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RAW MILK AS A RESERVOIR OF ANTIBIOTIC RESISTANT ENTEROPATHOGENIC *ESCHERICHIA COLI*

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ABSTRACT

Escherichia coli is a commensal in the intestine of humans and warm-blooded animals. Usually, they are harmless except certain strains like enteropathogenic, enteroinvasive, enteroaggregative, enterotoxigenic, enterohaemorrhagic and diffusely adherent *E. coli*. The present study was conducted to study the prevalence antibiotic resistant enteropathogenic *Escherichia coli* (EPEC) from raw cow milk in Ernakulam district of Kerala in India. A total of 100 raw cow milk samples were collected from various milk societies of the district. *Escherichia coli* was isolated by conventional culture technique on MacConkey agar. The culture and biochemical positive isolates were further confirmed by molecular technique by targeting genus specific 16S rRNA gene. The isolates obtained were further subjected to molecular confirmation by targeting eaeA and bfpA genes specific for enteropathogenic *E. coli*. The antibiotic sensitivity pattern of the confirmed isolates was tested against commonly used antibiotics by disc diffusion assay. Further molecular characterisation of the isolates was carried out by targeting dfrA1, dfrA10 for trimethoprim resistance, sul1 and sul2 for sulphamethoxazole resistance, and aadA1 and aadA2 for streptomycin resistance by Polymerase chain reaction (PCR).

Keywords: *E. coli*, raw cow milk, AMR

INTRODUCTION

Antibiotic resistance poses a critical global health challenge, necessitating urgent attention and comprehensive investigations to understand its multifaceted origins and potential reservoirs. In recent years, there has been a growing concern regarding the emergence and dissemination of

antibiotic resistance among foodborne pathogens, notably Enteropathogenic *E. coli* (Aslam *et al.,* 2018). *Escherichia coli* is a commensal in the intestine of humans and warm-blooded animals. Usually, they are harmless except certain strains like enteropathogenic, enteroinvasive, enteroaggregative, enterotoxigenic, enterohaemorrhagic and diffusely adherent *E. coli*. Enteropathogenic *E. coli* was first reported in 1945 by John Bray. The main lesion of this infection is the 'attaching-and-effacing' histopathology seen in bowel biopsy specimens. The plasmid carries the genetic code for a bundle-forming pilus (BFP), allowing EPEC to establish a specific attachment to enterocytes in the small intestine. Following this attachment, the outer membrane protein colonisation factor known as intimin plays a crucial role in promoting adherence. Encoded by the *eae* gene within the locus of enterocyte effacement (LEE) chromosomal island, intimin serves as an outer membrane protein colonization factor (Mueller and Tainter, 2023). Outbreaks of foodborne illness have been associated with milk and dairy products, although the frequency of such incidents is lower compared to outbreaks linked to meat and vegetables (Dell'Orco *et al*., 2019).

The use of antimicrobials in animal husbandry is a significant factor contributing to the emergence of antimicrobial resistance (AMR). It occurs when a microorganism becomes unresponsive to a drug that it was originally susceptible to. Issues such as over-prescription, improper dosages, and incorrect durations of medication further exacerbate this problem. The growing demand for food, driven by factors such as population growth, urbanisation, and increased income, has compelled farmers to produce more in limited spaces, intensifying the reliance on antimicrobial use in animal husbandry (Mutua *et al*., 2020). Sulphonamides, trimethoprim, and streptomycin represent antibiotics employed for the treatment of bacterial infections in cows. Sulphonamides, being the oldest and most widely utilised antibacterial agents in veterinary medicine, are favored for their cost-effectiveness and relative efficacy against common bacterial diseases in various species. They are commonly employed to manage systemic infections caused by susceptible bacteria. Trimethoprim, a diaminopyrimidine, efficiently inhibits dihydrofolate reductase in bacteria and protozoa, exhibiting greater efficacy in these organisms compared to mammalian cells. While trimethoprim alone may not be highly effective against bacteria, its combination with sulfonamides produces bactericidal effects. Streptomycin, an aminoglycoside antibiotic, finds application in the treatment of bacterial infections in cattle, addressing conditions

such as respiratory infections, mastitis, and metritis (Singh and Goyal, 2022). This study aims to address a critical gap in knowledge by exploring the prevalence and antibiotic resistance profiles and to assess the molecular basis of trimethoprim, sulphamethoxazole and streptomycin resistance in EPEC strains isolated from raw cow milk in the Ernakulam district of Kerala.

MATERIALS AND METHODS

Collection of milk samples

The samples were collected aseptically after thorough mixing of milk in individual cans, with a plunger and by transferring approximately 250 mL of milk to sterile sample containers.

Isolation and identification of E. coli by culture techniques

The isolation and identification of *E. coli* was done by conventional culture technique (Meng *et al*., 2001). Milk sample (0.1 ml) was transferred to 9.9 mL of Nutrient broth (NB), and incubated at 37°C for 24h. The samples after enrichment were streaked onto MacConkey agar (MCA) and incubated at 37°C for 24 h. Characteristic lactose fermenting bright pink-colored colonies surrounded by bile precipitate was selected based on morphology and subjected to biochemical tests.

DNA extraction and polymerase chain reaction

The DNA extraction was done by snap chill method (Swetha *et al*., 2015). Three sets of oligonucleotide primers targeting the conserved genus specific *16S*rRNA gene and virulence genes *viz*., *eae*A and *bfp*A for identification of enteropathogenic *E. coli* (EPEC) were used for the study. The primers used for identification are listed in table 1. A simple PCR and duplex PCR was performed in a final volume of 25µL reaction mixture using 3µL of extracted DNA as template for the amplification of *16S*rRNA gene and *eae*A and *bfp*A genes, respectively. The *16S*rRNA gene was amplified with initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 50 sec. and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The virulence genes were amplified with initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 40 sec, annealing at 58°C for 1 min and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min.

Antibiotic resistance profiling

All the isolates that were positive for virulence genes were subjected to antibiotic susceptibility test against amoxyclav (30µg), ampicillin (10 µg),

azithromycin (15 µg), cefixime (5 µg), cefotaxime (30 µg), cefoperazone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), co-trimoxazole (25 µg), doxycycline (30 µg), enrofloxacin (10 µg), streptomycin (10 µg), tetracycline (30 µg) and trimethoprim (5 µg) antibiotics by Kirby-Bauer disc diffusion method (CLSI, 2020).The isolates that showed resistance to trimethoprim, co-trimoxazole and streptomycin by phenotypic method were subjected to multiplex PCR for the detection of *dfr*A1 and *dfr*A10 for trimethoprim resistance, *sul*1 and *sul*2 for sulphamethoxazole resistance and *aad*A1 and *aad*A2 genes for streptomycin resistance separately. A final volume of 25µL reaction mixture using 3µL of extracted DNA as template,12.5 µL mastermix, 10 pmoles/ µL each of forward and reverse primer of resistance genes (*dfr*A1 and *dfr*A10 for trimethoprim resistance, *sul*1 and *sul*2 for sulphamethoxazole resistance and *aad*A1 and *aad*A2 genes for streptomycin resistance) and nuclease free water made upto 25µL total volume. The primers used for detection of *dfr*A1 and *dfr*A10, *sul*1 and *sul*2, *aad*A1 and *aad*A2 are shown in table 2.

The cyclic condition of duplex PCR for *dfr*A1 and *dfr*A10 genes included an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 45 sec, annealing at 64.5°C for 30 sec. and extension at 72°C for 45 sec, followed by a final extension at 72°C for 10 min. The *sul*1 and *sul*2 genes were amplified with an initial denaturation temperature of 95°C for 5 min, 35 cycles of denaturation at 94°C for 45 sec, annealing at 65.6°C for 45 sec. and extension at 72°C for 45 sec, followed by a final extension at 72°C for 5 min. The cyclic condition of duplex PCR for *aad*A1 and *aad*A2 genes included an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 58.5°C for 45sec. and extension at 72°C for 45 sec, followed by a final extension at 72°C for 10 min.

Table 1. Primers used for identification of *16S* rRNA, *eae*A and *bfp*A genes

Primer	Primer sequence	Size (bp)	Reference
16*SrRNA* F	5'ATCAACCGAGATTCCCCCAGT3'	231	Sun Dong-bo *et al.* (2011)
16*SrRNA* R	5'CACTATCGGTCAGT CAGGAG 3'		
*eae*A F	5' TCCTGGTTCCCTTATCAACG3'	209	Mathew (2021)
*eae*A R	5'GCGACCGCTACCAACATAG 3'		
*bfp*A F	5'AATGGTGCTTGCGCTTGCTGC3'	326	Aranda *et al.* (2004)
*bfp*A R	5'GCCGCTTTATCCAACCTGGTA3'		

Table 2. Primers used for identification of *dfr*A1 and *dfr*A10, *sul*1 and *sul*2, *aad*A1 and *aad*A2

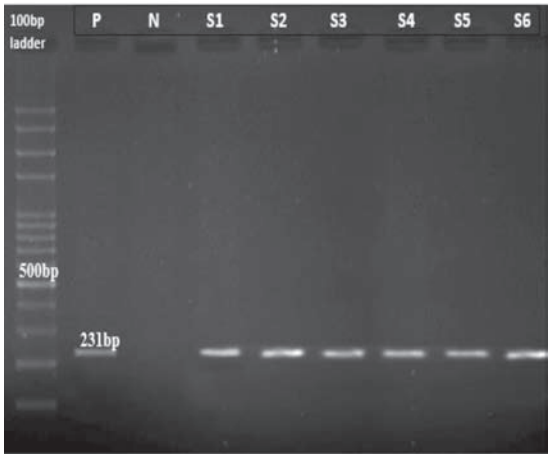
Primer	Primer sequence	Size (bp)	Reference
*dfr*A1 F	5' GGGAGCATTACCCAACCGAA3'	151	Mathew (2021)
*dfr*A1 R	5' CTCCCCACCACCTGAAACAA 3'		
*dfr*A10 F	5'AGAGCATTCGGTAATCAAGGCA3'	513	Mathew (2021)
*dfr*A10 R	5' ATCATTTGGCACCCCAACCA 3'		
*sul*1 F	5' TTCGGCATTCTGAATCTCAC3'	822	Van *et al*. (2007)
*sul*1 R	5' ATGATCTAACCCTCGGTCTC 3'		
sul2 F	5' GCGCTCAAGGCAGATGGCATT3'	285	Kerrn *et al.* (2002)
sul2 R	5' GCGTTTGATACCGGCACCCGT 3'		
*aad*A1 F	5' TATCCAGCTAAGCGCGAACT3'	490	Van *et al*. (2007)
*aad*A1 R	5' ATTTGCCGACTACCTTGGTC 3'		
*aad*A2 F	5' ATCATCCCGTGGCGTTATCC3'	372	Mathew (2021)
*aad*A2 R	5' CTGGGCAGGTAGGCGTTTTA 3'		

RESULTS AND DISCUSSION

Isolation and Identification of E. coli from raw cow milk

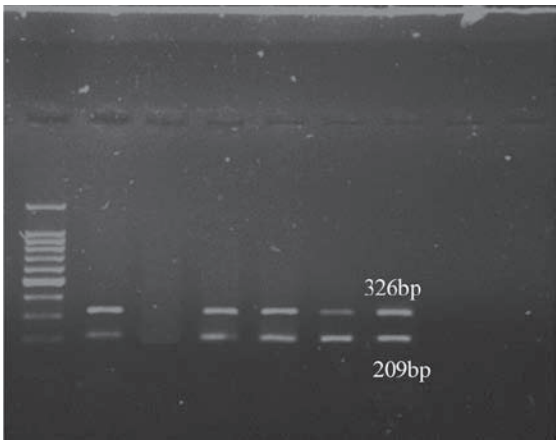
Out of 100 milk samples collected from Ernakulam districts, *E. coli* was isolated from 32 out of 100 milk samples (32 per cent) (Fig.1). Among this, 15 isolates were found to be enteropathogenic *E. coli* (46.87 per cent). Out of these 32 *E. coli* isolates 15 isolates were positive for *eae*A and 7 isolates were positive for *bfp*A genes. The amplicons for *eae*A and *bfp*A were obtained at 209 and 326 bp, respectively (Fig.2). All the samples which showed positive for *bfp*A were positive to *eae*A also. Similar study was done by Ombarak *et al*. (2016) reported the occurrence of 47.2 per cent of enteropathogenic *E. coli* in raw milk from Egypt. Whereas in the study of Junior *et al*. (2019) from Northeast Brazil, out of the 205 *E. coli* isolates from milk, only 9.75 per cent were enteropathogenic.

Out of these 15 isolates of EPEC, ABST study reveals that the highest resistance was shown by ampicillin and tetracycline. Similarly, 13 isolates (86 per cent) were resistant to co-trimoxazole, 9 isolates (60 per cent) were resistant to trimethoprim and 5 isolates (33.3 per cent) were resistant to streptomycin. Similar results were obtained in the study of Dyar *et al*. (2012) in which high level of resistance was shown by co-trimoxazole (87 per cent) followed by chloramphenicol (40 per cent). In study of Adzitey, (2020) from Ghana, reported that more than 80 per cent of *E. coli* isolates were susceptible to sulphamethoxazole and trimethoprim. The resistant isolates were further subjected to



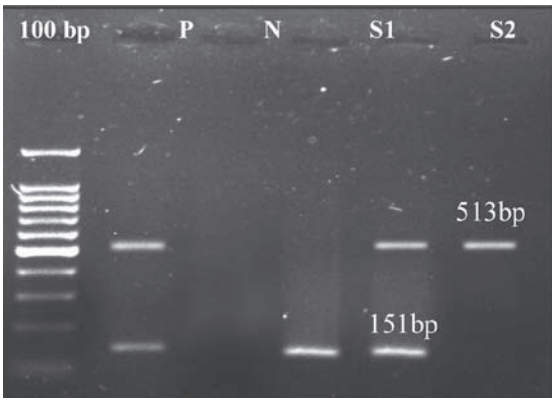
P- Positive Control
N- Negative Control
S1, S2, S3, S4, S5, S6 - Samples

Fig. 1 Detection of *16S r*RNA gene by PCR



P- Positive Control
N- Negative Control
S1, S2, S3, S4 -Samples

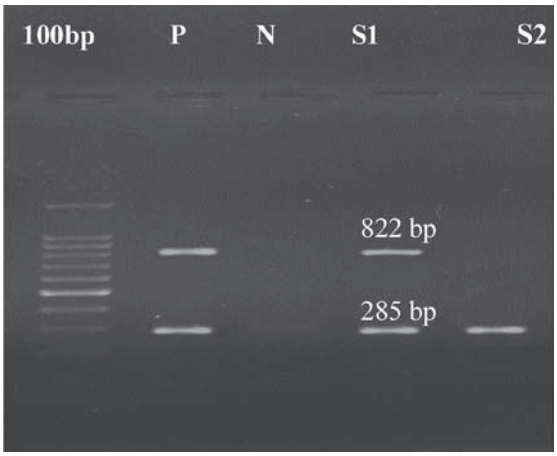
Fig. 2. Detection of *eae*A and *bfp*A genes by PCR



P- Positive Control
N- Negative Control
S1, S2, S3 - Samples

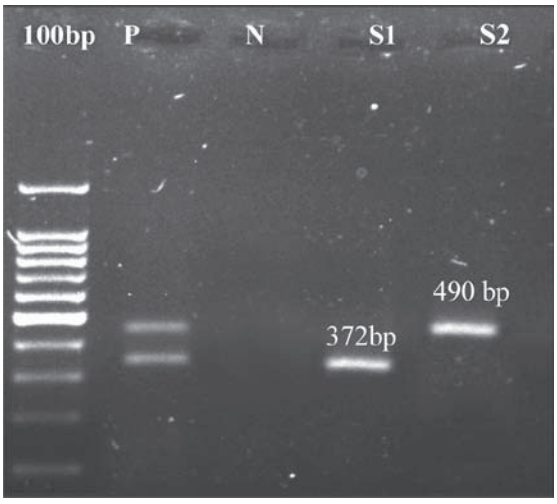
Fig. 3 Detection of *dfr*A1 and *dfr*A10 gene by PCR

PCR for the detection of resistant genes. From the nine phenotypic resistant isolates, five were positive for *dfr*A1 (55.55 per cent) and one was positive for *dfr*A10 (11.11 per cent). The amplicons for *dfrA1* and *dfr*A10 were obtained at 151 and 513 bp, respectively (Fig.3). This result was in accordance with the study of Sunde and Norstrom, (2006) where 79 per cent



P- Positive Control
N- Negative Control
S1, S2 - Samples

Fig. 4. Detection of *sul*1 and *sul*2 gene by PCR



P- Positive Control
N- Negative Control
S1, S2 - Samples

Fig. 5. Detection of *aad*A1 and *aad*A2 gene by PCR

of trimethoprim resistant isolates showed the presence of *dfr*A1. But, contrary to the result in the study of Liamthong (2008) at Thailand, only eight per cent were positive for *dfr*A1 and 40.2 per cent were positive for *dfr*A10 genes. Similarly, for the detection of *sul*1 and *sul*2 genes, of the 13 resistant isolates, 3 isolates were positive for *sul*1(23.07 per cent) and 7 were positive for *sul*2 genes (53.84 per cent). The amplicons for *sul1* and *sul2* were obtained at 822 and 285 bp, respectively (Fig.4.). This result was in accordance with the study of Tang *et al*. (2011) *sul*1 and *sul*2 were detected in 34.4 and 55.2 per cent of resistant isolates, respectively. But, comparatively higher occurrence of *sul*1 (40 per cent) was detected in the study of Hammerum *et al*. (2006) from Denmark. From the 5 streptomycin resistant isolates two (40 per cent) each were positive for *aad*A1 and *aad*A2 genes. The amplicons for *aadA1* and *aadA2* were obtained at 490 and 372

bp, respectively (Fig.5.). The results were in accordance with the study of Lay *et al*. (2012) where 42.9 per cent of the resistant isolates were positive for *aad*A2 genes.

The results of the study can be used to steer and encourage responsible antimicrobial use among farmers by formulating strategies to mitigate and diminish the potential risks associated with antimicrobial resistance.

CONCLUSION

The present study concludes that the milk acts as a carrier of multidrug resistant enteropathogenic *E. coli.* Since, antimicrobial resistance is an alarming public health concern, studies must focus on the prevalence of AMR in foodborne pathogens of animal origin. Antimicrobial resistance is a natural phenomenon. But, the indiscriminate use of antibiotics accelerates the process. So, awareness should be created among the personnels involved in animal husbandry and the general public about AMR and antibiotic stewardship and non-therapeutic use of antibiotics should be checked.

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Conflict of interest

The authors declare that they have no competing interest.

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