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A Pilot Study on β-lactamase Producing *Escherichia coli* from Bovine Milk, West Bengal

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Abstract

Drug resistant bacteria have become a significant global healthcare issue that requires close attention. With regards to antibiotic resistance, E.coli and its Enterobacteriaceae family are highly notorious for their ability to swiftly spread antibiotic resistance genes, thereby posing a major concern. In order to effectively address the spread of multi drug resistant bacteria, ongoing monitoring of the microflora in milk for the presence of drug resistant genes is essential. This continuous assessment will enable us to determine the frequency at which antibiotic resistant genes are found specifically in E.coli. As part of a recent study, a total of 288 E.coli colonies were isolated from bovine milk (n=144) to obtain a comprehensive understanding of the situation at hand. These isolates were thoroughly examined, resulting in the identification of multi drug resistance in a noteworthy percentage of the E.coli strains, specifically 24.3% of them. Further analysis to determine the types of resistance revealed that the majority of the resistance cases were attributed to $_{bla}$AmpC type beta-lactamases, accounting for approximately 65.03% of the observed instances. Additionally, a smaller percentage of resistance genes were seen to belong to 4.14% of the for $_{bla}$CTX-M and 7.14 % for $_{bla}$TEM. It is important to note that roughly 42.85% of the E.coli isolates were found to be AmpC type beta-lactamase producers. These findings shed a brighter light on the prevalence of drug resistance among the microbial population in bovine milk, with an emphasis on the concerning presence of drug resistance in E.coli. By expanding our knowledge in this specific area, we can successfully develop effective strategies and interventions to effectively combat and prevent the spread of antibiotic resistance. Thus, it becomes apparent that addressing this issue is of utmost importance for the overall health and well-being of the global population.

Keywords: Antibiotic resistance markers; ESBL; ACBL; Enterobacteriaceae; *Escherichia coli;* MDR

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INTRODUCTION

Over the years, the demand for cow milk has increased substantially, as it is a good source of protein and micronutrient. Being a country where a large number of people are vegetarian, India has witnessed rapid expansion is supply chain of milk and milk products across the country, particularly in urban areas. However, shelf-life of milk is very poor as it may be spoiled due to bacterial contamination if not stored or handled properly. There are several reports of milk-borne outbreaks of shiga toxin producing or

enterohemorrhagic *E. coli* [1, 2]. With increasing report of antibiotic resistance, it has become imperative to understand the antimicrobial resistance profile of the milk-borne pathogens and *E. coli*, being a commensal and indicator pathogen may be investigated to understand the resistance characteristics and antimicrobial-resistance genes circulating in milk and milk products. The number of microorganisms that produce extended-spectrum beta-lactamases (ESBLs) is increasing, making high-generation cephalosporins useless. When second- or third-generation antibiotics are used extensively to treat bacterial infections, these pathogens provide a significant challenge to the management of general infections [3]. Antibiotics that are often used as a last option, such as carbapenems, are utilized more frequently during treatment of ESBL *E. coli* because of its general insensitivity to many of them. Drug resistance spreads because, *E. coli* strains bearing resistance genes are easily able to transfer those genes to other pathogens [4]. Therefore, a comprehensive examination into the presence of *E. coli* that produces ESBLs is required in the food processing supply chain or in the food we consume regular basis, which may originate from these robust farm animals. The current work aims to identify and characterize *E. coli* that produces ESBL and ACBL from raw milk samples from different parts of West Bengal (by identifying the beta-lactamase genes in the isolates), followed by additional characterization and an understanding of their patterns of antibiotic resistance in vitro.

MATERIAL AND METHODS

Sampling and isolation of *E.coli* bacteria

In total, One hundred and forty-four bovine milk samples (n=144) were aseptically collected during our study period (Oct, 2015 to Mar, 2019) from cows spanning seven districts of West Bengal district and immediately carried to the laboratory maintaining proper cold chain. Briefly 5 ml of bovine milk sample was spun down and then inoculated in EC broth (BD DIFCO USA) for further inoculation, MacConkey Agar media is used (BD DIFCO USA) to obtain pink rose red colonies. The same were then re-inoculated on EMB Agar (BD DIFCO USA) for confirmation of *E.coli* by green metallic sheen appearance. For each sample, two purified colonies with such appearance (n=288) were further streaked in slant for future use followed by Bio chemical assay performed for *E.coli* identification such as Indole, Methyl Red, Voges-Proskauer and citrate tests (IMVIC TEST) following previous worker [5].

Molecular confirmation of *E.coli*

Further the isolates were subjected to molecular conformation by using multiplex PCR targeting highly conserved regions of *E.coli* like *lacY*, *lacZ*, *cydA*, *uidA* and *phoA* [6].

Screening for Antibiotic Susceptibility

Following the recommendations provided by the Clinical Laboratory Standards Institute (CLSI-2018), the susceptibility to antimicrobials was ascertained by an agar diffusion test with antimicrobial agents impregnated paper discs (BD Difco/Himedia). Ampicillin (10 µg), amoxicillin and clavulanic acid (20/10 µg), amikacin (30 µg), enrofloxacin (5 µg), cefpodoxime (10 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), trimethoprim-sulfamethoxazole (25 µg), imipenem (10 µg), nalidixic acid (30 µg), tetracycline (30 µg), cefotaxime (30 µg), aztreonam (30 µg), ceftazidime (30 µg), and *E. coli* ATCC 25922 were utilized as negative controls in this study. To interpret the results, a CLSI reference chart was utilized.

Phenotypic Screening for ESBL Production Among *E.coli* Isolates

Using the combination disc approach, isolates with decreased cephalosporin susceptibility were examined for the presence of ESBL with Mueller-Hinton agar (Bd-Difco Laboratories, USA), the combination disc method was performed with discs containing 30 µg of ceftazidime, cefotaxime alone, and clavulanic acid (30 µg/10 µg). A 5 mm or greater increase in the zone of inhibition in the presence of clavulanic acid relative to ceftazidime or cefotaxime alone was considered a good indicator for the development of ESBLs [7].

Phenotypic confirmation of AmpC type β lactamase production

AmpC type β lactamase (ACBL) production was determined among MDR isolates by combination disc method similar to ESBL detection but here cefoxitin (30 µg) and cefoxitin-cloxacillin (30/200 µg) disc were used. A 4 mm or greater increase in the zone of inhibition when cefoxitin and cloxacillin were present compared to cefoxitin alone was thought to be a sign of ACBL production [7].

Detection of Antibiotic Resistance Genes in ESBL Producing Organisms

All MDR isolates were tested in multiplex PCR format to identify the various beta-lactamase (TEM, SHV, OXA varients and CTX-M family), carbapenemase (GES, PER, VEB, IMP, VIM, SIM, KPC and NDM-1) and AmpC type beta lactamase along with its family (ACC, FOX, MOX, DHA, CIT, EBC) [8].

RESULTS

Isolation of *E.coli*

All of the samples collected yielded pink rose red colonies on MacConkey agar and such colonies were picked and re-inoculated on EMB Agar (BD DIFCO USA), those that gave a green metallic sheen were considered as *E.coli*. For each sample that gave a green metallic sheen, two purified colonies (n=288) were further subjected to biochemical assay for identification of *E.coli*. All of those samples that exhibited Indole and Methyl Red test positive, Voges-Proskeur and Citrate test negative and changed the colour of Triple Sugar Iron slants yellow, indicating positive results were confirmed as *E.coli* phenotypically.

Molecular confirmation of *E.coli*

All the purified isolates from EMB Agar that were phenotypically confirmed as *E.coli* were subjected to molecular confirmation by using multiplex PCR targeting highly conserved regions of *E.coli*(6). Out of the 288 (n=288) isolates 98 isolates were confirmed as *E.coli* by yielding distinctive five bands reserved of highly conservative genes for *E.coli*.

Antimicrobial Susceptibility Tests

All the confirmed *E.coli* isolates (n=98) were subjected to antimicrobial susceptibility test against different groups of antibiotics. The highest percentage of isolates (63.28%) were resistant to amoxicillin and clavulanic acid combination, followed by cefotaxime (62.24%). Resistance to ampicillin made up a percentage of 54.08% while 51.02% were resistant to cefpodoxime and nalidixic acid. Resistance to ceftazidime and trimethoprim-sulfamethoxazole contributed a percentage of 36.73%. Resistance were frequently noted against tetracycline, enrofloxacin, imipenem, ceftriaxone, amikacin and chloramphenicol were 48.97%, 39.79%, 38.77%, 34.69%, 21.42%, 18.36% respectively. The detailed result of antimicrobial susceptibility is given in the Table 1.

Table 1. Antimicrobial susceptibility pattern.

Drug	Resistant	Intermediate resistant	Susceptible
AMC	63 (63.28%)	25 (25.5%)	10 (10.20%)
CTX	61 (62.24%)	12 (12.2%)	25 (25.52%)
AMP	53 (54.08%)	31 (31.63%)	14 (14.28%)
CPD	50 (51.02%)	17 (17.34%)	45 (45.91%)
NA	50 (51.02%)	29 (29.54%)	19 (19.38%)
TE	48 (48.97%)	5 (5.10%)	45 (45.91%)
EX	39 (39.79%)	31 (31.63%)	28 (28.57%)
IPM	38 (38.77%)	25 (25.51%)	35 (35.75%)
CAZ	36 (36.73%)	32 (32.65%)	30 (30.61%)
COT	36 (36.73%)	17 (17.34%)	45 (45.91%)
CTR	34 (34.69%)	23 (23.46%)	41 (41.83%)
AK	21 (21.42%)	20 (20.42%)	57 (58.16%)
C	18 (18.36%)	16 (16.32%)	64 (65.30%)

AMC- Amoxicillin-clavulanic acid; CTX- Cefotaxime; AMP- Ampicillin; CPD- Cefpodoxime; NA-Nalidixic acid; TE-Tetracycline; EX-Enrofloxacin; IPM- Imipenem; CAZ- Ceftazidime; COT- Trimethoprim-sulfamethoxazole; CTR- Ceftriaxone; AK- Amikacin; C- Chloramphenicol.

Phenotypic Confirmation of ESBL Production

All the isolates with reduced susceptibly with more than two groups of antimicrobials were tested for ESBL production by CDDST.11 *E.coli* isolates were confirmed as positive for ESBL production based on the positive result by combination disc diffusion tests.

Phenotypic Confirmation of AmpC Type β Lactamase Production

The isolates that were resistant to more than two groups of antimicrobials were subjected to ACBL test. 5 isolates were found positive for ACBL production in our study.

Screening of ARG's Among ESBL and ACBL Producers

The MDR isolates were checked for the presence of $_{bla}$ESBL genes ($_{bla}$TEM, $_{bla}$SHV,$_{bla}$CTX-M). The predominant type $_{bla}$ESBL gene was $_{bla}$TEM i.e. seven isolates making up a total of 7.14% followed by $_{bla}$CTX-M gene i.e. four isolates making up a total of 4.08%. The isolates harbouring $_{bla}$CTX-M gene were further categorised by CTX-M grouping (CTX-M 1/2/9 &CTX-M8/25) and all four isolates were of the $_{bla}$CTX-M type 1. Amongst the 11 isolates that were phenotypically identified as ESBL producers, only four isolates carried the $_{bla}$ESBL genes out of which all of them were $_{bla}$CTX-M genes.

DISCUSSION

The detection of ESBL producers in bovine milk is a matter of significant concern for public health. It is essential to address this issue promptly and effectively to prevent the potential transmission of antibiotic-resistant genes (ARGs) and protect public health. Although the Indian population generally does not consume raw milk, the presence of ESBL-producing bacteria in bovine milk still poses a risk [9]. This risk should not be overlooked, as it could contribute to the spread and emergence of drug-resistant bacteria [10]. One particular concern is the elevated resistance to amoxicillin-clavulanic acid (AMC) in ESBL producers. This antibiotic is often recommended for the treatment of ESBL infections, and its reduced efficacy due to resistance is worrisome. The overproduction of AmpC β-lactamase or inhibitor-resistant β-lactamase might be responsible for this phenomenon, making it crucial to identify and address the mechanisms behind this resistance [9]. Several studies have provided evidence of contamination of bovine milk with resistant strains of bacteria. The majority of these bacteria displayed resistance to one or more antibiotics, with ampicillin and amoxicillin-clavulanic acid being the most commonly resistant antibiotics. These findings indicate a higher resistance to cephalosporin and penicillin antibiotics compared to other classes of antibiotics. Interestingly, chloramphenicol showed the least resistance among the tested antimicrobials, only at 18.36% [10]. This pattern of resistance is not unique to bovine milk. A similar resistance pattern was observed in phenotypic resistance of Enterobactriaceae from organized and backyard duck farms. These bacteria showed resistance to ampicillin, ampicillin/cloxacillin, and ceftriaxone, but susceptibility to chloramphenicol [11]. The beta-lactamase-producing *E.coli* in ducks also displayed resistance to cefotaxime and ampicillin, but susceptibility to chloramphenicol [12]. Among the *E.coli* isolates, the most prevalent beta-lactamase gene found was blaTEM, followed by the $_{bla}$CTX-M gene. The $_{bla}$CTX-M type 1 was the most common subtype, consistent with previous research [13]. It is worth noting that a study conducted in northeast India reported a higher prevalence of genotypic ESBL-producing organisms compared to phenotypic detection, which contrasts with the findings of this study [14]. In this study, only a small percentage (4.08%) of the isolates were confirmed to be both phenotypically and genotypically ESBL-producing *E.coli*. Additionally, 65.03% of the isolates were found to carry the $_{bla}$AmpC gene, while only 5.10% tested positive for ACBL production on a phenotypic level. These findings suggest a higher prevalence of AmpC-type β-lactamase genes compared to bla ESBL genes in farm animals, specifically bovine [14].

Given these concerning findings, it is crucial for the relevant authorities to implement and enforce strict control measures to prevent the spread and emergence of drug-resistant bacteria derived from bovine milk. These control measures should involve monitoring and surveillance of ESBL producers,

implementing appropriate antibiotic stewardship programs, and promoting effective hygiene and sanitation practices on farms. Only through comprehensive and collaborative efforts can we mitigate the risks associated with antibiotic resistance and protect public health.

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REFERENCES

1. Hickey CD, Sheehan JJ, Wilkinson MG, Auty MAE. Growth and location of bacterial colonies within dairy foods using microscopy techniques : a review. 2015;6(February):1–8.
2. Enterohemorrhagic E. Enterohemorrhagic E. coli (EHEC) pathogenesis. 2012;2(July):1–7.
3. Tenover FC, Mohammed MJ, Gorton TS. Detection and Reporting of Organisms Producing Extended-Spectrum ␣ -Lactamases : Survey of Laboratories in Connecticut. 1999;37(12):4065–70.
4. Hu Y, Yang X, Li J, Lv N, Liu F, Wu J, et al. The Bacterial Mobile Resistome Transfer Network Connecting the Animal and Human Microbiomes. 2016;82(22):6672–81.
5. Kar D, Bandyopadhyay S, Bhattacharyya D, Samanta I, Mahanti A, Nanda PK, et al. Molecular and phylogenetic characterization of multidrug resistant extended spectrum beta-lactamase producing Escherichia coli isolated from poultry and cattle in Odisha, India. Infect Genet Evol [Internet]. 2015;29:82–90. Available from: http://dx.doi.org/10.1016/j.meegid.2014.11.003
6. Modak R, Mitra S Das, Krishnamoorthy P, Bhat A, Banerjee A, Gowsica BR, et al. Histone H3K14 and H4K8 hyperacetylation is associated with escherichia coli induced mastitis in mice. Epigenetics. 2012;7(5):492–501.
7. Koovapra S, Bandyopadhyay S, Das G, Bhattacharyya D, Banerjee J, Mahanti A, et al. Molecular signature of extended spectrum β-lactamase producing Klebsiella pneumoniae isolated from bovine milk in eastern and north-eastern India. Infect Genet Evol [Internet]. 2016;44:395–402. Available from: http://dx.doi.org/10.1016/j.meegid.2016.07.032
8. Dallenne C, da Costa A, Decré D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. J Antimicrob Chemother. 2010;65(3):490–5.
9. Bitrus AA, Mshelia PA, Kwoji ID, Goni MD, Jajere SM. Extended-spectrum beta-lactamase and ampicillin Class C beta-lactamase-producing Escherichia coli from food animals: A review. Int J One Heal. 2019;5:65–75.
10. Ghosh S, LaPara TM. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J. 2007;1(3): 191–203.
11. Banerjee A, Bardhan R, Chowdhury M, Joardar SN, Isore DP, Batabyal K, et al. Characterization of beta-lactamase and biofilm producing Enterobacteriaceae isolated from organized and backyard farm ducks. Lett Appl Microbiol. 2019;69(2):110–5.
12. Ma J, Liu JH, Lv L, Zong Z, Sun Y, Zheng H, et al. Characterization of extended-spectrum β-lactamase genes found among Escherichia coli isolates from duck and environmental samples obtained on a duck farm. Appl Environ Microbiol. 2012 May;78(10):3668–73.
13. Manchanda V, Singh NP. Occurrence and detection of AmpC β-lactamases among Gram-negative clinical isolates using a modified tree-dimensional test at Guru Tegh Bahadur hospital, Delhi, India. J Antimicrob Chemother. 2003;51(2):415–8.
14. Bora A, Hazarika NK, Shukla SK, Prasad KN, Sarma JB, Ahmed G. Prevalence of blaTEM , blaSHV and blaCTX-M genes in clinical isolates of Escherichia coli and Klebsiella pneumoniae from Northeast India. Indian J Pathol Microbiol. 2014;57(2):249–54.

