Prevalence and molecular characterization of extended-spectrum β-Lactamases (bla\textsubscript{TEM}) producing \textit{Escherichia coli} isolated from humans and foods of animal origin in Chhattisgarh, India

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ABSTRACT

The present study describes the prevalence of extended spectrum b-lactamases (ESBL) producing \textit{E. coli} in raw chevon, milk and human samples in different districts of Chhattisgarh state. A total of 330 samples comprising of chevon (n=126), raw milk (n=104), human urine and stool (n=100) were collected from Bilaspur, Durg, Raipur, Rajnandgaon and Dhamtari districts of Chhattisgarh and processed for isolation of \textit{E. coli}. The biochemically confirmed \textit{E. coli} isolates were further screened of for the presence of \textit{bla}\textsubscript{TEM} gene by PCR amplification. Analysis of samples indicated an overall prevalence of 31.52%. The highest prevalence of \textit{E. coli} was recorded in fresh chevon samples (38.09%) followed by human urine samples (37.14%), human stool samples (30%) followed by milk samples (20.19%). \textit{In –vitro} antibiotic sensitivity test of \textit{E. coli} isolates revealed that all isolates to be highly sensitive towards imipenem, gentamicin, ciprofloxacin, amoxyclylvin, ampicillin, oxytetracyclin. The highest numbers of \textit{E. coli} isolates were found resistant to erythromycin, cefotaxim, ceftazidine, cephalxin and cifixime. The 49 \textit{E. coli} isolates were found to have Multiple Antibiotic Resistance (MAR) index more than 0.2, thus indicating indiscriminate use of antimicrobials. The 44 (42.3%) isolates were identified as presumptive ESBL producers and out of them 39.4% isolates were found to harbour the \textit{bla}\textsubscript{TEM} gene on their plasmid DNA indicating the presence of multidrug resistant ESBL producing \textit{E. coli} in foods of animal origin and human samples.

Key words: \textit{bla}\textsubscript{TEM}, \textit{E. coli}, Extended-spectrum b-lactamases, Foods of animal origin Prevalence, Human, Molecular characterization.

INTRODUCTION

Infectious diarrhoea is one of the leading causes of mortality in humans and amongst the various pathogens, \textit{Escherichia coli} is mainly responsible for causing diarrhoea in India (Lanjewar et al., 2010). \textit{E. coli} is usually a commensal bacterium of humans and animals but its pathogenic variants cause intestinal and extraintestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis, and septicemia (Von Baum and Marre, 2005). The pathogenic strain of \textit{E. coli} O157:H7 causes high mortality with symptoms of bloody diarrhoea, abdominal cramps, nausea, fever, hemorrhagic colitis and hemolytic uraemic syndrome (Reuben et al., 2013). In developing countries, up to 70% cases of diarrhoeal disease are associated with consumption of contaminated food, especially the foods of animal origin. With an increase in consumption of foods of animal origin, the risk of foodborne diseases to humans also increases and remains a major public health problem in developing countries like India. \textit{E. coli} can contaminate the foods of animal origin viz. raw milk, meat and their products etc and contribute to human foodborne diseases (Gundogan and Yakar, 2007; Haryani et al., 2007).

Beside the high prevalence of \textit{E. coli} in foods of animal origin, antimicrobial resistance (AMR) in bacteria has emerged as a serious problem in both human and veterinary medicine worldwide. AMR among bacteria is mainly due to overuse or misuse of different antimicrobials in food animal production systems. Food borne diseases associated with resistant bacteria in humans are difficult to treat, resulting in longer hospitalization, higher morbidity and mortality rates, decreased productivity and increased costs.

One of the currently most important AMR mechanisms in \textit{Enterobacteriaceae} is production of extended spectrum b-lactamases (ESBL) enzymes. These enzymes mediate resistance to extended-spectrum (third generation) cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone) and monobactams (e.g., aztreonam) but do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g., meropenem or imipenem) (CDC, 2010). ESBLs are plasmid mediated and the genes encoding these enzymes are easily transferable among different groups of bacteria (Todar, 2012). They are not only detected in humans (Pitout et al., 2009), but also in a wide range of animal species.

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ranging from companion animals (Carattoli et al., 2005) to food animals (Horton et al., 2011). The emergence of ESBLs in the food-producing animals and in food products like meat, fish and raw milk have been reported from different parts of globe (Geson et al., 2012). The poor hygienic practices further enhances the possibility of transfer ESBL producer strains to the GIT of consumers (Overdevest et al., 2011).

The data on ESBL-producing bacteria in food animals in India are limited. Till date, no authentic reports are published regarding the association of ESBLs producing enteric bacteria in humans and foods of animal origin in the state of Chhattisgarh, India. Therefore, this study was conducted to estimate the prevalence of E. coli and their AMR pattern as well as to investigate the prevalence of ESBL-producing E. coli in raw chevon, milk and human samples collected from different districts of Chhattisgarh.

MATERIALS AND METHODS

Sample collection: A total of 330 samples comprising of chevon meat, raw milk, human urine and stool were collected by simple random technique during July 2013 to April 2014, from Bilaspur, Durg, Raipur, Rajnandgaon and Dhamtari districts of Chhattisgarh, India. The chevon meat samples (n=126) were aseptically collected from retail meat shops in sterile polythene bags and transported in refrigerated conditions to the laboratory as soon as possible and processed within 5 hrs. Milk samples (n=104) were aseptically collected from the dairies, vendors and hotels/restaurants using sterile milk sample bottles. All milk samples were stored at 4°C and were cultured within 5 hrs. Human urine and stool samples (n=100) were collected from hospitals, polyclinics, diagnostic centers situated in the study area. Human patients suffering from urinary tract infection were given dry test tube and requested to provide 10-20 ml urine sample. The first urine passed by the patient at the beginning of the day was collected for examination. The human stool samples were collected using sterilized dry absorbent cotton swab following the protocol of Cheesbrough (2006).

Isolation and biochemical characterization: For isolation, ten grams of raw chevon meat, 1 ml of raw milk, 1 gram of human stool and 1 ml of human urine samples were taken and inoculated in sterilized MacConkey’s lactose broth (HiMedia, India) and incubated at 37°C for 24 hrs. Thereafter, a loop-full culture was taken and streaked onto MacConkey’s Lactose Agar (MLA) (HiMedia, India) and incubated at 37°C for 24 hrs. The suspected E. coli colonies, pink to red in colour were picked up and further streaked onto Eosin methylene blue (EMB) (HiMedia, India) agar and incubated at 37°C for 24 hrs. The dark centred and flat colonies with metallic sheen were considered as E. coli and were further confirmed by using KMB001 HiMotility Biochemical kit for Escherichia coli (HiMedia, India). The eleven tests viz. Motility, Indole, Citrate utilization, Glucorinidase, Nitrate reduction, O-Nitrophenyl β-D-Galactopyranoside (ONPG), Lysin decarboxylase, Lactose, Glucose, Sucrose and Sorbitol were also performed.

Antimicrobial susceptibility test and Phenotypic detection of ESBL producers: All biochemically confirmed E. coli isolates were examined for their in vitro antimicrobial drug susceptibility pattern on Mueller-Hinton agar (HiMedia, India) in accordance with CLSI (2010) against ampicillin, amoxicillin/clavulanic acid, cefotaxim, ceftazidime, cephalaxin, cefixime, ciprofloxacin, erythromycin, gentamicin, imipenem, meropenem and oxytetracyclin (HiMedia, India). The diameter of the zones of complete inhibition was measured and compared with the zone size interpretation chart and were graded as sensitive, intermediate and resistant. The Multiple Antibiotic Resistance (MAR) Index was also calculated for all E. coli isolates, by applying formula a/b where “a” is the number of antibiotics to which an isolate was resistant and “b” is the number of antibiotics to which the isolates were exposed (Krumperman, 1983).

E. coli isolates showing resistance to the extended spectrum cephalosporin group of antibiotics were selected for confirmation of ESBLs production. The ceftazidime (30 µg) and Ceftazidime+Tazobactum (30/30 µg) (HiMedia, India) discs were placed on the inoculated MHA plate at a distance of 30 mm apart and incubated overnight. The bacterial isolates resistant to ceftazidime and sensitive to Ceftazidime +Tazobactum with diameter of more than 5 mm were considered as presumptive ESBL producers.

Molecular characterization of ESBL genotypes: All the presumptive ESBL producing E. coli isolates were screened for the presence of blaTEM gene by PCR amplification following the protocol described by Sharma et al. (2010) with some modifications. The plasmid DNA incorporated in PCR reactions was extracted from bacterial cells by using GeneJET Plasmid Miniprep Kit #K0502#, #K0503, Thermo Scientific (USA). Purity and concentration of DNA was detected by 0.8% agarose gel electrophoresis and stored at -20°C. Recommended primer set of a blaTEM forward primer (16bp): 5’-AAAAATTCTTGAAGACG-3’ and a blaTEM reverse primer (17 bp): 5’- TTACCAATGCTTAATCA-3’ were used to obtain a predicted product size of 1080 bp. The primers of blaTEM gene used in present study were obtained from Imperial Life Sciences (P) Limited, Gurgaon, Haryana, India. PCR reactions were performed in a total volume of 25 µl containing 10X PCR Buffer (Tris with 15 mM MgCl2), 100 mM of each deoxyribonucleotide triphosphate (dNTP), 1µM of each oligonucleotide primers, 1U Taq Polymerase and 3 µl of Template DNA. PCR amplification was done by using thermocycler (Mastercycler, Eppendorf, Germany) and cycles were performed with initial denaturation of 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for
2 min. Final extension was done at 72°C for 10 min. After the completion of reaction cycles, the amplified products were electrophoresed on 1.5 % agarose gel stained with ethidium bromide (0.5 mg/ml). The images of ethidium bromide stained DNA bands were analysed using UV transilluminator (Biometra) and digitized using a Gel Documentation System (Gel Doc™ XR, Biorad, USA). All the biologicals used during the present study were procured from Thermo Scientific (USA), Genetix (India) and Bangalore Genei (India).

RESULTS AND DISCUSSION

In the present study, a total of 104 isolates of *E. coli* were isolated with an overall prevalence of 31.52% (Table 1). The highest prevalence of *E. coli* was recorded in fresh chevon meat samples followed by human urine samples, human stool samples and milk samples. The prevalence of *E. coli* in chevon meat samples recorded was 38.09%, which is in agreement with earlier findings (Ali *et al.*, 2010). However higher prevalence rates of 57% (Amin and Borah, 2002), 76% (Gangil *et al.*, 2011) and 100% (Chaubey *et al.*, 2004) were reported by different investigators. In present study, 20.19% milk samples were found positive for *E. coli*, however higher prevalence rates of 33.96% (Rashid *et al.*, 2013), 38% (Thaker *et al.*, 2012) and 43.3% (Rasheed *et al.*, 2014) were reported from different regions. On the contrary lower prevalence rate of 15.5% (Gundogan and Avci, 2013) was recorded in raw milk samples in previous reports. The 37.14% of human urine samples during the study were found positive for *E. coli*, which is in conformity with earlier reports (Shafiyabi *et al.*, 2014). However, higher prevalence rates of 42.9% (Khater and Sherif, 2014), 54.6% (Manikandan and Amsath, 2014) and 61% (Akram *et al.*, 2007) were reported by other researchers. During the study, 30% of human stool samples were found positive for *E. coli*, which is in agreement with earlier reports (Kumar *et al.*, 2014).

In present study, all *E. coli* isolates recovered from chevon, milk and human samples were found highly sensitive towards Imipenem (79.80%) followed by Gentamicin (75.96%), Ciprofloxacin (58.65%), Amoxycyclav (52.88%), Ampicillin (51.92%), Oxytetracyclin (51.92%), Meropenem (49.03%), Cifixime (48.07%), Cephalexin (42.30%), Cefotaxim (30.76%), Ceftazidime (30.76%) and lowest sensitivity towards Erythromycin (24.03%) (Table 2). The highest numbers of *E. coli* isolates were found resistant to Erythromycin, Ampicillin and Cefotaxim (41.34%), followed by Ceftazidime (40.38%), Cephalexin, Ciprofloxacin, Meropenem (39.42%), Oxytetracyclin (37.50%), Amoxycyclav (33.65%) with minimum resistant to Imipenem (2.88%) (Table 2). Results of present study are in agreement with earlier findings (Ibrahim *et al.*, 2012, Manoharan *et al.*, 2011). Imipenem is an antibiotic which is recently introduced and possibly resistance against it is not

<table>
<thead>
<tr>
<th>Samples</th>
<th>Source of samples</th>
<th>No. of samples analyzed</th>
<th>No. of samples positive for <em>E. coli</em> (% Prevalence)</th>
<th>No. of samples positive for <em>E. coli</em> and blaTEM gene on Plasmid DNA</th>
<th>No. of samples positive for ESBL presumptive producers</th>
<th>blaTEM gene on producers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chevon meat</td>
<td>Local markets</td>
<td>126</td>
<td>8 (16.67%)</td>
<td>7 (14.58%)</td>
<td>8 (16.67%)</td>
<td>7 (14.58%)</td>
</tr>
<tr>
<td>Milk</td>
<td>Vendor, Dairies, Restaurants</td>
<td>104</td>
<td>21 (20.19%)</td>
<td>21 (20.19%)</td>
<td>21 (20.19%)</td>
<td>21 (20.19%)</td>
</tr>
<tr>
<td>Human Urine</td>
<td>Hospitals, Polyclinics, Diagnostic centers</td>
<td>70</td>
<td>26 (37.14%)</td>
<td>26 (37.14%)</td>
<td>26 (37.14%)</td>
<td>26 (37.14%)</td>
</tr>
<tr>
<td>Human stool</td>
<td>Hospitals, Polyclinics, Diagnostic centers</td>
<td>30</td>
<td>9 (30.00%)</td>
<td>9 (30.00%)</td>
<td>9 (30.00%)</td>
<td>9 (30.00%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>330</td>
<td>104 (31.52%)</td>
<td>104 (31.52%)</td>
<td>104 (31.52%)</td>
<td>104 (31.52%)</td>
</tr>
</tbody>
</table>
developed among *E. coli* isolates. Several other researchers have also reported similar resistance/sensitivity patterns of *E. coli* isolates against these antibiotics (Sharma et al., 2010, Thaker et al., 2012). Among the *E. coli* isolates, the highest value of MAR index reported was 1 for one isolate and the lowest MAR index reported was 0.16 for four isolates, two each from meat and milk. Out of 104 *E. coli* isolates, 49 were found to have MAR index more than 0.2, thus indicating indiscriminate use of antimicrobials. The findings of present study are comparable with reports of Jaulkar et al. (2011).

Out of the 104 *E. coli* isolates, 44 isolates (42.3%) were identified as presumptive ESBL producers and examined for the presence of *bla*<sub>TEM</sub> gene by PCR. The 41 (39.4%) isolates were found to harboured the *bla*<sub>TEM</sub> gene (1080 bp) on their plasmid DNA (Fig. 1, Table 1). The highest prevalence of *bla*<sub>TEM</sub> gene was recorded in human urine samples (80.76%) followed by human stool samples (77.78%), milk samples (28.57%) and chevon meat samples (14.58%) (Table 1).

The relatively recent emergence of *E. coli* as a food borne pathogen has a significant impact on the food quality. This possesses a number of undesirable characteristics that combine to make it one of the most serious threats to food safety in recent years. *E. coli* in milk can enter through teat canal, infected udder, milker, extraneous dirt or untreated water. Raw chevon available in open air at local retail shops without appropriate temperature control and inadequate sanitary conditions favor the contamination by *E. coli* and other organisms. Antimicrobial drugs have played an indispensable role in decreasing illness and death associated with infectious diseases in animals and humans. However, selective pressure exerted by use of antimicrobial drugs seems to be major reason for the emergence and spread of drug-resistance traits among pathogenic and commensal bacteria. Thus suggests that antibiotic sensitivity testing should be carried out regularly in routine practice. It is important to emphasize the spread of *bla*<sub>TEM</sub> gene among nonpathogenic *E. coli* isolates recovered from foods or from the intestinal environment of human and healthy animals. Therefore, study in depth should be carried out about the distribution and evolution of *bla* genes in isolates from different ecosystems.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxyclav</td>
<td>35 (33.65%)</td>
<td>14 (13.46%)</td>
<td>55 (52.88%)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>43 (41.34%)</td>
<td>07 (06.73%)</td>
<td>54 (51.92%)</td>
</tr>
<tr>
<td>Cefotaxim</td>
<td>43 (41.34%)</td>
<td>29 (27.88%)</td>
<td>32 (30.76%)</td>
</tr>
<tr>
<td>Ceftazidine</td>
<td>42 (40.38%)</td>
<td>30 (28.84%)</td>
<td>32 (30.76%)</td>
</tr>
<tr>
<td>Cephalaxin</td>
<td>41 (39.42%)</td>
<td>19 (18.26%)</td>
<td>44 (42.30%)</td>
</tr>
<tr>
<td>Cifixime</td>
<td>41 (39.42%)</td>
<td>13 (12.50%)</td>
<td>50 (48.07%)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>41 (39.42%)</td>
<td>02 (01.92%)</td>
<td>61 (58.65%)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>43 (41.34%)</td>
<td>36 (34.41%)</td>
<td>25 (24.03%)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>13 (12.50%)</td>
<td>12 (11.53%)</td>
<td>79 (75.96%)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>03 (02.88%)</td>
<td>18 (17.30%)</td>
<td>83 (79.80%)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>41 (39.42%)</td>
<td>12 (11.53%)</td>
<td>51 (49.03%)</td>
</tr>
<tr>
<td>Oxytetracyclin</td>
<td>39 (37.50%)</td>
<td>11 (10.57%)</td>
<td>54 (51.92%)</td>
</tr>
</tbody>
</table>

Table 2: Antibiotic sensitivity pattern of *E. coli* isolates

The findings of present study are comparable with reports of Jaulkar et al. (2011).

The relatively recent emergence of *E. coli* as a food borne pathogen has a significant impact on the food quality. This possesses a number of undesirable characteristics that combine to make it one of the most serious threats to food safety in recent years. *E. coli* in milk can enter through teat canal, infected udder, milker, extraneous dirt or untreated water. Raw chevon available in open air at local retail shops without appropriate temperature control and inadequate sanitary conditions favor the contamination by *E. coli* and other organisms. Antimicrobial drugs have played an indispensable role in decreasing illness and death associated with infectious diseases in animals and humans. However, selective pressure exerted by use of antimicrobial drugs seems to be major reason for the emergence and spread of drug-resistance traits among pathogenic and commensal bacteria. Thus suggests that antibiotic sensitivity testing should be carried out regularly in routine practice. It is important to emphasize the spread of *bla*<sub>TEM</sub> gene among nonpathogenic *E. coli* isolates recovered from foods or from the intestinal environment of human and healthy animals. Therefore, study in depth should be carried out about the distribution and evolution of *bla* genes in isolates from different ecosystems.

Fig 1: Agarose Gel Electrophoresis showing amplified PCR product of *bla*<sub>TEM</sub> gene

Lane M: 100bp DNA Ladder
Lane 3, 4, 7: coli isolates with *bla*<sub>TEM</sub> positive amplicons (1080 bp)
Lane 1, 2, 5, 6: E coli isolates with no amplicons
REFERENCES


