RESEARCH ARTICLE

Prevalence of Antimicrobial Resistance in *Escherichia coli* isolated from Dairy Supply Chain by Phenotypic Methods

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Abstract

A total of 139 *E. coli* positive isolates from 122 raw milk (57.2%), 10 pasteurized milk (33.3%), and 7 human handlers (35%) were identified by phenotypic methods and 22 of the isolates were further confirmed by PCR. All 22 *E. coli* (100%) isolates were found resistant to penicillin, oxacillin, erythromycin, and clindamycin. Amongst *E. coli* isolates, antibiotics resistance was detected towards cefotaxime and amoxyclave (18.7% each) followed by ampicillin (7.98%), trimethoprim (15.82%), tetracycline (10.79%), nalidixic acid (7.91%), and piperacillin (7.79%). Out of 139 isolates, 7.91% isolates were found to be MDR (multidrug resistant) and 4 isolates were confirmed as extended spectrum β-lactamase (ESBL) producers.

Keyword: Antimicrobial resistance, *E. coli*, ESBL, Milk, Multidrug-resistant.

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Introduction

Antimicrobial resistant (AMR) bacterial pathogens are the greatest universal epidemiological menace in this 21st century (Prestinaci *et al*., 2015). Presently, there are no ordinances or laws are available for the regulation of antibiotic usage in non-curative purposes and an advent of resistance is due to antibiotic abuse in the livestock segment as one of the important non-calculated crises in India. One of the most familiar ethical consequences confronted in milk production and management is mastitis. Bacteria isolated from the milk of mastitis dairy animals have been demonstrated to harbor a broad range of bacterial resistance to generally habituated antibiotics. Antimicrobial resistant bacteria (AMRB) incepted from the livestock can spread to consumers all the way through eating livestock products or to agrarian laborers through direct contacts with the livestock. The most wide-spread facultative anaerobic bacteria in the gut of animals and humans are *E. coli* (Rasheed *et al*., 2014) and it is also one of the etiological factors for mastitis (Zadoks *et al*., 2011). The emergence of AMR *E. coli* against the first line of antimicrobial has complicated the treatment of infection (Sabate *et al*., 2008). The genes encoding extended for spectrum ß-lactam (ESBL) and Metallo-β-lactam (MBLs) resistance have been documented from various pathogenic bacteria encountered in Indian subcontinent. In case of MBLs resistance, New Delhi Metallo-β-lactamases (NDMs) in *Klebsiella pneumonia* and *E. coli* are recently identified resistance, and the most conferred broad resistance found is Carbapenems (Yong *et al*., 2009). As per survey report made by Indian Council of Medical Research (ICMR) during the year 2005 to 2009, the sale of units of antibiotics was enhanced by about 40% and especially cephalosporin's antibiotic sale was increased up to 60% over the last five-year period (GARP, 2011).

Recently, a renowned economist Lord Jim O'Neill predicted that if India doesn't balance its effort to tackle the menace of AMR, otherwise by 2050, it would cause death of estimated millions of populations. Recently, ICMR and ICAR in India have corroborated a national network on AMR in food-borne zoonotic pathogens laboratories established at academic institutes/veterinary labs, targeting medically important index microbes/foodborne zoonotic pathogens (Laxminarayan and Chaudhury, 2016). Therefore, this study was undertaken on AMR *E. coli* in dairy production chain to establish the impact of resistance in species frequently allied with infection in the management of diseases in dairy animal in the area of northern part of India.

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Materials And Methods

Procurement and Maintenance of Culture

The cultures for this study included *E. coli* ATCC 25922 procured from American Type Culture collection and checked for purity. Cultures were revived in nutrient broth by streaking on Violet red bile agar (VRBA) followed by incubation for overnight at 37°C. A single pure colony from VRBA after microscopic examination was picked up and maintained on nutrient agar slant by routine sub-culturing after every fortnight. All the experiments were conducted using overnight grown cultures. All confirmation media and components of biochemical identification were procured from Himedia lab (Mumbai, India). All media chemicals including Muller Hinton Agar and antimicrobial agents including discs were procured from Himedia lab (Mumbai, India).

Sampling

A total of 190 samples comprising raw milk (n=100), pasteurized milk (n=50), infant food (n=20) and human handler swab samples (n=20) were collected aseptically from in and around Karnal city, Haryana, India. The collected samples were packed, labeled and transported in a cool pack to the National Referral Centre for milk quality and safety (NRCMQS), ICAR-NDRI, Karnal (Haryana). Further, milk samples were cold stored until microbiological processing.

Isolation and Identification of *E. coli*

The isolation and identification of *E. coli* in collected milk samples were carried out as per the Bureau of Indian Standard (BIS) 1976, *i.e.,* IS 5887 (Part 1).

Antimicrobial Susceptibility Test

Antimicrobial susceptibility tests were done on Mueller-Hinton Agar (Hi-media lab) using the Kirby-Bauer disk diffusion method (Bauer *et al.,* 1996). The antimicrobial agents used were ampicillin (AMP-30 µg), piperacillin (PI-30 µg), penicillin (P-10 µg), cefotaxime (CTX-30 µg), ceftazidime (CAZ-30 µg), gentamicin (GEN-10 µg), netilmicin (NET-10 µg), amikacin (AK-30 µg), chloramphenicol (C-30 µg), tetracycline (TE-30 µg), nalidixic acid (NA-30 µg), ciprofloxacin (CIP-5 µg), nitrofurantoin (NIT-100 µg), trimethoprim (TR-5 µg), oxacillin (OX-1 µg), cefepime (CPM-30 µg), ceftriaxone (CTR-30 µg), clindamycin (CD-2 µg), ofloxacin (OF-5 µg), meropenem (MRP-10 µg), ertapenem (ETP-10 µg), ampicillin-sulbactam (A/S-10/10), amoxiclav (AMC-30 µg), and erythromycin (E-15 µg) (Himedia). Resistance data were interpreted according to National Committee for Clinical Laboratory Standards (NCCLS, 1993). Reference strain of *E. coli* ATCC 25922 was used for quality control for antimicrobial susceptibility tests (CLSI, 2007). The results were interpreted according to CLSI guidelines (CLSI, 2007).

Extended Spectrum β-Lactamase (ESBL) Confirmatory Test

Detection of ESBLs was performed using the double disc synergy test (DDST) using amoxicillin/clavulanate, ceftazidime, ceftriaxone, aztreonam, and cefotaxime as described by Drieux *et al*. (2008). Cefotaxime (30 µg) or Ceftazidime discs (30 µg) with or without Clavulanate (10 mcg) were used for phenotypic confirmation of ESBL positive isolates (CLSI, 2012 guidelines). A difference in the zone of inhibition of 5 mm of either of cephalosporin discs and their Clavulanate containing discs indicates the production of ESBL. The test was considered as positive when a decreased susceptibility to cefotaxime was combined with a clear-cut enhancement of the inhibition zone of cefotaxime in front of the clavulanate-containing disk, often resulting in a characteristic shape-zone referred to as 'champagne-cork' or 'keyhole (Drieux *et al*., 2008). Further, overnight grown ESBL +ve isolates whose turbidity was adjusted to 0.5 McFarland solutions (Himedia Lab) was streaked on the Hicrome ESBL agar plates followed by incubation at 37°C for 24 h. The development of pink or purple colored colonies on the Hicrome ESBL agar plates was considered as positive for ESBL.

Results and Discussion

Prevalence of *E. coli* by Culture-based Techniques

Out of 100 raw milk samples, 60 samples were presumptively found positive and these positive samples were further confirmed on MacConkey agar with pink-red colored colonies with bile salt precipitation. From the MacConkey agar plates, 213 characteristic colonies of *E. coli* (multiple colonies 2-5) were selected for streaking on Eosin methylene blue (EMB) agar. Out of 213 colonies, only 122 colonies of *E. coli* shown characteristic green metallic sheen color on EMB agar. In the case of pasteurized milk, only 10 samples were found positive for *E. coli* by presumptive test (PMT). From 10 positive samples from PMT, about 30 characteristics colonies were obtained by streaking on MacConkey agar plates, but only 10 isolates had shown characteristic green metallic sheen colored colonies on EMB and these 10 isolates were selected for biochemical identification. However, in the case of infant foods, none of the samples was found positive for *E. coli*. Out of 20 swabs of human handler, only 5 samples were found positive for *E. coli* and from 5 positive samples, 20 characteristics colonies (4/plate) were selected from MacConkey agar plates. Among 20 colonies, only 7 showed characteristic color on EMB and these were selected for further biochemical identification. Overall from 263 colonies selected from 190 samples, only 139 isolates (122 from raw milk, 10 from pasteurized milk, zero from infant food and 7 from human hand swab) showing green metallic sheen on EMB agar plates were further evaluated for biochemical confirmation.

The prevalence rate of *E. coli* in raw milk, pasteurized milk, and human handler's swab samples were found to be 57.27%, 33.33%, and 35%, respectively (Fig. 1). However, no *E. coli* were found in any of the infant food samples. All the 139 *E. coli* colonies were further established by Hicrome ECD agar with blue colored colonies and by NDRI two-stage enzyme assays wherein all the isolates had shown specific enzyme-substrate interaction with yellow color in first stage and blue-green color in the second stage of the assay which confirms that all the isolated colonies of *E. coli* had β-D-glucuronidase enzyme activity. Badri *et al*. (2017) shown lower prevalence rate (33%, 38%) of *E. coli* in raw milk samples in the USA and Sudan, respectively. The deviation in the frequency rate of *E. coli* may be endorsed to the differences in hygiene conditions applied while milking, geographic site and seasonal variation (Gundogan and Avci, 2014). In the case of human handlers, the incidence of *E. coli* was about 35%. Gwida and EL-Gohary (2013) have reported a prevalence rate of *E.coli* in 18.8% samples.

Antibiotic-Resistant Pattern of *E. coli* Isolates

All the phenotypically confirmed *E. coli* (n=139) isolates were tested for their antibiotic sensitivity against 24 different antibiotics and all 139 isolates of *E. coli* were resistant towards penicillin, oxacillin, clindamycin, and erythromycin. Among these isolates, 18.7% of *E. coli* showed resistance towards amoxiclav and cefotaxime antibiotics. The resistance was also observed in the presence of ampicillin, trimethoprim, tetracycline, nalidixic acid and piperacillin in 17.98%, 15.82%, 10.79%, 7.91%, and 7.19% of *E. coli*, respectively. Almost equal percentages of isolates (3.59%) had shown resistance to chloramphenicol and gentamicin antibiotics. Resistance was also observed in the presence of a third (ceftriaxone) and fourth (cefepime) generation cephalosporins in 2.87% of *E. coli* isolates. Out of 139 isolates, only 2.16% isolates had shown resistance to nitrofurantoin and amikacin antibiotics. However, resistance to ciprofloxacin was observed only in 1.43% of *E. coli* isolates. In the case of ceftazidime and ofloxacin, only 0.71% of isolates showed resistance (Fig. 2).

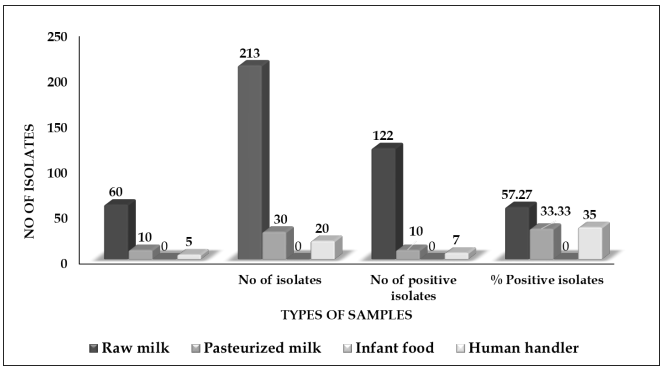

Fig. 1: Prevalence of *E. coli* in different samples of raw, pasteurized milk, infant milk foods and human handler in dairy supply chain environment

All *E. coli* isolates showed resistance towards penicillin, oxacillin, clindamycin, and erythromycin. About 38 (27.33%) of the isolates showed resistance to multiple antibiotics ranging from 3 to 8 different antibiotics (Fig. 3).

Among these isolates, 7.91% (11/139) of the isolates were found to be multidrug resistance (MDR), resistance for 3 to 6 different groups of antibiotics (Fig. 4). Another four isolates (3.27%) which were from raw milk showed resistance towards ESBL antibiotics. Our results were in close association with the findings of Rasheed *et al*. (2014), who also revealed a resistance in 14.7% of the isolates and the resistance may be due to attainment of plasmids, transposons, and Class 2 integron (Scott *et al*., 2009).

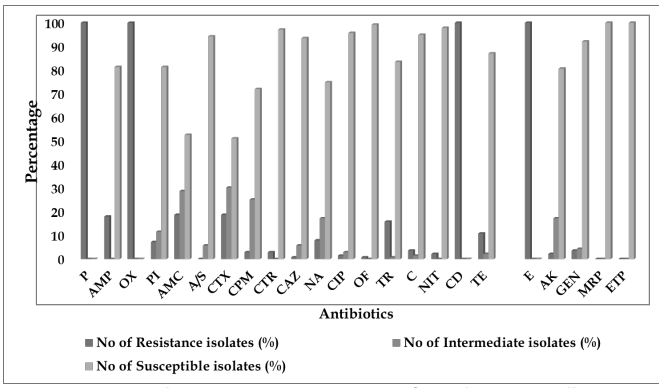

Fig. 2: Antibiotic resistance pattern of *E. coli*. (P: penicillin; AMP: ampicillin, OX: oxacillin; PI: piperacillin, CTX: cefotaxime; CAZ: ceftazidime; GEN: gentamicin, NET: netilmicin; AK: amikacin; C: chloramphenicol, TE: tetracycline, NA: nalidixic acid, CIP: ciprofloxacin; NIT: Nitrofurantoin; TR: trimethoprim; CPM: cefepime, CTR: ceftriaxone, CD: clindamycin; OF: ofloxacin; MRP: meropenem, ETP: ertapenem, A/S ampicillin-sulbactam; AMC: amoxiclav and erythromycin)

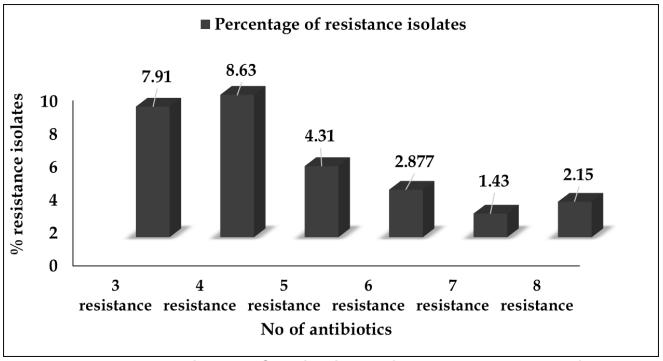

Fig. 3: Prevalence of multiple antibiotic resistant *E. coli*

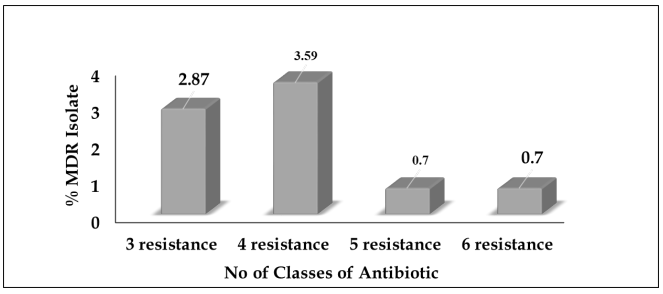

Fig. 4: Prevalence of Multi Drug resistance *E. coli* in milk

Table 1: Diameter of zone of inhibition (in mm) for ESBL positive *E. coli* isolates

	KF-7994-3	*Kn33RMD-1*	*Kj14M-2*	*KJ12M-1*
Name of antibiotic	*Diameter of zone of inhibition (in mm)*			
CTX	10	13	No zone	10
CPM	14	18	14	15
CAZ	16	25	17	17
CTR	No zone	22	No zone	No zone

Extended-Spectrum β-Lactamase (ESBL) Isolates

By AbST method, *E. coli* isolates from raw milk were found positive for ESBL groups by disk diffusion assay. These 4 positive isolates were further validated phenotypically as ESBL isolates using double disk diffusion assay and streaking on Hi-Chrome agar. From the double disk diffusion assay for these four suspected isolates wherein, all four *E. coli* isolates had shown a zone of inhibition around ≤ 27 mm diameter for CTX, three out of four isolates had shown a zone of inhibition of ≤ 22 mm diameter for CAZ and one of the isolate had shown a zone of inhibition of 25 mm, four isolates had a zone diameter of ≤ 25 mm for CTR and in the case of CPM, ≤ 18 mm inhibition zone diameter was observed in all four isolates (Table 1). Gundogan and Avci (2013) reported a 10% (2/20) prevalence of ESBL positive *E. coli* in milk which is slightly higher than our current findings. Badri *et al*. (2017) also revealed the prevalence rate of ESBL producing *E. coli* as 29.3% (17/22) in raw milk, which is much higher than in the current study.

Conclusions

The prevalence of AMR *E. coli* was more observed in raw milk in contrast to pasteurized milk, human handlers, and infant food samples. In case of pasteurized milk samples, resistance may be due to post-pasteurization contamination either from human handlers, unhygenic environment condition, and poor storage condition. Multi or multiple drug resistance was more prevalent in *E. coli,* wherein *E. coli* has shown resistance towards cefepime, gentamicin, erythromycin, trimethoprim, chloramphenicol, ciprofloxacin, tetracycline, which may indicate the presence of multiple-drug resistance genes on similar mobile genetic element.

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