Genotypic identification of extended spectrum β -lactamase producing *Escherichia coli* in dairy supply chain

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Abstract: Foodborne illnesses due to antibiotic-resistant bacteria represents a major public health problem in both developed and developing countries. Among 190 samples, 139 *Escherichia coli* positive isolates from raw milk, pasteurized milk, and human handlers were identified by phenotypic methods and genotypic methods. All *E. coli* isolates were found to be resistance to penicillin, oxacillin, erythromycin and clindamycin. The dominant type of resistance to cefotaxime and amoxiclavate identically detected in 18.7% isolates followed by ampicillin in 17.98%, trimethoprim 15.82%, tetracycline 10.79%, nalidixic acid 7.91%, and piperacillin 7.79%. Four isolates have shown resistance (2.87%) to Ceftriaxone and ceftazodime, Cefotaxime and one isolate has shown resistance to Cefepime. Further, all four isolates were confirmed as extended spectrum β -lactamase (ESBL) producer by double disc diffusion test and ESBL chromogenic medium. Later, all four isolates were evaluated by PCR and they are observed as carrier of blaCTX M gene which is responsible for ESBL antibiotic resistance in *E. coli* but blaTEM and blaSHV genes were absent in all four ESBL isolates. Based on the above findings, it is concluded that ESBL antibiotic resistance in *E. coli* were more prevalent in milk and this may due to spread and acquirement of antibiotics resistance gene by plasmid and mobile genetic elements.

Keyword: Antibiotic resistance, *E. coli*, Milk, ESBL

Introduction

The enzyme responsible for degradation of oxyimino- β -lactam antibiotics is Extended-spectrum β -lactamases (ESBL) and these antibiotics are used in the treatment of various serous humans and animal infections (Palmeira and Ferreira, 2020). ESBL were first identified in the year 1983 in the family Enterobacteriaceae and they are responsible for serious risk to human health may be due to treatment failure in various severe cases of infections in 2013 (Adeolu et al. 2016). These enzymes are encoded by plasmids that confer resistance to the penicillins; to first-, second-, third-, and fourth-generation cephalosporins; and to aztreonam but not to carbapenems (EFSA, 2011). Human-to-human spread of these type of enzyme producing bacterial pathogens have been demonstrated in public hospital settings, representing that human colonization is a pool for dissemination (Liebana et al. 2013). Furthermore, various reports are available for the isolation and identification of ESBL-producing bacteria from foods and food animals, suggesting the likely role of the food production chain as a pool for this group of pathogenic bacteria (EFSA, 2019, Odenthal et al. 2016). There are various data's are available regarding robust correlation between the incidence of ESBL-producing bacteria in foods (Algeria et al. 2020) and the prevalence of infections in humans may be anticipated that food obtained from animals may be infected with ESBL producers which contributing to the transmission within the population (EFSA, 2011). Furthermore, ESBL producers also can the resistant bacteria can fetch additional genes having some virulence property; it is notable that strains of *Escherichia coli* (STEC) are known to be food-borne pathogens, have been confirmed as ESBL-producers, representing that the transference of the extracellular DNA from commensals to foodborne pathogenic strains is possible (Torpdahl et al. 2013).

The occurrence of ESBL-producing *Escherichia coli* is extensively growing throughout India. These pathogens pose a major threat to the treatment of infection and may cause tricky in the management of infections. This may create unnecessary problem with the widespread use of second- or third-generation antibiotics for the monitoring and treatment of bacterial infections (Van Hoek et al. 2015). ESBL *E. coli* is typically unaffected to routinely used antibiotics instigating a surge in the use of almost

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all antimicrobials (i.e., carbapenems) in treatment. The *E. coli* strains harboring those resistance genes can easily spread to other pathogens leading to the spread of resistance (Odenthal et al. 2016). Hence, the presence of ESBL-producing *E. coli* in the dairy supply chain maybe arriving from healthy animals is the fact which has to be suitably premeditated. Therefore, in our present study, we are evaluating the occurrence of ESBL-producing *E. coli* in dairy supply chain.

Materials and Methods

Procurement and Maintenance of culture

The standard culture used in our study was *Escherichia coli* ATCC 25922 was purchased from American Type Culture collection. The culture was activated in nutrient broth followed by streaking on Violet red bile (VRBA) agar 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 media chemicals including Muller Hinton Agar and antimicrobial agents including discs were procured from Himedia lab (Mumbai, India).

Detection of *E. coli* using PCR

Species specific primers were used in Colony polymerase chain reaction (PCR) includes Forward: GGTAACGTTTCTACCGCAGAGTTG/Reversed: CAGGGTTGGTACTGTCACTACG, target gene of *E. coli* as phoA with a size of 468 bp (Shome et al. 2011) using a protocol given by Godambe et al. (2017).

Antimicrobial susceptibility tests (AST)

Antimicrobial susceptibility tests was carried out on Mueller-Hinton Agar (Hi-media, Mumbai, India) using the Kirby-Bauer disk diffusion method (Bauer et al. 1996). The data were evaluated and inferred according to National Committee for Clinical Laboratory Standards (NCCLS 1993). Reference strain of *E. coli* ATCC 25922 was used as a quality control strain for studying AST (CLSI 2007). The resistance strains were evaluated for minimum inhibitory concentration (MIC) using micro-dilution methods followed data inferred as per CLSI guidelines (CLSI 2007).

Extended Spectrum β -lactamase (ESBL) confirmatory test

The double disc synergy test (DDST) was performed for ESBL using amoxicillin/clavulanate, ceftazidime, ceftriaxone, aztreonam, and cefotaxime (Jarlier et al. 1988; Drieux et al. 2008). Further, phenotypic confirmation of ESBL positive isolates was carried out using CLSI (2012) guidelines. The test is considered as positive when a decreased susceptibility to cefotaxime is

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). A ratio of ceftazidime or cefotaxime MIC to ceftazidime or cefotaxime-clavulanic acid MIC equal to or greater than eight indicated the presence of ESBL (Drieux et al. 2008).

Phenotypic identification of ESBL in *E. coli*

An overnight grown culture of ESBL +ve isolate whose turbidity was adjusted to 0.5 McFarland solutions (Himedia Lab, Mumbai, India) 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 considered as positive for ESBL.

Identification of ESBL by PCR

The following ESBLs resistance determinants were investigated by PCR for bla-CTXM, bla-TEM and bla-SHV gene (Karczmarczyk et al. 2011). Colony PCR were performed as per protocol given by Godambe et al. (2017) by targeting ESBL encoding genes [Primer Sequence (5'-3') such as CTX-M universal- F- CGATGTGCAGTACCAGTAA; CTX-M universal-R- TTAGTGACCAGAATCAGCGG, blaSHV- F- TTATCTCCCTGTTAGCCACC; blaSHV- R- GATTTGCTGATTTTCGCTCGG, blaTEM- F- GCGGAACCCCTATTTG and blaTEM- R- ACCAATGCTTAATCAGTGAG] (Olesen et al. 2004) with a genome size of 585 bp for CTX-M (Batchelor et al. 2005) and 795 bp for blaSHV (Weill et al. 2004).

Results and Discussion

Detection of *E. coli* in dairy supply chain

In our study, the prevalence of *E. coli* in raw milk samples were found to be 57.27%, 20% in pasteurized milk samples, and 25% in swab of human handler working in dairy farms and plants by biochemical identification test such as Indole, Methyl red, Vogues Prausker test and citrate utilization test. From the above, 139 isolates were selected and confirmed as *E. coli* using specific enzyme substrate interaction using two stage enzyme assay and Hichrome ECD agar wherein all isolates have shown characteristic enzyme reaction and characteristic blue colored colonies on Hichrome ECD agar (Fig. 1). Later, these isolates were shown characteristic 468 bp amplicon bands on gelelectrophoresis based on the use of species specific primer. The amplification was seen in the reference strain *E. coli* ATCC 25922 which was used as positive control. Almost similar prevalence rate of *E. coli* were reported by Soomro et al. (2002) wherein they found 57 and 52% prevalence of *E. coli* in raw milk respectively. Tadesse et al. (2012) and Badri et al. (2017) have shown lower prevalence rate (33%, 38%) of *E. coli* in raw milk samples respectively. The variation in

Fig. 1. Rapid detection of *E. coli* isolates on Hichrome ECD/MUG agar

prevalence rate of *E. coli* may be attributed to lapses in clean milk production, different geographic location and season, fecal contamination of milk, and due to poor hygiene and sanitary practices followed while milking and further handling (Thaker et al. 2013). The prevalence of *E. coli* in pasteurized milk may be due to post processing contamination and poor hygienic management subsequently the milk is pasteurized (Ali and Abdelgadir, 2011)

Antibiotic susceptibility test

Among 139 *E. coli* isolates, all isolates have shown resistance towards penicillin (P), oxacillin (OX) and erythromycin (E), respectively based on inhibition zone diameter by AST followed

by 26 isolates have shown resistance towards amoxicillin (MC) and Cefotaxime (CTX), 25 isolates to ampicillin (AMP), 22 isolates to trimethoprim (TR), 15 isolates to tetracycline (TET), 11 isolates to nalidixic acid (NA), 10 isolates to piperacin (PI), 5 isolates to gentamycin (GEN) and chloramphenicol (C), 4 isolates ceftriaxone (CTR) and cefepime (CPM), 3 isolates to nitrofurantoin (NIT) and amikacin (AK), 1 isolate has shown resistance towards ceftazidime (CAZ) and ofloxacin (OF), and none of the isolates have shown resistance towards ampicillin-sulbactam (A/S), meropenem (MRP), ertapenem (ERP) and netilmicin (NET), respectively (Table 1). Based on the AST, it was clear that four isolates were showed resistance towards ESBL and none were showing resistance to carbapenem group of antibiotics. The

Table 1 Prevalence of antibiotics resistant pattern of *E. coli*

Name of antibiotics	No of Resistance isolates	No of Intermediate isolates	No of Susceptible isolates
P	139 (100%)	0	0
AMP	25 (17.98)	1 (0.71%)	113 (81.29%)
OX	139 (100%)	0	0
PI	10 (7.19%)	16 (11.51%)	113 (81.29%)
AMC	26 (18.7%)	40 (28.77%)	73 (52.51%)
A/S	0	8 (5.75%)	131 (94.24%)
CTX	4 (2.87%)	35 (25.17%)	100 (71.94%)
CPM	4 (2.87%)	35 (25.17%)	100 (71.94%)
CTR	4 (2.87%)	0	135 (97.12%)
CAZ	1 (0.71%)	8 (5.75%)	130 (93.52%)
NA	11 (7.91%)	24 (17.26%)	104 (74.82%)
CIP	2 (1.43%)	4 (2.87%)	133 (95.68%)
OF	1 (0.71%)	0	138
TR	22 (15.82%)	1 (0.71%)	116 (83.45%)
C	5 (3.59%)	2 (1.43%)	132 (94.96%)
NIT	3 (2.15%)	0	136 (97.84%)
CD	139 (100%)	0	0
TE	15 (10.79%)	3 (2.15%)	121 (87.05%)
E	139 (100%)	0	0
AK	3 (2.15%)	24 (17.26%)	112 (80.57%)
GEN	5 (3.59%)	6 (4.31%)	128 (92.08%)
MRP	0	0	139 (100%)
ETP	0	0	139 (100%)
NET	0	0	139 (100%)

P: Penicillin-G, AMP: Ampicillin, OX: Oxacillin, PI: Piperacin, AMC: Amoxicillin, A/S: Ampicillin-sulbactam, CTX: Cefotaxime, CPM: Cefepime, CTR: Ceftriaxone, CAZ: Ceftazidime, NA: Nalidixic Acid, CIP: Ciprofloxacin, OF: Ofloxacin, TR: Trimethoprim, C: Chloramphenicol, NIT: Nitrofurantoin, CD: Clindamycin, TE: Tetracycline, E: Erythromycin, AK: Amikacin, GEN: Gentamycin, MRP: Meropenam, ETP: Erthapenem, NET: Netilmicin

dominant types of resistance was observed in our results are in close association with the result of Rasheed et al. (2014) wherein he was reported 14.7% of the isolate from raw milk were showing resistance towards ESBL. This may be due acquiring of mobile genetic element such as plasmids, transposons, and Class 2 integrons (Singh et al. 2005).

ESBL *E. coli*

Based on diameter of zone of inhibition by AST methods four isolates of *E. coli* have found positive for ESBL. Further, these 4 positive isolates were confirmed phenotypically as ESBL using double disk diffusion test (DDDT) and Hi-Chrome ESBL agar base (Fig. 2). All four ESBL positive isolates have shown an inhibition zone diameter of ≤ 27 mm for CTX and CTR, followed by three isolates have shown an inhibition zone diameter of ≤ 22 mm for CAZ and CPM (Table 2). Overall prevalence of ESBL positive *E. coli* in raw milk samples was 3.27%. No ESBL positive isolates were obtained from pasteurized milk and human handlers swab samples. All the ESBL positive isolates by DDDT and

Chromogenic ESBL medium have shown identical resistance towards (P, AMP, PI, CTX, CTR, CAZ, and CPM) and (P, PI, CTR, CPM, TE) by 4 and 2 *E. coli* isolates, respectively. Duan et al. (2006) reported a 3.1% prevalence of ESBL producers among *E. coli* isolates from dairy cattle. In a Turkish study reported by Kucukbasmaci et al. (2008) reported 2.1% prevalence of ESBL producing Enterobacteriaceae isolated from dairy cattle. Gundogan and Avcı (2013) reported 10% (2/20) prevalence of ESBL positive *E. coli* in milk which is slightly higher than current study. The prevalence rate of ESBL producing *E. coli* was 29.3% (17/22) in raw milk which is much higher than the current study (Badri et al. 2017).

PCR identification of ESBL in *E. coli*

All four ESBL positive isolates were further confirmed as ESBL *E. coli* by genotypic methods using colony PCR wherein all 4 isolates have shown bands for bla CTXM gene yielded 885 bp amplicon on agarose gel (Fig. 3). However, no amplified products were obtained with bla-SHV and bla-TEM primer. This indicates

Fig. 2 Confirmation of ESBL +ve *E. coli* isolates by DDDT and ESBL chromogenic agar. a. double disc diffusion test b. ESBL +ve *E. coli* on Hichrome ESBL agar c. Diameter of zone of inhibition in ESBL +ve silates by DDDT.

Fig. 3 ESBL producing isolates with primers specific for bla-CTXM and bla-SHV gene. Lane 1 to 4: ESBL producing isolates with bla-CTXM primer; Lane 5 to 9: ESBL producing isolates with bla-SHV primer; Lane 10: Negative control for bla-CTXM primer; Lane 11: Negative control for bla-SHV primer

that all four ESBL producing isolates were harboring bla-CTX-M gene which encodes ESBL in *E. coli* isolates. Similar findings were reported by Batabyal et al. (2018) regarding the prevalence of bla^{CTX-M} gene in 12 ESBL *E. coli* among 22 isolates obtained from West Bengal. Ghatak et al. (2013) have reported a one isolate

was harboring New Delhi metallo β lactamase gene (*bla*_{NDM}) and another isolate was carrying ESBL gene – *bla*_{CTX-M}. Further, Dhara and Tripathi (2014) has reported ESBL *E. coli* were found positive for bla CTX M-3 gene (18 nos), bla CTX M-9 gene (6 nos), bla SHV gene: (5 nos) and bla TEM gene: (5 nos) and may cause

health risk to consumers due to contamination by ESBL producing *E. coli*, their pathogenicity and treatment failure as a result of antibiotic resistant.

Conclusions

Based on the above findings it is concluded that four *E. coli* isolates have shown resistance to ESBL antibiotics like CTX, CTR, CAZ and CPM may indicate presence of multiple drug resistance gene on same mobile genetic elements. Further, all four ESBL positive *E. coli* isolates were harboring CTX-M gene which is linked with dairy animal. It also concluded that the prevalence of ESBL *E. coli* in raw milk may due to transmission and acquisition of antibiotics resistance gene by plasmid and mobile genetic elements.

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