



Phenotypic and molecular characterization of ESBLs producing *Escherichia coli* in bovine faecal and milk samples of North Gujarat

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ABSTRACT

Extended-spectrum β -lactamases (ESBLs) producing *E. coli* seems to be emerging in veterinary science impacting major threat to public health due to resistance to golden age antibiotics. In this study a total of 109 samples (42 faecal and 67 mastitis milk) of bovines were collected from different regions of North Gujarat. The samples were cultured and identified by standard procedures. The screening for ESBLs production was performed by using Cefotaxime and Cefotaxime+Clavulanate (Combination disc screening method). A total of 71 *E. coli* isolates were recovered from 109 samples processed, out of which thirty (42.25%) isolates (17 from milk and 13 from faecal) were positive for ESBLs showing multiple resistance to the antibiotics used. The ESBL confirmed isolates were further processed for detection of *bla*CTX-M, *bla*TEM, and *bla*SHV genes. Major gene detected was *bla*TEM in 17 (23.94%) *E. coli* isolates. Antibiotic resistance pattern of *E. coli* isolates was studied against eleven commonly used antimicrobial drugs in the northern region of Gujarat. The results recorded resistance to following antibiotics: tetracycline (100%), ampicillin/sulbactam (83.10%), amoxiclav and gentamicin (83.10%), chloramphenicol (57.74%), ceftriaxone (66.19%), cefoperazone (66.19%), ciprofloxacin (74.65%), amikacin (57.74%), enrofloxacin (74.65%) and, levofloxacin (74.65%).

Keywords: BlaTEM, Bovines, ESBLs producing *E. coli*, Mastitis milk, North Gujarat

Most of the bacterial pathogens associated with human enteric illness are either of animal origin that can be transmitted directly or indirectly to humans, e.g. through food of animal origin, contaminated water or from a common reservoir (Schlundt *et al.* 2004, Newell *et al.* 2010). This type of mode of horizontal gene transfer facilitates the spread of resistance genes in and between various bacterial species. Extended spectrum beta-lactamase (ESBLs), plasmid-encoded enzyme, inactivates a broad range of β -lactam antibiotics such as oxyimino-cephalosporins including cefotaxime. Added to this, ESBL producers are also resistant to other antibiotics such as fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole (Machado *et al.* 2008, Pasom *et al.* 2013, Rath *et al.* 2014). Beta-lactam antimicrobial agents are the most commonly used drugs for treating the bacterial infections. As per WHO, third and fourth generation cephalosporins are critically important antimicrobials, their therapeutic effectiveness and short withdrawal period make them increasingly important in dairy farming, providing a selection pressure for the emergence of bacterial resistance against these compounds by production of ESBLs (Snow *et al.* 2012, Liebana *et al.* 2013).

Since, late 1990's, ESBLs producing Enterobacteriaceae,

in particular *E. coli* has emerged globally. Initially, ESBL producing bacteria were only observed in human medicine (Olowe *et al.* 2012, Ogbolu *et al.* 2013) but the occurrence of these bacteria in companion animals firstly and increasingly in livestock nowadays, has initiated monitoring studies more focused on livestock (Smet *et al.* 2010). It has been documented that food-producing animals are important reservoirs for ESBL-producing Enterobacteriaceae (Carattoli 2008, EFSA 2011, Smet *et al.* 2010). Recent studies raised severe concerns about the role of farm animals, especially dairy cattle, on dissemination of ESBL-producing Enterobacteriaceae and/or their mobile genetic elements via the food chain (Brinas *et al.* 2002, Aarestrup *et al.* 2006, Meunier *et al.* 2006, Dahmen *et al.* 2013, Liebana *et al.* 2013, Schmid *et al.* 2013).

ESBL determinants have been detected not only in clinical isolates but also in commensal bacteria from humans and, animals and in isolates from products of the food chain and sewage, revealing distribution and suggesting the presence of environmental reservoirs for these resistance determinants (Brinas *et al.* 2002).

Currently, there is limited information available on ESBL producing *E. coli* in veterinary science and the possible contribution. The objective of the present study was to detect ESBL producing *E. coli* in the faecal and milk samples of bovines from different regions of North Gujarat.

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Table 1. Details of primer sequence used for detection of *bla*SHV, *bla*TEM and *bla*CTX-M genes

Target gene	Primer sequence	Amplicon size (bp)	References
<i>bla</i> SHV	F: 'ATG CGT TAT ATT CGC CTG TG' R: 'TGC TTT GTT ATT CGG GCC AA'	747	Paterson <i>et al.</i> 2003
<i>bla</i> TEM	F: 'TCG CCG CAT ACA CTA TTC TCA GAA TGA' R: 'ACG CTC ACC GGC TCC AGA TTT AT'	445	Monstein <i>et al.</i> 2007
<i>bla</i> CTX-M	F: 'ATG TGC AGY ACC AGT AAR GTK ATG GC' R: 'TGG GTR AAR TAR GTS ACC AGA AYC AGC GG'	593	Boyd <i>et al.</i> 2004

MATERIALS AND METHODS

A total of 109 samples (42 faecal and 67 mastitic milk) were collected from bovines into sterile capped universal bottles with sterile spatula and were transported to the laboratory immediately. Isolation of *E. coli* isolates were carried out by culturing of samples on MacConkey's agar and incubated for 18–24 h at 37°C. The identification of *E. coli* was carried out by culturing on eosin methylene blue agar and incubated for 18–24 h at 37°C. Presumptive characteristic of *E. coli* isolates were identified and confirmed using arrays of biochemical tests (IMViC). The study was carried from August, 2017 to October, 2018 in the Department of Veterinary Public Health and Epidemiology, College of Veterinary Science and Animal Husbandry, SDAU, Sardarkrushinagar.

Antimicrobial susceptibility of pure colonies to ampicillin/sulbactam (A/S) (20 µg), amoxiclav (AMC) (30 µg), ceftriaxone (CTR) (30 µg), cefoperazone (CPZ) (75 µg), ciprofloxacin (CIP) (5 µg), chloramphenicol (C) (30 µg), amikacin (AK) (30 µg), enrofloxacin (EX) (10 µg), levofloxacin (LE) (5 µg), gentamicin (GEN) (10 µg) and tetracycline (TE) (10 µg) was determined and interpreted by the disc diffusion method following the guidelines of Clinical Laboratory Standard Institute (CLSI) (CLSI, 2012).

Phenotypic detection of ESBL production is done by combination disc screening method. Cefotaxime and cefotaxime+clavulanate (Hi Media, INDIA) discs were applied on freshly prepared Muller Hinton agar and the plates were further incubated aerobically at 37°C. A final measurement of the zone of inhibition was made after overnight incubation. ESBL producers were defined as having a differential zone diameter of $\geq +5$ mm (Cefotaxime+clavulanate zone - cefotaxime zone).

The DNA of the ESBL confirmed isolates by combination disc screening method was extracted separately using a DNA extraction kit (Qiagen, Germany). Molecular confirmation of ESBL producing *E. coli* was done by detection of *bla*CTX-M, *bla*TEM, and *bla*SHV genes by multiplex polymerase chain reaction (m-PCR) as described by Monstein *et al.* (2007) with some modifications using specific primers. The recommended oligonucleotide primers specific for the *bla*SHV, *bla*TEM, and *bla*CTX-M genes used in the m-PCR assay and expected amplicon sizes are depicted in Table 1.

RESULTS AND DISCUSSION

In the present study, out of 109 samples, 71 (65.13%)

isolates were identified as *E. coli*, giving greenish metallic sheen on EMB agar (Fig. 1) and through biochemical characterization. The highest prevalence of *E. coli* was observed in faecal samples (83.33%) followed by mastitic milk samples (53.73%).

All the 71 *E. coli* isolates that were subjected to antimicrobial testing (Fig. 2) and interpreted as resistant, intermediate and sensitive following the guidelines of CLSI (2012) is depicted in Table 2 (Fig. 3). Resistance to tetracycline (TE) (100%), ampicillin/sulbactam (A/S) (83.10%), amoxycav (AMC) (83.10%) and gentamicin (GEN) (83.10%) was found to be higher as compared to



Fig. 1. Greenish metallic sheen on EMB agar.

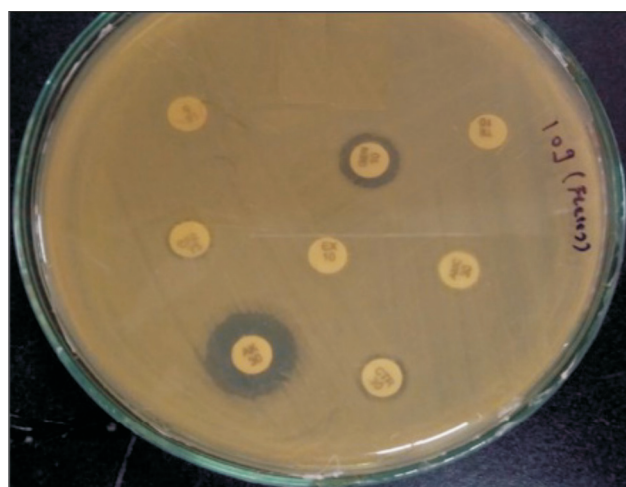
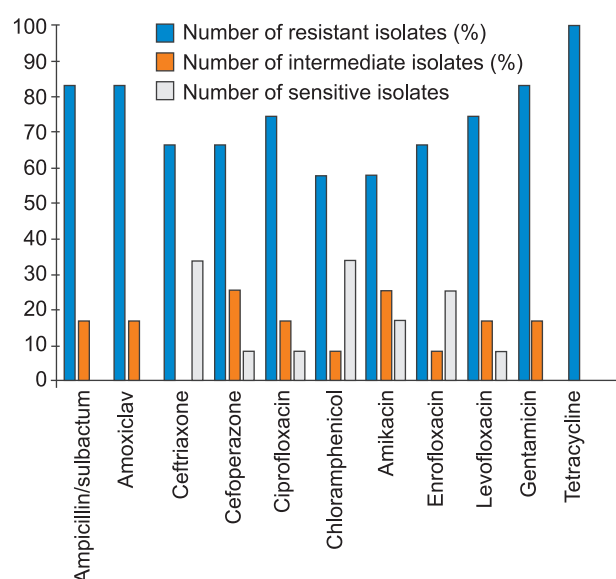
Fig. 2. *In vitro* antimicrobial drug sensitivity test for ESBLs producing *E. coli*.

Table 2. Antibiotic sensitive pattern of *E. coli* isolates (n=71)

Antibiotic	Resistant isolates (%)	Intermediate isolates (%)	Sensitive isolates (%)
Ampicillin/Sulbactam (10/10 mcg)	83.10	16.90	0
Amoxycylav (20/10 mcg)	83.10	16.90	0
Ceftriaxone (30 mcg)	66.19	0	33.81
Cefoperazone (75 mcg)	66.19	25.35	8.45
Ciprofloxacin (5 mcg)	74.65	16.90	8.45
Chloramphenicol (30 mcg)	57.74	8.45	33.81
Amikacin (30 mcg)	57.74	25.35	16.90
Enrofloxacin (5 mcg)	66.20	8.45	25.35
Levofloxacin (5 mcg)	74.65	16.90	8.45
Gentamicin (10 mcg)	83.10	16.90	0
Tetracycline (30 mcg)	100.00	0.00	0

Fig. 3. Antibiotic sensitive pattern of *E. coli*.

other antibiotics. Comparatively resistance to chloramphenicol (C) (57.74%) was recorded to be lower.

Antibiotic resistance is an emerging threat in human medicine as well as in veterinary medicine (Witte 1998, Lawson 2008). Antibiotic resistance has put deleterious effect on livestock disease management thus in turn on the productivity of the animals.

Recently, there has been increased resistance to beta lactam antibiotics due to plasmid mediated acquisition of ESBL producing genes predominantly by *E. coli*. Although most of the studies conducted on ESBL producers are confined to human beings within the hospital premises, focus should also be given on its occurrence and dissemination in food producing animals. Reports by several researchers also indicated its presence in chicken meat and raw milk (Geser *et al.* 2012, Lyhs *et al.* 2012).

Phenotypic detection of ESBL producing *E. coli* is detected in 30 (42.25%) isolates (17 from mastitis milk and 13 from faecal samples) out of the 71 isolates by combination disc screening method (Fig.4). Similarly,

higher prevalence of 71% ESBLs was recorded by Olugbenga *et al.* (2015) from bovine faecal samples. All the 30 ESBL producers were resistant (100%) to ceftriaxone, ampicillin/sulbactam, amoxiclav, cefoperazone, ciprofloxacin, chloramphenicol, amikacin, enrofloxacin, levofloxacin, gentamicin and tetracycline as revealed by disc diffusion assay. These observations reiterate the findings in other studies that have reported increased antibiotic resistance amongst different bacterial species especially *E. coli* isolated from cattle and other animals at an alarming rate (Kozak *et al.* 2009, Ajayi *et al.* 2011). The conclusive finding in the present study is multiple drug resistant against commensal *E. coli* in bovines to most commonly used antibiotics such as tetracycline (100%), ampicillin/sulbactam (83.10%), amoxiclav (83.10%), and gentamicin (83.10%). This finding correlates with similar results obtained from other studies that have reported some levels of multiple antibiotic resistance by *E. coli* from cattle, meat products and other animals (Saenz *et al.* 2001, Umolu *et al.* 2006). Chromosomal AmpC β -lactamase hyperproduction by *E. coli* isolates (Livermore 1995) and plasmid-mediated β -lactamases (Sanders and Sanders 1992, Zhou *et al.* 1994) are documented as the main cause for the increasing resistance to aminopenicillins, β -lactam- β -lactamase inhibitor combinations, narrow-spectrum cephalosporins, and cephamycins to a greater extent and, resistance to carboxy- and ureidopenicillins and extended-spectrum cephalosporins to a lesser extent. Amongst, the plasmid-mediated β -lactamases, more often TEM-1 hyperproduction and its inhibitor-resistant variant (IRT enzymes) are found to be responsible for β -lactam- β -lactamase inhibitor combination resistance, a major threat to therapeutic success and occasionally OXA type is reported for the same (Martinez *et al.* 1987, Zhou *et al.* 1994, Wu *et al.* 1994, Stapleton *et al.* 1995).

Out of 71 *E. coli* isolates, ceftriaxone, chloramphenicol, enrofloxacin, cefoperazone, levofloxacin, ciprofloxacin were found to be sensitive in 24 (33.81%), 24 (33.81%), 18 (25.35%), 6 (8.45%), 6 (8.45%), 6 (8.45%) isolates, respectively. Also, few isolates were found to be of intermediate resistance to ampicillin/sulbactam (16.90%), amoxiclav (16.90%), cefoperazone (25.35%), ciprofloxacin (16.90%), chloramphenicol (8.45%), amikacin (25.35%), enrofloxacin (8.45%), levofloxacin (16.90%) and

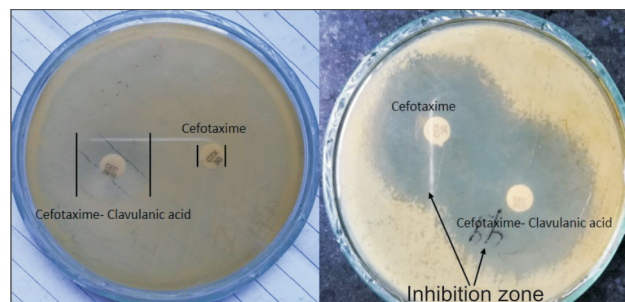


Fig. 4. Detection of ESBLs producing *E. coli* by combination disc method, A. Positive for ESBLs production. B. Negative for ESBLs production.

gentamicin (16.90%).

All the 30 isolates were further subjected to m-PCR for the detection of beta-lactamase genes viz. *blaSHV*, *blaTEM* and *blaCTX-M*. The genes *blaTEM* were detected in 17 isolates. Similarly high prevalence of *TEM-52* was detected in food animals by various researchers (Costa *et al.* 2004, Machado *et al.* 2008). *CTX-M* and *SHV* genes were not detected in any of the collected samples.

Similar study carried out in China by Yuan *et al.* (2009) showed higher prevalence of *blaTEM* gene followed by *blaCTX-M* with no samples detected positive for *blaSHV* gene in chickens. These findings suggested that *blaTEM* was more common amongst the ESBL genes in these isolates. In this study, the occurrence of ESBL producing *E. coli* was as high as 23.94% as ESBL genes were detected in 17 out of 71 isolates screened. Several studies on high prevalence of ESBL-producing *E. coli* isolates in faecal samples from food producing animals has also been found in other European countries (Blanc *et al.* 2006, Moreno *et al.* 2007, Smet *et al.* 2008, Falgenhauer *et al.* 2019). The wide dissemination of ESBL-positive *E. coli* among faecal isolates of food-producing animals is a serious issue of food safety and it is important to analyze the factors that could contribute to this situation. One possible origin of this problem might be the use of broad-spectrum cephalosporins in animal production. Other possible factor would be overcrowding in herds, facilitating the transmission of resistant bacteria amongst the animals. It is difficult to know the specific contribution of each factor to the global problem of the wide dissemination of ESBL in the animal ecosystem, and all these factors should be further studied with larger sample size. Previous reports also refer to a relatively higher frequency of the presence of ESBL-producing *E. coli* isolates in samples of healthy humans (Miro *et al.* 2005, Moubareck *et al.* 2005, Pallecchi *et al.* 2007, Valverde *et al.* 2004). An important issue to be elucidated is whether presence of ESBL-producing bacteria in the intestinal tract of healthy animals and humans has occurred in an independent way, or by contrast, both ecosystems interrelated in their evolution.

In conclusion, increasing prevalence of ESBL producers in fecal and milk samples of normal animals is pointing towards the hypothesis of animals might be becoming carriers or even reservoirs of the same thus contributing to silent spread of these multidrug resistant bacteria amongst the animals at different places, borders and boundaries. Globally occurring climate changes had put effect on the genes causing mutations amongst organisms leading to resistance. These resistant traits call for gross surveillance in community settings because ESBLs gene is a growing threat worldwide for available antibiotics but its epidemiological effects and evaluation are still underestimated with low awareness.

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