et al*. (2012) [4]. Shiga toxin producing *E. coli* are the one mainly associated with the food borne infections and having public health importance.

Isolation and identification of *E. coli*

The prevalence of *E. coli* in the present study was estimated to 38.4% in the raw milk, the results are in accordance with the results of Vahedi *et al.* (2013) [26] where 42% prevalence was reported. In contrary to the present study, slightly lower prevalence of 33.96% and 31.6% was reported by Mohd *et al.* (2013) [15] and Nanu *et al*. (2007) [18] respectively.

Molecular characterization of *E. coli*

In this study, a universal stress protein (*usp*A) gene of *E. coli*, important for activities like adhesion and motility, was targeted for rapid detection by PCR. Thirty five out of 48 (72.91%) isolates, were positive for species-specific *usp*A gene which is similar to findings of Fazal *et al*. (2022) [12] who had reported 78% prevalence, while lower prevalence of 53.5% was reported by Vendramin *et al*. (2014) [27].

Molecular detection of virulence genes of *E. coli*

In the present study, the prevalence of STEC was found to be 14.4% (18 of 125 raw milk samples), which is similar to the works of Vernozy Rozand *et al*. (2005) [28] and Mohd *et al.* (2013) [15] where prevalence of STEC was reported to be 13% and 6.98% in raw milk products samples and raw milk samples respectively.

Out of 35 PCR positive *E. coli* isolates, 14 (40%) isolates harboured *stx*1 gene and 8 (22.8%) isolates harboured *stx*2 gene. This is almost identical to the findings of Momtaz *et al.* (2012) [16] who found 46.29% and 20.37% prevalence of *stx*1 and *stx*2 genes respectively. Mohd *et al.* (2013) [15] and Farzan *et al.* (2012) [11] discovered a similar predominance of *stx*1 producing strains. Virpari *et al.* (2013) [29] and Sabry *et al.* (2008) [25] both reported a predominance of *stx*2 producing strains.

Antibiotic sensitivity of *E. coli*

E. coli possess different antibiotic resistance genes, including beta-lactam antibiotic resistance genes. Presence of beta-lactam antibiotic resistance genes are responsible for production of beta-lactamase enzymes in *E. coli* (Putra *et al*., 2020 and Effendi *et al*., 2021) [23, 10]. Phenotypically, 25.7% (9/35) of *E. coli* isolates produced ESBL. This is almost identical to studies of Joseph *et al.* (2022) [14] and Badri *et al.* (2017) [2] who found 26.75% and 29.3% ESBL prevalence in bovine milk samples respectively. Presence of ESBL producing *E. coli* in milk indicates unhygienic milking or environmental pollution (Okeke *et al*., 2005) [19].

Therefore, strict hygiene and managemental practices *viz.*, 'washing the hands with soap and water, washing the teats and udder with sanitizing solutions, thoroughly drying of teats and udder with individual towels, using effective germicidal teat dip to reduce the number of pathogenic bacteria from the udder skin' should be adopted for herd management; milch animal management; during pre and post milking activities. (Jones *et al*., 2006) [13].

Conclusion

Human-consumed milk can become tainted with *E. coli* through indirect means such as contaminated water, farm equipment, or udder and teats contaminated with animal faeces. Antibiotic-resistant bacteria can spread due to the presence of ESBL-producing *E. coli* and STEC in raw milk, which is a major public health risk. Therefore, hygienic practices should be employed at farm level and pollution free farm environment has to be maintained to reduce the prevalence of these pathogenic bacteria.

Author's contribution

K. Pavani carried out the entire investigation, including sample collection and processing. PVS helped in design and analysis using Epi Info™ software. With assistance from PVS and PSB, KP wrote the first draft of the manuscript and corrected it. C. Siva Swetha and A. Jagadeesh Babu contributed to the study's design, coordination, and scientific discussion in addition to writing the final text. The final manuscript was read and approved by all authors.

Acknowledgements

The authors are thankful to Dr. C. Siva Swetha, Assistant Professor and Dr. A. Jagadeesh Babu, Professor and Head, Dept. of Veterinary Public Health & Epidemiology, College of veterinary science, Tirupati for providing guidance, constant encouragement and necessary resources required to conduct this research.

Competing Interests

The authors state that they do not have any competing interests.

References

1. Ashenafi M, Beyene F. Microbial load, microflora and keeping quality of raw and pasteurized milk from a dairy farm. Bulletin of animal health and production in Africa; c1994.
2. Badri AM, Ibrahim IT, Mohamed SG, Garbi MI, Kabbashi AS, Arbab MH. Prevalence of extended spectrum beta lactamase (ESBL) producing *Escherichia coli*, and Klebsiella pneumoniae isolated from raw milk samples in Al Jazirah state, Sudan. Mol. Biol. 2017;7(1):201.
3. Batabyal K, Banerjee A, Pal S, Dey S, Joardar SN, Samanta I, *et al*. Detection, characterization, and antibiogram of extended-spectrum beta-lactamase *Escherichia coli* isolated from bovine milk samples in West Bengal, India. Veterinary world. 2018;11(10):1423.
4. Bavaro MF. *E. coli* O157: H7 and other toxigenic strains: the curse of global food distribution. Current gastroenterology reports. 2012;14:317-323.
5. Brett KN, Hornitzky MA, Bettelheim KA, Walker MJ, Djordjevic SP. Bovine non-O157 Shiga toxin 2-containing *Escherichia coli* isolates commonly possess stx 2-EDL933 and/or stx 2vhb subtypes. Journal of Clinical Microbiology. 2003;41(6):2716-2722.
6. Chen J, Griffiths MW. PCR differentiation of *Escherichia coli* from other Gram-negative bacteria using primers derived from the nucleotide sequences flanking the gene encoding the universal stress protein. Letters in applied microbiology. 1998;27(6):369-371.
7. Diliello LR. Methods in Food and Dairy Microbiology. AVI Publishing Co. Inc. Westport Connt. USA, 1982, 39.
8. Devleesschauwer B, Pires SM, Young I, Gill A, Majowicz SE. Associating sporadic, foodborne illness

caused by Shiga toxin-producing *Escherichia coli* with specific foods: a systematic review and meta-analysis of case-control studies. Epidemiology & Infection. 2019, 147.
9. Edwards PR, Ewing WH. Identification of Enterobacteriaceae. Emerg. Infect. Dis. 1972;12:154-159.
10. Effendi MH, Tyasningsih W, Yurianti YA, Rahmahani J, Harijani N, Plumeriastuti H. Presence of multidrug resistance (MDR) and extended-spectrum beta-lactamase (ESBL) of *Escherichia coli* isolated from cloacal swab of broilers in several wet markets in Surabaya, Indonesia. Biodiversitas. 2021;22(1):304-310.
11. Farzanh R, Rahimi E, Momtaz H. Virulence properties of Shiga toxin-producing *Escherichia coli* isolated from Iranian raw milk and dairy products. Slovenian Veterinary Research, 2012, 49(4).
12. Fazal MA, Nath C, Islam MS, Hasib FY, Reza MMB, Devnath HS, *et al*. Isolation and identification of multidrug-resistant *Escherichia coli* from cattle, sheep, poultry and human in Cumilla, Bangladesh. Malaysian Journal of Microbiology. 2022;18(2):227-234.
13. Jones GM, Bailey TL. Understanding the basics of mastitis. Virginia Cooperative Extension, Publication No. 404-233. Virginia State University; c2006. p. 1-7.
14. Joseph J, Kalyanikutty S. Occurrence of multiple drug-resistant Shiga toxigenic *Escherichia coli* in raw milk samples collected from retail outlets in South India. Journal of Food Science and Technology. 2022;59(6):2150-2159.
15. Mohd R, Kotwal SK, Malik MA, Maninder S. Prevalence, genetic profile of virulence determinants and multidrug resistance of *Escherichia coli* isolates from foods of animal origin. Veterinary World. 2013;6(3):139-142.
16. Momtaz H, Farzan R, Rahimi E, Safarpoor Dehkordi F, Souod N. Molecular characterization of Shiga toxin-producing *Escherichia coli* isolated from ruminant and donkey raw milk samples and traditional dairy products in Iran. The Scientific World Journal; c2012.
17. Nachin L, Nannmark U, Nyström T. Differential roles of the universal stress proteins of *Escherichia coli* in oxidative stress resistance, adhesion, and motility. Journal of bacteriology. 2005;187(18):6265-6272.
18. Nanu E, Latha C, Sunil B. Quality assurance and public health safety of raw milk at the production point. American Journal of Food Technology. 2007;2(3):145-152.
19. Okeke IN, Laxminarayan R, Bhutta ZA, Duse AG, Jenkins P, O'Brien TF, *et al*. Antimicrobial resistance in developing countries. Part I: recent trends and current status. The Lancet infectious diseases. 2005;5(8):481-493.
20. Oliver SP, Jayarao BM, Almeida RA. Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. Foodbourne Pathogens & Disease. 2005;2(2):115-129.
21. Paterson DL, Bonomo RA. Extended-spectrum β-lactamases: A clinical update. Clinical microbiology reviews. 2005;18(4):657-686.
22. Paton AW, Paton JC. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx 1, stx 2, eaeA, enterohemorrhagic *E. coli* hlyA, rfb O111, and rfb O157. Journal of clinical microbiology. 1998;36(2):598-602.
23. Putra AR, Effendi MH, Koesdarto S, Suwarno S, Tyasningsih W, Estoepangestie AT. Detection of the extended spectrum β-lactamase produced by *Escherichia coli* from dairy cows by using the Vitek-2 method in Tulungagung regency, Indonesia. Iraqi J Vet Sci. 2020;34(1):203-207.
24. Rodrigues C, Joshi P, Jani SH, Alphonse M, Radhakrishnan R, Mehta A. Detection of β-lactamases in nosocomial gram negative clinical isolates. Indian journal of medical microbiology. 2004;22(4):247-250.
25. Sabry AH, Elmalt LM. Informally raw milk and kareish cheese investigation on the occurrence of toxigenic *Escherichia coli* in qena city, egypt with emphasis on molecular characterization. Ass. Univ. Bull. Environ. Res. 2008;11(2):35-42.
26. Vahedi M, Nasrolahei M, Sharif M, Mirabi AM. Bacteriological study of raw and unexpired pasteurized cow's milk collected at the dairy farms and super markets in Sari city in 2011. Journal of preventive medicine and hygiene. 2013;54(2):120.
27. Vendramin T, Kich DM, Molina RD, Souza CFVD, Salvatori RU, Pozzobon A, *et al*. Molecular screening of bovine raw milk for the presence of Shiga toxin-producing *Escherichia coli* (STEC) on dairy farms. Food Science and Technology. 2014;34:604-608.
28. Vernozy-Rozand C, Montet MP, Berardin M, Bavai C, Beutin L. Isolation and characterization of Shiga toxin-producing *Escherichia coli* strains from raw milk cheeses in France. Letters in applied microbiology. 2005;41(3):235-241.
29. Virpari PK, Nayak JB, Brahmbhatt MN, Thaker HC. Study on isolation, molecular detection of virulence gene and antibiotic sensitivity pattern of *Escherichia coli* isolated from milk and milk products. Veterinary World, 2013, 6(8).