Isolation and Molecular Characterization of Extended Spectrum Beta Lactamase Producing *Escherichia coli* from Milk

Dhara Paghdar[1]*, Jitendra Nayak[1], R.A. Mathakiya[2], B.C. Parmar[3], H.K. Gida[1] and P.P. Bhavsar[3]

[1]*Department of Veterinary Public Health & Epidemiology, Anand Veterinary College, Anand, INDIA*
[2]*Department of Veterinary Microbiology, Anand Veterinary College, Anand, INDIA*
[3]*Departent of Livestock Products Technology, Anand Veterinary College, Anand, INDIA*

*Correspondig author: D Paghdar; E-mail: dhara.1898@gmail.com

ABSTRACT

Milk plays a major role as a source of nutrition in the diet but contaminated milk can be a source of harmful bacteria. *Escherichia coli* is opportunistic pathogen and is responsible for a wide range of infections. The prevalence of pathogenic multi-drug resistant extended-spectrum β-lactamase (ESBL)-producing *E. coli* is increasing and becoming a global concern. A study was carried out to isolate ESBL producing *E. coli* from 150 milk samples from Anand and around villages. Total 94(62.66%) samples were found positive as *E. coli* by isolation on MacConkey and Eosin Methylene Blue agar which were confirmed by primary & biochemical tests including Gram's staining. Antibiotic sensitivity test (ABST) was performed against 6 antibiotics and isolates found resistant to Aztrionem: 58(61%), Cefoxitin: 20(21%), Ceftriaxone: 56(59%), Ceftazidime: 62(65%), Cefpodoxime: 34(44.73%) & Ceftazidime + Clavulanic acid: 8(8.5%). A total 34(36.17%) ESBL producing *E. coli* were phenotypically confirmed by ABST and Epsilometer test. Genotypic confirmation of 34 isolates was done by PCR and isolates found positive for $bla_{CTX\,M\text{-}3}$ gene: 18(52.94%), $bla_{CTX\,M\text{-}9}$ gene 6(17.64%), bla_{SHV} gene: 5(14.70%) and bla_{TEM} gene: 5(14.70%). In summary, analyzed milk samples were found to have a health risk for consumers due to contamination by ESBL producing *E. coli*, their pathogenicity and treatment failure as a result of antibiotic resistance.

Keywords: Extended Spectrum Beta Lactamase (ESBL), *Escherichia coli*, Milk, Antibiotic resistance.

Milk is considered as nature's single most complete food and important part of the diet (Haug *et al*., 2007). India is the largest milk producing countries in the world with dairy industry playing an important role in the rural economy generating huge self-employment (Batabyal *et al.,* 2018). Contamination of milk can occur due to several causes, moreover; its high nutritive value makes it an ideal medium for the rapid multiplication of bacteria, particularly under unhygienic production and storage at ambient temperatures (Kim *et al*., 1983; OECD, 2005). Pathogens that have been involved in foodborne outbreaks include *Escherichia coli* (*E. coli*), *Salmonella* and *Staphylococcus aureus*. The presence of these pathogenic bacteria in milk emerged as major public health concerns, especially for those individuals who still drink raw milk (Riser, 1998). *E. coli* is mainly responsible for causing diarrhea, urinary tract infection (UTI), hemolytic uremic syndrome (HUS), and hemorrhagic colitis (HC) (Lanjewar *et al.,* 2010).

Extensive reviews of multidrug resistant (MDR) commensal *E. coli* in animals and its impact on public health has been published recently (Szmolka and Nagy, 2013). One of the most important AMR (Antimicrobial resistance) mechanisms in *Enterobacteriaceae* family is production of extended-spectrum β-lactamase (ESBL) enzymes. ESBLs are plasmid as well as chromosome mediated and they are the results of the mutation of *TEM-1* and *TEM-2* and *SHV-I* genes (Nathisuwan, 2001). ESBL producers are resistant against wide variety of beta

lactam antibiotics including penicilins, 3rd generation cephalosporins and aztreonem. The emergence of ESBL producing *E. coli* in the food-producing animals and in foods of animal origin is a growing problem worldwide (Geser *et al.,* 2012). Thus, this study was carried out to investigate the microbiological quality and safety of raw cow milk in Anand (Gujarat, India) and around villages.

MATERIALS AND METHODS

Sample collection

A total 150 milk samples were collected from all the milking animals in the dairy cattle farms with at least 10 milking animals in a sterile container in aseptic condition using all the precautions (A- Navali: 25, B- Sai dairy farm: 50, C- Chikhodara: 25 , D- Bedva: 25, E – Mogar: 25). All the collected samples were further sent to the laboratory in an icebox within 1 h of collection and tested immediately upon arrival for further study.

Isolation and Identification

All the samples were first inoculated on MacConkey Agar (MCA) and incubated at 37°C for 24 hours. Plates showing pink colonies (lactose fermenting) were considered as positive and further transferred to Eosin Methylene Blue Agar (EMB) agar. Dark colonies with greenish metallic sheen after 24 hours of incubation at 37°C were considered to be of typical *E. coli,* were transferred to nutrient agar (NA) slants for further identification and characterization.

Biochemical characterization

Biochemical tests like Indole, Methyl-red, Voges Proskaeur, citrate utilization and TSI tests were employed for identification of *E. coli,* as per Edwards and Ewing, (1972).

Serotyping of *E. coli* isolates

Cultures identified as *E. coli* were serotyped at National Salmonella and Escherchia Centre (NSEC), Central Research Institute (CRI), Kasauli (Himachal Pradesh, India).

Antibiotic sensitivity test

Phenotypic confirmation of ESBL producing *E. coli* isolates was done using *in vitro* antibiotic sensitivity test by disc diffusion method using the discs supplied by HiMedia Laboratories Pvt. Ltd., Mumbai (India). Isolates were subjected to antimicrobial sensitivity tests against 6 antibiotics of 3rd generation cephalosporins viz.; Cefoxitin (30 μg), Ceftriaxone (30 μg), Ceftazidime (30 μg), Cefpodoxim (30 μg), Aztrionem (30 μg) and Ceftazidime + Clavulanic acid (30/10 μg). A difference of ≥ 5 mm between the zone diameters of ceftazidme and Ceftazidime + Clavulanic acid disc is measured to phenotypically confirm the ESBL production by the *E. coli* isolates under study.

Epsilometer test

All the *E. coli* isolates were tested again for phenotypic confirmation of ESBL producing *E. coli* by E test using ESBL strip which carries two gradients; on the one

Table 1: Primer sequences for $bla_{CTX\ M-3}$, $bla_{CTX\ M-9}$, bla_{SHV}, bla_{TEM}

Target genes	Primer sequence (5'→ 3')	Product size	References
$bla_{CTX\ M-3}$	F : CGTCACGCTGTT GTT AGG AA	780 bp	Kim *et al.* 2005
	R : ACG GCT TTC TGC CTT AGG TT		
$bla_{CTX\ M-9}$	F : GCGCATGGTGACAAAGAGAGTGCAA	876 bp	Yu *et al.* 2007
	R : GTTACAGCCCTTCGGCGATGATTC		
bla_{SHV}	F : TCGCCTGTGTATTATCTCCC	768 bp	Shehata *et al.* 2016
	R : CGCAGATAAATCACCACAATG		
bla_{TEM}	F : GAGTATTCAACATTTTCGT	698 bp	Shehata *et al.,* 2016
	R : ACCAATGCTTAATCAGTGA		

(F) = Forward primer; (R) = Reverse primer.

Table 2: Steps and conditions of thermal cycling for different primer pairs in PCR

Primers	Cycling conditions				
(Forward and Reverse)	Initial denaturation	Denaturation	Annealing	Extension	Final extension
bla CTX M-3 (F)	94 °C	95 °C	55 °C	72 °C	72 °C
bla CTX M-3 (R)	5 min	1 min	2 min	1 min	10 min
bla CTX M-9 (F)	94 °C	95 °C	50 °C	72 °C	72 °C
bla CTX M-9 (R)	5 min	1 min	1 min	1 min	7 min
bla SHV (F)	95 °C	94 °C	65 °C	58 °C	72 °C
bla SHV (R)	15 min	30 sec	2 min	30 sec	1 min
bla TEM (F)	95 °C	94 °C	65 °C	58 °C	72 °C
bla TEM (R)	15 min	30 sec	2 min	30 sec	1 min
	Repeated 35 cycles				

end, ceftazidime and on the opposite end ceftazidime plus clavulanic acid. MIC is interpreted as the point of intersection of the inhibition ellipse with the E test strip edge. Ratio of ceftazidime MIC and ceftazidime clavulanic acid MIC ≥ 8 indicates the presence of ESBL (Bush and Jacoby, 1995).

Polymerase chain reaction

Genotypic confirmation of ESBL genes was done for all the phenotypically confirmed ESBL producing *E. coli* using the PCR protocols separately standardized for the detection of different ESB genes viz.; bla_{CTXM-3}, $bla_{CTX M-9}$, bla_{SHV}, bla_{TEM}. The PCR was standardized for the detection of four genes following the methodology as described in table 2 with suitable modifications (Kim *et al.*, 2005, Yu *et al.*, 2007 and Shehata *et al.*, 2016).

RESULTS AND DISCUSSION

Isolation and Identification

A total 94 (62.66%) *E. coli* isolates were recovered from 150 raw milk samples which were collected from Anand and around villages (Navali-17, Sai dairy farm-32, Chikhodara-14, Bedva-16, Mogar-15). All the positive isolates showed typical characteristics of *E. coli* viz., greenish metallic sheen on EMB agar (Fig. 2), positive indole and methyl red, negative VP and citrate utilization test and yellow slant, yellow butt and no H_2S production on TSI agar.
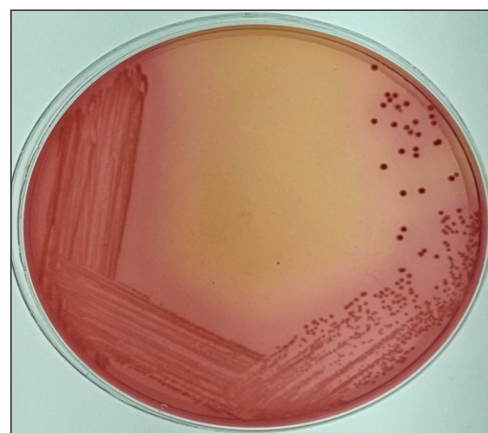
Fig. 1: Lactose fermenting pink colonies of gram negative bacteria on MacConkey agar plate
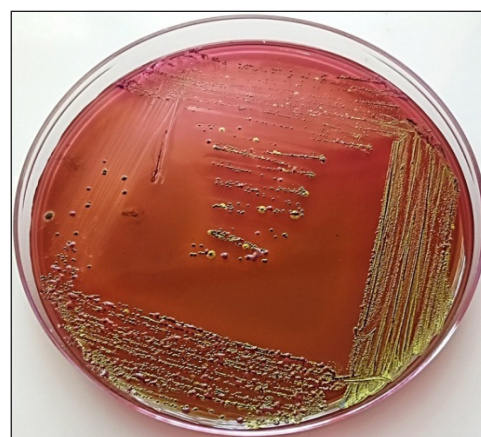
Fig. 2: Greenish metallic sheen producing colonies of *E. coli* on Eosin Methylene Blue agar plate

Serotyping of *E. coli* isolates

Cultures identified as *E. coli* were serotyped at National Salmonella and Escherchia Centre (NSEC), Central Research Institute (CRI), Kasauli (Himachal Pradesh, India). Out of 94 *E. coli* isolates 60 (63%), 28 (29%) isolates were unypable (UT) and 6 (6%) were found to be rough. A total 14 different serotypes were found in the present study namely; O83, O49, O157, O103, O22, O34, O26, O145, O146, O2, O141, O119, O84 and O120.

Antibiotic sensitivity test

In vitro antimicrobial susceptibility test (ABST) was performed for all 94 *E. coli* isolates by disk diffusion method and results were as shown in Table 3. Maximum isolates (84.04%) were sensitive to antibiotic Ceftazidime + Clavulanic acid combination while maximum isolates (65.95%) were resistance to Ceftazidime. A total 34 (36%) isolates out of 94 isolated were found positive as ESBL producing *E. coli* when the difference in the inhibition zone was measured between ceftazidime and ceftazidime + cavulanic acid; organisms giving the difference in inhibition zone ≥ 5 were considered as positive ESBL producers.

**Table 3:** *In vitro* antimicrobial drug resistance pattern of *E. coli* isolates

| Antimicrobial agents	Sensitive	Intermediate	Resistant
Ceftazidime	12 (12.76%)	20 (21.27%)	62 (65.95%)
Aztreonem	21 (22.34%)	15 (15.95%)	58 (61.70%)
Ceftriaxone	16 (17.02%)	22 (23.40%)	56 (59.57%)
Cefpodoxime	36 (38.29%)	24 (4.53%)	34 (36.17%)
Cefoxitin	29 (30.85%)	45 (47.87%)	20 (21.27%)
Ceftazidime + Clavulanic acid	79 (84.04%)	07 (7.44%)	08 (8.5%)

Epsilometer test

Out of 94 *E. coli* isolates, 34 (36.00%) isolates were phenotypically confirmed as ESBL producer through E test. Here for the interpretation ESBL producing *E. coli* give ratio of MIC of Mix +/ MIC of Mix ≥ 8 is considered as positive ESBL producers.

Polymerase chain reaction

Out of 34 phenotypically confirmed ESBL producing *E. coli* isolates, 18 (52.94%) isolates were positive for $bla_{CTX M-3}$ gene, 6 (17.64%) isolates were positive for $bla_{CTX M-9}$, 5 (14.70%) isolates were positive bla_{SHV} & also 5 (14.70%) isolates were positive bla_{TEM} genes.
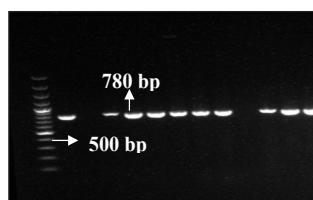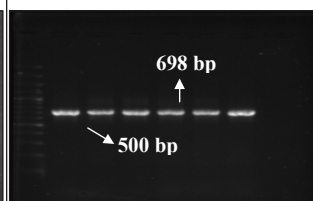

Fig. 3: PCR amplified product (780bp) for $bla_{CTX M-3}$ gene
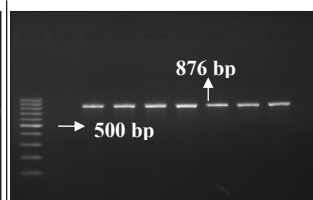
Fig. 4: PCR amplified product (876bp) for $bla_{CTX M-9}$ gene
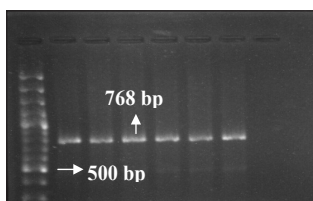
Fig. 5: PCR amplified product (768bp) for bla_{SHV} gene

Fig. 6: PCR amplified product (698bp) for bla_{TEM} gene

Here, in the present study 62.66% isolates from raw milk samples revealed characteristic features of *E. coli* which is in agreement with the prior findings; 65% by Chye *et al*. (2004), 52% by Virpari *et al*. (2013), 65% by Soomro *et al*. (2002), 63% by Uddin *et al*. (2011).

The serotypes reported by the National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, were also supported by Manna *et al*. (2006), Miszczycha *et al*. (2013), Hardik *et al*. (2017) who reported *E. coli* serotype O157, O83, O84, O145 and O103 in their studies. Elhadidy & Mohammed (2013) and Blanco *et al*. (2004) reported the presence of serotypes O22, O120, O2, O141 O119, O34 and O49 from *E. coli* isolated from raw milk samples which is in agreement to the present study.

Gashe *et al*. (2018) reported 60% resistance against ceftazidime and 59% against ceftriaxone in a prior study. Xu *et al*. (2018) reported 20% resistance against cefoxitin, Jena *et al*. (2018) reported 61% resistance against aztreonem and 36% against cefpodoxime in theie studies. Bhattacharya *et al*. (2015) observed higher degree (84.04%) of sensitivity of *E. coli* isolates when Ceftazidime

+ Clavulanic acid combination was used. Results of all these studies support the findings of the present study.

In the present study, total 34 (36%) *E. coli* isolates were found positive through E test which is in agreement to prior study by Cormican *et al.* (1996) where 82 (36%) *E. coli* isolates were found positive as ESBL producers out of 225.

In the present study, 18 (52%), 6 (17%), 5 (14%) and 5 (14%) isolates were positive for *bla* $_{CTX M-3}$, *bla* $_{CTX M-9}$, *bla* $_{TEM}$ and *bla* $_{SHV}$ genes respectively. Similar results were reported by Tekiner and Ozpinar (2016); where 53% prevalence of *bla* $_{CTX M-3}$, 12% for *bla* $_{TEM}$ & *bla* $_{SHV}$ genes, and 16% for *bla* $_{CTX M-9}$ was observed.

CONCLUSION

Analysed raw milk samples poses high health risk to the consumers because of such high prevalence (94%) of *E. coli* from milk. A high degree of the resistance (80%) was observed by ESBL producing *E. coli* towards beta lactam antibiotics which can be considered a reason of the treatment failure and economic losses thus it rises great public health concerns. It is suggested to administer beta lactam antibiotic along with sucidal inhibitors like Clavulanic acid, Tazobactam, and Salbactam for cost effective treatment and to meet the purpose of public health.

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