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, while 100% 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 (8.0%); 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 blaCTX, blaSHV and blaTEM genes in E. coli isolates was 37.97%, 6.89% and 3.44%, respectively. The co-existence of blaCTX and blaTEM, blaSHV and blaTEM and blaCTX and blaSHV, was found 17.24%, 6.89% and 3.44% in E. coli isolates, respectively. Citrobacter isolates harboured single (blaCTX 3.44%) as well as multiple genes (blaCTX, blaSHV 3.44%) and (blaCTX,blaTEM 6.89%) while Klebsiella isolates showed only blaCTX gene (6.89%). Only one E. coli isolate (3.44%) in the present study harboured all three genes.

Keywords
ESBL, Enterobacteria, Milk, Meat, Environment

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 Methylen Blue Agar and Brilliant Green Agar media were used for isolation as well as differentiation of lactose fermenters and lactose non-fermentors belonging to Enterobacteriaceae. 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 ≤ 27 mm and for ceftazidime ≤ 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 ≥ 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 MgCl₂, 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, blaCTX gene (37.93%) was predominantly present followed by blashv (6.89%) and blatem (3.44%). The co-existence of blactx with blatem and blashv was recorded in 5(17.24%) isolates and 1(3.44%) isolate, respectively. The blashv and blatem 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 blactx, blachv and blatem, blactx and blashv, respectively. In ESBL positive Klebsiella isolates, only blactx 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 Karachi. 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 occurrence 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 Gundagon 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 blaCTX gene (37.93%) followed by blaSHV (6.89%) and blaTEM (3.44%). Similarly, Le et al., (2015) also reported that approximately 40% of the ESBL E. coli isolates harboured ESBL genes with predominance of blaCTX gene (37.93%) followed by blaSHV (6.89%) and blaTEM (3.44%). Likewise, Shahid et al., (2009) also found majority of Citrobacter harbouring blaCTX-M gene (67.5%) followed by blaTEM (40%) and blaSHV gene (25%).

On the contrary, Tekiner et al., (2015) reported predominance of blaTEM gene (7.3%) in Citrobacter isolates with co-existence of blaTEM and blaSHV genes in 5.5% isolates. The blaCTX 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 blaCTX 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 |
|------|------------------|--------------|-----------|
| blaTEM | F-ATGAGTATTCACATTTCGCCGAGCCACCTAT | 851bp | Grimm et al., 2004 |
| blaCTX | F-CGCTTTGCGATGTCAGGGGTTG | 551bp | Paterson et al., 2003 |
| blaSHV | F-GCAAAAACGGCCTGGATTACGCGTTCGTTGTGCT | 940bp | Grobner et al., 2009 |

Table.2 PCR cycling conditions used for ESBL gene amplification

| Parameters | blaTEM (temp,time) | blaCTX-M  (temp,time) | blaSHV  (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 |
|--------------------------------------------|-------------|---------|--------------|------------|------------|---------|
| 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 | 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)           |
In India, there are several reports suggesting large percentage of enterobacteria to be resistant to third generation cephalosporins with predominance of bla\textsubscript{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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