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Occurrence of Extended–Spectrum Beta- Lactamases (ESBLS) Producing Enterobacteria in Animal Products and their Environment

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A B S T R A C T

The present study was undertaken to evaluate the status of ESBL producing *Enterobacteriaceae* in foods of animal origin and their environment. A total of 125 samples were collected comprising 95 animal products (40 raw milk, 25 milk products, 15 raw meat and 15 meat products) and 30 environmental samples. The isolation rate was recorded 93.95% in food samples with *Citrobacter* (38.41%) being the dominant flora, while100% in environmental samples with the dominance of *E. coli* (89.18%). Of all the ESBL producers, 24.29% were found positive by phenotypic method while 16.38% were found positive by PCR. The phenotypic test revealed highest occurrence of ESBL producers in environmental samples (56.76%) followed by milk (24.44%), meat (16.0%), meat products (15.0%) and milk products (8.00%). Similarly, PCR assay also recorded highest occurrence in the environment (48.65%) followed by raw meat (8.0%) and raw milk (2.0%) samples; however none of the ESBL genes was detected in milk and meat products. ESBL genes positive isolates belonged to the genera *Escherichia*, *Klebsiella* and *Citrobacter*. The frequency of bla_{CTX}, bla_{SHV} and bla_{TEM} genes in *E. coli* isolates was 37.97%, 6.89% and 3.44%, respectively. The co-existence of bla_{CTX} and bla_{TEM}, bla_{SHV} and bla_{TEM} and bla_{CTX} and bla_{SHV}, was found 17.24%, 6.89% and 3.44% in *E. coli* isolates, respectively. *Citrobacter* isolates harboured single (bla_{CTX} 3.44%) as well as multiple genes (bla_{CTX} +bla_{SHV} 3.44%) and (bla_{CTX}+bla_{TEM} 6.89%) while *Klebsiella* isolates showed only bla_{CTX} gene (6.89%). Only one *E. coli* isolate (3.44%) in the present study harboured all three genes.

Introduction

ESBLs are the rapidly evolving β-lactamases (Paterson and Bonomo, 2005) with an ability to hydrolyze penicillins, first, second, and third generation cephalosporin, and aztreonam but can be inhibited by β-lactamase inhibitors such as clavulanic acid (Jacoby and Medeiros, 1991; Bush *et al.*, 1995).The extensive use of such antibiotics in food animals has resulted in the development of resistance and food animal serve as a reservoir of resistant strains for human and animal population. Food may get contaminated with these strains during

animal slaughtering, milking or processing. Consequently, without good hygienic practices, foods may act as a vehicle for transfer of β-lactam resistant bacteria to the consumers (Overdevest *et al.,* 2011). Some recent studies have documented frequent occurrence of ESBL producers in poultry (Kolar *et al.,* 2010; Overdevest *et al.,* 2011), dairy and meat products (Gundogan and Yakar, 2007; Gundogan *et al.,* 2011). Due to paucity of data from this region of UP, the present study aimed to assess the occurrence of ESBL-producing enterobacteria in milk, meat and their products as well as farm animal's environment.

Materials and Methods

Samples Collection

A total of 125 samples from food animal and their environment were collected in the present study. The food samples comprising of raw milk (40), milk products (30), raw meat (15) and meat products (15) were collected from different shops of Kumarganj and Lucknow (UP). Processed milk product's samples included ice cream, dahi, chhena, paneer, rasgulla, peda and barfi; while meat product's samples included beef kabab, mutton kabab, chicken tikka, chicken roll, biryani and roasted chicken. Raw milk samples were also procured from instructional livestock farming complex (I.L.F.C.), Teaching veterinary clinical complex (T.V.C.C.) of College of Veterinary Science & Animal Husbandry, animal farms nearby Kumarganj.

Samples were collected aseptically and transported under refrigerated condition to the laboratory. Total 30 environmental samples which included floor swabs representing the animal farm environment were collected from I.L.F.C. and animal farms nearby Kumarganj, U.P.

Isolation and identification of *Enterobacteria*

The samples were processed for isolation of *Enterobacteria* as per the procedures described by Cruickshank *et al.,* (1975). Mac Conkey Lactose Agar, Eosin Methylene Blue Agar and Brilliant Green Agar media were used for isolation as well as differentiation of lactose fermenters and lactose non-fermentors belonging to *Enterobacteriacae*. All the samples diluted in peptone water were grown in improvised media i.e. MacC-CTX broth and MacC-CTX agar to selectively culture the drug resistance organism and eliminate the susceptible organism so as to minimise the growth of all other organism. The identification of enterobacteria was done on the basis of morphology, growth and biochemical characteristics as per the method described by Edwards and Ewing (1972). The biochemical tests included catalase, oxidase, indole, methyl red, Voges Proskauer, citrate, urease, triple sugar iron agar and sugar fermentation tests.

Identification of ESBL producers

Screening of ESBL producing isolates of *Enterobacteriaceae* was done by disk diffusion method as prescribed in CLSI guidelines (2009). The isolates were tested against two antibiotics viz. cefotaxim and ceftazidime and presumed as ESBL producers if the zone diameter for cefotaxim was $\leq$ 27 mm and for ceftazidime $\leq$ 22 mm. These ESBL producing *Enterobacteria* were confirmed by combination disks test as per the procedure of CLSI (2009) with slight modification. The ESBL kit I and kit III of Hi media Laboratories were used for phenotypic confirmation of ESBL producers as per the manufacturer's instruction. The test organisms were considered as ESBL positive if a $\geq$ 5 mm increase in zone diameter was observed for two or more antimicrobial agents

tested in combination with clavulanic acid versus its zone when tested alone.

Molecular characterization of ESBL producers

The ESBL genes were targeted for molecular characterization of ESBL producers using published primer sequence (Table 1) synthesized by Bangalore Genei (India). The DNA templates were prepared using snap-chill method as described by Franco *et al.*, (2008). The PCR assay was performed in 20 µl final volume containing 10µl of master mix, 2µl of forward and reverse primer (100pmol), 2µl of $MgCl_2$,, 2µl of DNA template and 2 µl of nuclease free water. The ESBL genes viz. *bla* $_{TEM,}$ *bla* $_{CTX-M}$ and *bla* $_{SHV}$ were targeted by PCR using the conditions given in the table 2. The amplified PCR products were run in 1.5% agarose gel and visualized and analyzed using gel documentation system (Uvi tech, UK).

Results and Discussion

Food animals are increasingly being recognized as a reservoir for ESBL-producing strains. Worldwide studies have revealed that ESBL producing isolates such as *E. coli* and *Klebsiella* can contaminate foods of animal origin and contribute to diseases and spoilage (Gundogan and Yakar, 2007; Haryani *et al.*, 2007). In the present study, processing of 125 samples yielded 186 isolates, of which 177 (95%) were screened out as members of *Enterobacteriaceae* family. The isolates grew luxuriantly and selectively on MLA showing typical morphology. The small round rose pink colonies were regarded as of *E. coli* and *Citrobacter*, while the light pink mucoid colonies were regarded as of *Enterobacter* and *Klebsiella*. The pale colourless colonies on MLA were presumed as of *Salmonella* and *Proteus***.** Further identification and differentiation of enterobacteria was done using selective medium like EMB and BGA. The tiny metallic sheen colonies on EMB were considered as *E. coli,* while purple dark centred colonies with mucoid rim were regarded as either *Enterobacter* or *Klebsiella*; however, the colonies of *Klebsiella* appeared smaller than *Enterobacter*. The lactose non-fermenters isolates were grown on BGA and the isolates revealing light pinkish colonies with dark pinkish background of the media were presumed as *Salmonella.* The isolates showing swarming characteristic on nutrient agar plates were considered as *Proteus.* Further identification and differentiation of bacterial isolates was done on the basis of motility, staining and biochemical characteristics. Based on these characteristics, 68 isolates were identified as *Citrobacter* spp., 54 isolates as *E. coli*, 30 isolates as *Enterobacter* spp., 15 isolates as *Klebsiella* spp., 6 isolates as *Salmonella* and 4 isolates as *Proteus* spp (Table 1). Isolation rate of enterobacteria was found to be 100% from environmental samples and raw meat samples, while raw milk, milk products and meat products revealed 97.82 %, 89.28%, 90.90% isolation rate, respectively (Table 2). Thus overall isolation rate of enterobacteria from foods of animal origin was found to be 93.95%.

The enterobacterial isolates were subjected to ESBL screening using cefotaxime in growth medium and all the presumptively positive ESBL producers were further confirmed by phenotypic double disc diffusion assay. The highest prevalence of ESBL producers was seen in environmental samples (56.76%) followed by milk (24.44%) and milk products (8.0%), meat (16.0%) and meat products (15.0%). PCR assay recorded highest prevalence (48.65%) in the environment samples followed by raw meat (8.0%), raw milk samples (2.0%). None of the isolates from milk and meat products revealed ESBL genes (Table 2). All the enterobacterial

isolates tested positive for ESBL genes belonged to 3 different genera viz. *Escherichia*, *Citrobacter* and *Klebsiella*. Proportionate study of ESBL and Non-ESBL producers among the enterobacterial isolates revealed highest distribution rate in *E. coli* (74.91%) followed by *Klebsiella* (15.38%), *Citrobacter* (6.25%). However, rest of the enterobacteria i.e. *Enterobacter*, *Salmonella* and *Proteus* were found to be non-ESBL producers. Source wise distribution study revealed that *E. coli* were found in highest proportion in environmental isolates (55.17%, 16) followed by raw milk (17.24%, 5) and raw meat isolates (6.90%, 2). All 4 ESBL positive *Citrobacter* were isolated from raw milk with 13.79% prevalence while 2 ESBL positive *Klebsiella* isolates were recovered from the environment with 6.89% prevalence. However, none of the ESBL positive *E. coli*, *Citrobacter* and *Klebsiella* could be recovered from milk and meat products.

The distribution study of ESBL genes (Fig. 1, 2 and 3) among enterobacterial isolates revealed that out of 29, occurrence of ESBL genes was highest in *E. coli* (12.99 %, 23), followed by *Citrobacter* (2.25%, 4) and *Klebsiella* (1.12%, 2). Among *E. coli* isolates, bla_{CTX} gene (37.93%) was predominantly present followed by bla_{SHV} (6.89%) and bla_{TEM} (3.44%). The co-existence of bla_{CTX} with bla_{TEM} and bla_{SHV} was recorded in 5(17.24%) isolates and 1(3.44%) isolate, respectively. The bla_{SHV} and bla_{TEM} gene combination was detected in 2 isolates with 6.89% prevalence. Only one isolate of *E. coli* carried all the three genes with 3.44% prevalence. The frequency rate of ESBL genes in *Citrobacter* was found to be 3.44%, 3.44% and 6.89% for bla_{CTX}, bla_{CTX} and bla_{TEM}, bla_{CTX} and bla_{SHV}, respectively. In ESBL positive *Klebsiella* isolates, only bla_{CTX} gene was detected with 6.89% prevalence (Table 3). None of the isolates of *Enterobacter*, *Salmonella* or *Proteus* were found positive for ESBL genes.

Foods may act as a vehicle for transfer of β-lactam resistant bacteria to the consumers without good hygienic practices (Overdevest *et al.,* 2011).The present study was conducted with the aim to assess the occurrence of ESBL-producing enterobacteria in different types of foods of animal origin sold out in retail market in UP as well as in their environment. The overall isolation rate of enterobacteria from foods of animal origin was found to be 93.95% while all the environmental samples (100%) were found to harbour enterobacteria. Our finding corroborated with the observation of Tham *et al.,* (2012) where 82.7% food sample swabs exhibited characteristic growth of enterobacteria while Khan *et al.,* (2015) reported 51.85% occurrence of enterobacteria in food items from Karanchi. However, Geser *et al.,* (2012) reported that no ESBL producing enterobacteria could be isolated from foods of animal origin from Switzerland. These geographic differences may be attributed to variation in hygienic standards. Among the various food products analysed in present study, isolation rate of enterobacteria was 97.82%, 89.28%, 100% and 90.90% in raw milk, milk products, raw meat and meat products, respectively. Of 177 isolated strains of the family *Enterobacteriaceae*, the dominant bacterial flora was *Citrobacter* (38.41%) followed by *E. coli* (30.50%), *Enterobacter* (16.94%), *Klebsiella* (8.47%), *Salmonella* (3.38%) and *Proteus* (2.25%). *Enterobacteriaceae* contamination observed in this study clearly highlights breakdown of hygienic handling practices at different stages of the production, processing and distribution chain. Our findings were in conformity with the observations of Fadel and Ismail (2009) and Saikia and Joshi (2010) who also reported enterobacteria in most of the milk products and meat products, respectively. Likewise, Yusha *et al.,* (2010) also reported *Citrobacter* as predominant organisms (31.25%) in food. However, Shahid *et al.,* (2009) reported

Citrobacter as second most dominant organism from food specimens (meat and milk products) sold out in Indian markets. In most of the studies carried out on animal food products, the dominant bacterial flora appeared to be either *E. coli* (Jensen *et al.,* 2006; Kumar *et al.,* 2011; Tekiner *et al.,* 2015) or *Klebsiella* (Kim *et al.,* 2005; Shahid *et al.,* 2009; Gundogan and Avci, 2013). The reason behind could be that these are common inhabitants of gastrointestinal tract and most widely distributed environmental contaminants. In environmental swab samples, *E. coli* was the most dominant organism (89.18%) followed by *Enterobacter* (5.40%) and *Klebsiella* (5.40%) which coincided with the observations of Mesa *et al.,* (2006).

All the presumptive ESBL enterobacterial isolates were subjected to double disc diffusion assay for phenotypic confirmation. The highest occurrence of ESBL producers was seen in environmental samples (56.76%) followed by milk (24.44%), meat (16%), and meat products (15%) and milk products (8%). Similarly, Mesa *et al.,* (2006) also recorded the highest prevalence of ESBL producers in farm samples (80-100%) as compared to food samples (0.40%) by E-test. Polymerase chain reaction characterized merely 29 isolates as ESBL producers and majority were recorded in environment samples (48.65%) followed by raw meat (8.0%) and raw milk (2.0%). Likewise, Gundagon and Avci (2013) tested presence of ESBL producers in animal foods and reported more number of ESBL producers from meat products than milk and milk products. The relatively high occurrence of ESBL producers in floor samples is not surprising as there is indiscriminate use of antibiotics in veterinary practices, and non ESBL producers may acquire the plasmid from ESBL producers living in the same environment. Moreover, it is striking that none of the ESBL was found in milk products and meat products. The non occurrence of ESBL producers in milk and meat products in our study might be attributed to high processing temperature and low moisture content of these products. Present findings were found in agreement with the observations of Geser *et al.,* (2012) as 26.9% fecal samples of farm animals yielded ESBL and only 1.5% mastitic milk isolates were found ESBL producers but none was isolated from either minced meat or bulk tank milk samples. The relatively high occurence of ESBL in raw milk than raw meat in our study might be attributed to mastitic milk samples from the animals undergoing treatment.

In the present study, the frequency of ESBL producing *E. coli* (79.31) was highest as compared to other enterobacteria (*Citrobacter*, 13.79 % and *Klebsiella*, 6.89 %), which was similar to those reported by Tekiner *et al.,* (2015) where the most prevalent ESBL producer was *E. coli* (44 of 55), followed by six *Citrobacter* spp., five *Enterobacter* and 2 *Klebsiella*. Similar pattern of observations was reported by various co-workers from different parts of the world (Mesa *et al.,* 2006; Geser *et al.,* 2012 and Gondagon and Avci, 2013). The proportionate study revealed that approximately half of the *E. coli* (42.59%) isolates were ESBL producers while majority of the isolates of *Citrobacter* (94.12%) and *Klebsiella* (86.66%) were non ESBL producers. There are evidences reporting an increase in prevalence of ESBL-producing *E. coli* in foods (Duan *et al.,* 2006; Coque *et al.,* 2008, Hiroi *et al.,* 2012). ESBL-producing *E. coli* associated mortality is three-times higher than non ESBL producing *E. coli* (Melzera and Petersen, 2007).

Genotypic analysis in the present study, showed that the ESBL genes carrying isolates belonged to only 3 genera of family *Enterobacteriaceae* i.e. *Escherichia,*

Citrobacter and *Klebsiella*. These isolates carried *bla* genes alone as well as in combination. The maximum number of *E. coli* isolates harboured ESBL genes with predominance of bla_{CTX} gene (37.93%) followed by bla_{SHV} (6.89%) and bla_{TEM} (3.44%). Similarly, Le *et al.*, (2015) also reported that approximately 40% of the ESBL *E. coli* isolates belonged exclusively to the CTX-M group and only 3.5 % belonged to the TEM group. Whereas, Tekiner *et al.*, (2015) reported predominance of bla_{TEM} genes followed by bla_{CTX} and bla_{SHV} in *E. coli*. Some of *E. coli* isolates in the present study showed co-existence of bla_{CTX} and bla_{TEM} (17.24%), bla_{CTX} and bla_{SHV} (3.44%), bla_{SHV} and bla_{TEM} (6.89%) and only one isolate (3.44%) exhibited multiple genes. These findings were in accordance with the observations of Tekiner *et al.*, (2015). *Citrobacter* obtained in the present study also exhibited predominance of bla_{CTX} gene distributed either alone (3.44%) or in combination with bla_{SHV} (6.89%) and bla_{TEM} (3.44%). Likewise, Shahid *et al.*, (2009) also found majority of *Citrobacter* harbouring bla_{CTX-M} gene (67.5%) followed by bla_{TEM} (40%) and bla_{SHV} gene (25%).

On the contrary, Tekiner *et al.*, (2015) reported predominance of bla_{TEM} gene (7.3%) in *Citrobacter* isolates with co-existence of bla_{TEM} and bla_{SHV} genes in 5.5% isolates. The bla_{CTX} gene (6.89%) was also dominant in *Klebsiella* isolates obtained in the present study as none of the other gene was detected. Similar to our finding, previous workers have also reported the predominance of bla_{CTX} gene in *Klebsiella* isolated from different sources (Hiroi *et al.*, 2011; Tekiner *et al.*, 2015) (Table 4 and 5).

Table.1 Primers sequence used for identification of ESBL genes

Gene	Sequence (5'→3')	Product size	Reference
bla_{TEM}	F-ATGAGTATTCAACATTTCCG R-TTAATCAGTGAGGCACCTAT	851bp	Grimm *et al.*, 2004
bla_{CTX}	F-CGCTTTGCGATGTGCAG R-ACCGCGATATCGTTGGT	551bp	Paterson *et al.*, 2003
bla_{SHV}	F-GCAAAACGCCGGGTTATTC R-CGTTAGCGTTGCCAGTGCT	940bp	Grobner *et al.*, 2009

Table.2 PCR cycling conditions used for ESBL gene amplification

Parameters	bla_{TEM} (temp,time)	bla_{CTX-M} (temp,time)	bla_{SHV} (temp,time)
Initial denaturation	94°C, 5 min.	94°C, 5 min.	95°C, 5 min.
Number of cycle	35	35	35
Denaturation	94°C, 30 sec.	94°C, 30 sec.	94°C, 30 sec.
Annealing	50°C, 35 sec	55°C, 35 sec	58°C, 40 sec
Elongation	72°C, 40 sec.	72°C, 40 sec.	72°C, 45 sec.
Final extension	72°C, 5 min.	72°C, 5 min.	72°C, 5 min.
Final Hold	10°C, 5 min.	10°C, 5 min.	10°C, 5 min.

Table.3 Isolation rate of Enterobacteria in various animal products and their environment

Sources (n= Enterobacterial isolates number)	*Citrobacter*	*E. coli*	*Enterobacter*	*Klebsiella*	*Salmonella*	*Proteus*
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Raw Milk n=45	18(40)	10(22.22)	11(24.44)	4(8.88)	1(2.22)	1(2.22)
Milk Products n=50	29(58.00)	4(8.00)	8(16.00)	5(10.00)	2(4.00)	2(4.00)
Raw Meat n=25	12(48.00)	3(12.00)	5(20.00)	2(8.00)	2(8.00)	1(4.00)
Meat Products n=20	9(45.00)	4(20.00)	4(20.00)	2(10.00)	1(5.00)	Nil
Environment n=37	Nil	33(89.18)	2(5.40)	2(5.40)	Nil	Nil
Total=177	**68(38.41)**	**54(30.50)**	**30(16.94)**	**15(8.47)**	**6(3.38)**	**4(2.25)**

Table.4 Prevalence of ESBL Enterobacteria in animal foods and their environment

Source (n= no. of isolates)	Enterobacteria	ESBL positive isolates	
		Phenotypic test	Molecular test
Raw Milk n=46	45 (97.82%)	11(24.44%)	9 (2.0%)
Milk Products n=56	50 (89.28%)	4 (8.0%)	Nil
Raw Meat n=25	25 (100%)	4 (16.0%)	2 (8.0%)
Meat Products n=22	20 (90.90%)	3 (15.0%)	Nil
Environment n=37	37 (100%)	21(56.76%)	18 (48.65%)
Total (n=186)	177 (95.16%)	43 (24.29%)	29 (16.38%)

Table.5 Distribution of ESBL genes among Enterobacteria

Types of ESBL genes	ESBL Producers		
	E. coli No. (%)	*Citrobacter* No. (%)	*Klebsiella* No. (%)
CTX (n=14)	11 (37.93)	1 (3.44)	2(6.89)
SHV (n=02)	2 (6.89)	Nil	Nil
TEM (n=01)	1 (3.44)	Nil	Nil
CTX and TEM (n=06)	5 (17.24%)	1 (3.44)	Nil
CTX and SHV (n=03)	1 (3.44)	2 (6.89)	Nil
TEM and SHV (n=02)	2 (6.89)	Nil	Nil
CTX,TEM and SHV (n=01)	1 (3.44)	Nil	Nil
Total ESBL isolates= 29	**23(79.31)**	**4 (13.79)**	**2 (6.89)**

Figures

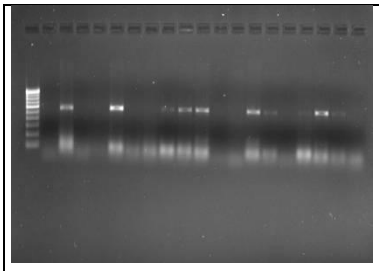

Fig 1: *bla*$_{CTX}$ gene (551 bp)

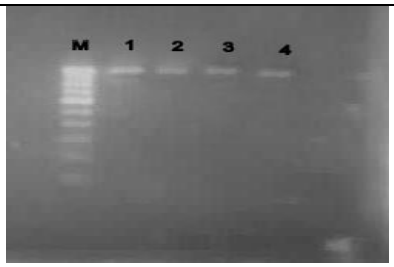

Fig 2:*bla*$_{SHV}$ gene (940 bp)

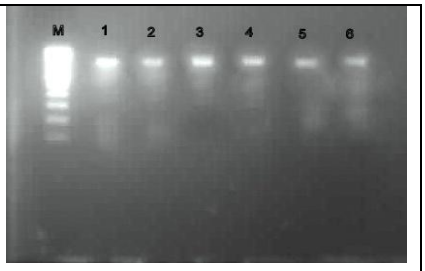

Fig 3: *bla*$_{TEM}$ gene (851bp)

In India, there are several reports suggesting large percentage of enterobacteria to be resistant to third generation cephalosporins with predominance of *bla*$_{CTX}$ gene (Shukla *et al.,* 2004; Grover *et al.,* 2006; Kumar *et al.,* 2006). This widespread occurrence of ESBL-producing *Enterobacteria* suggests that the community could act as a reservoir and that food could contribute to the spread of these strains. The present study reveals that ESBL-producing *E. coli, Citrobacter* and *Klebsiella* spp. can be transmitted by meat as well as milk. The increasing prevalence of resistance in the isolates from animal origin may have important therapeutic implications, therefore continuous monitoring of ESBL-producing enterobacteria is required at animals, human and environment interface.

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