

Extended Spectrum Beta Lactamases and Class-I Integrons Producing *Escherichia coli* in Pigs of North Eastern States of India

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ABSTRACT

The aim of this study was to determine the prevalence of extended spectrum beta lactamases (ESBLs) associated genes and integron elements in *Escherichia coli* from faeces of pigs in North eastern (NE) states of India. A total of 790 faecal samples were collected from pigs maintained under organized as well as individual house hold irrespective of age, sex and with or without history of diarrhea from all the eight states of NE region. A total of 2291 *E. coli* were isolated and identified. All the isolates were subjected to antimicrobial susceptibility test against 18 antimicrobial agents by disk diffusion method. The selected ESBLs genes (*bla_{SHV}*, *bla_{TEM}*, *bla_{CMY}* and *bla_{CTX-M}*) and integron (*IntI1* and *IntI2*) genes were detected by specific PCR assay. A total of 366 (15.98%) and 80 (3.49%) isolates were positive for *IntI1* and ESBLs, respectively. Twenty four (1.05%) isolates positive for *IntI1* were carrying multiple ESBLs genes, and individually 0.17%, 1.92%, 0.17% and 0.17% isolates were positive for *bla_{SHV}*, *bla_{TEM}*, *bla_{CMY}* and *bla_{CTX-M}* genes, respectively. Class 2 integrons (*IntI2*) were not detected in any of the *E. coli* isolates under the study. It may be concluded that *E. coli* isolates with multiple ESBL genotypes have a greater opportunity to carry Class 1 integron and can be a potential to exhibit stronger multi-drug resistance activity.

Keywords

E. coli, ESBLs, Integrons, Pigs, India

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Introduction

Antimicrobial resistance (AMR) is a potential threat to human and animal health. Antimicrobial agents are extensively used in livestock due to increasing demand for animal protein. Inappropriate use of antibiotics appears to be the major cause of increase in AMR bacteria. In *Enterobacteriaceae* family,

Escherichia coli are the most common commensal bacteria in the gastrointestinal tract of humans and animals (Chen *et al.*, 2019). *E. coli* have a considerable potential of accepting and transferring plasmids, which under stress readily transfers it to other species. Therefore, it is considered an important reservoir of transferable antibiotic resistance (Chamosa *et al.*, 2017;

Leungtonkam *et al.*, 2018). The distribution of broad-spectrum beta-lactams resistant enterobacterial strains along with co-resistance to other antibiotic families are emerging as a potential threat to animal and public health (EFSA, 2013). These resistance genes are greatly enhanced, when they are trapped in a mobile gene cassette, the so called integron (White *et al.*, 2001). Integrons are conserved DNA sequences that provide an efficient means for capturing and spreading of antimicrobial resistance genes (Peymani *et al.*, 2012).

The component of an integron includes integrase gene (*IntI*), attachment site (*AttI*), and promoter (Pant) region, which promotes the expression of any suitably integrated gene(s). Integrase is a member of the tyrosine site specific recombinase family that catalyzes the excision and integration of DNA units by performing two consecutive strand breakages and rejoining steps (Ahangarzadeh *et al.*, 2011).

Four classes of integrons so far identified are distinguished by their respective integrase (*Int*) genes. Most of the resistance integrons found in clinical isolates of *Enterobacteriaceae* are class 1 integrons, which are highly associated with widespread incidence and spread of antibiotic resistance to antimicrobial agents (Ghaly, *et al.*, 2017). Integrons are of clinical importance, because the use of only one antibiotic may activate the expression of a whole gene cassette. There is paucity of information so far on detection of integrons in multi drug resistant (MDR) isolates of *E. coli* from pigs, particularly in India. Therefore, the present study was aimed to determine the prevalence of *ESBL* genes and the frequency of class 1 and 2 integrons in *E. coli* isolated from pigs in the NE states and also to investigate the association between *ESBL* genes and existence of integrons.

Materials and Methods

Isolation and Identification of *E. coli*

A total of 790 fresh faecal samples were collected randomly from pigs of all the eight NE states maintained under organized as well as individual house hold. Samples were collected irrespective of age, sex and history of diarrhea of the animals. All the samples were collected using sterilized absorbent cotton swab under aseptic conditions. However, for collection of samples from distant locations, a sterilized swab dipped in brain heart infusion broth (HiMedia, Mumbai) was used as transport medium and transported to the laboratory under cold chain for further processing. The organisms were isolated and identified as per standard bacteriological techniques including cultural characteristics and biochemical tests (Quinn *et al.*, 2004).

Antimicrobial susceptibility assay

All the isolates were subjected to antimicrobial susceptibility assay by disc diffusion method on Mueller-Hinton agar (HiMedia, Mumbai) plate as per the recommendation of Clinical Laboratory Standard Institute (CLSI, 2018) against 18 commercially available antibiotic discs: amoxicillin (AMX, 30 mcg), ampicillin (AMP, 10 mcg), aztreonam (Az, 30 mcg), cefalexin (CN, 30 mcg), cefexime (CFM, 30 mcg), cefotaxime (CTX, 30 mcg), ceftazidime (CAZ, 30 mcg), ceftriaxone (CTR, 30 mcg), ciprofloxacin (CIP, 5 mcg), co-trimoxazole (COT, 1.25/23.75 mcg), gentamicin (GEN, 10 mcg), imipenem (IPM, 10 mcg), nalidixic acid (NA, 30 mcg), piperacillin (PI, 100 mcg), streptomycin (S, 10 mcg), sulphafurazole/sulfisoxazole (SF, 300 mcg), tetracycline (TE, 30 mcg) and trimethoprim (TR, 30 mcg). Further, the isolates exhibiting resistance to the extended-spectrum cephalosporin group of antibiotics were screened for *ESBL*

production using a double disk synergy test (DDST) for cefotaxime (30 mcg), amoxicillin (30 mcg) and ceftazidime (30 mcg) alone as well as cefotaxime/clavulanate (30/10 mcg), amoxicillin/clavulanate (30/10 mcg) and ceftazidime/clavulanate (30/10 mcg) combination as per the recommendation of CLSI (2018). Difference in zone diameters with and without clavulanic acid was measured. *E. coli* ATCC 25922 was used as control organisms. An increase of ≥ 5 mm in inhibition zone diameter around antimicrobial agent tested in combination with clavulanic acid versus its inhibition diameter zone tested alone was confirmed as potent ESBLs producing isolates.

Detection of ESBL genes by PCR

Presence of selected ESBLs (*bla_{SHV}*, *bla_{TEM}*, *bla_{CMY}* and *bla_{CTX-M}*) genes were detected by PCR assay using specific primers (Table-1). Bacterial DNA was prepared from all the isolates, which were positive for ESBLs production phenotypically as described earlier (Dutta *et al.*, 2013). PCR was carried out in a thermal cycler (Eppendorf, Germany) and visualized under UV transilluminator followed by documentation using Gel documentation system (Alpha Imager, USA) as described elsewhere (Dutta *et al.*, 2013). All the PCR products were purified and subsequently sequenced by Sanger's method at University of Delhi, South Campus, Department of Biochemistry, Benito Juarez Road, New Delhi-110021. The DNA sequences were analysed for genetic relatedness with published sequences and submitted to Genbank, NCBI.

Detection of class 1 and 2 integrons by PCR

All the isolates positive for *ESBL* genes were further screened for the presence of class 1 (*IntI1*) and 2 (*IntI2*) integrons as well as its gene cassettes 5CS/3CS and TiB/TiF by PCR

assay using specific primers (Table-1). Further, the amplification of variable region of class 1 and class 2 integrons were performed using the primers 5'-CS/3'-CS and Ti-F/Ti-B, as per the procedures described previously (Zhang *et al.*, 2004).

Results and Discussion

Multi Drug Resistant (MDR) bacteria, particularly the enteric bacteria including *E. coli* are becoming a great threat globally. In India, although sporadic reports of MDR bacteria in animals are available but there is very little information available on association of class I integrons and *ESBLs* genes in *E. coli* of animal origin. The present study was focused to investigate the prevalence of Class I integrons and *ESBLs* producing *E. coli* isolates from pigs of NE states of India with the broad objective to improve the practice of antimicrobials use in clinical practice, farm biosecurity, epidemiological studies and also safeguarding against the zoonotic outbreaks by MDR bacteria in human and animal population. A total of 2291 bacterial isolates recovered from 790 faecal samples were identified as *E. coli* on the basis of standard cultural characteristics and biochemical tests. All the isolates exhibited small, bright pink colonies on MacConkey's (MLA) agar and a characteristic metallic sheen on eosin methylene blue (EMB) agar medium. Biochemically, all isolates were positive for indole and methyl red tests and negative for oxidase, Voges-Proskauer and citrate utilization tests. Also, all the isolates fermented glucose, sucrose and lactose with production of gas. The antimicrobial resistance pattern of *E. coli* isolated is depicted in Table-2. All the isolates showed resistance to at least 3 antimicrobial agents with highest resistance to amoxycillin (84.81%) and lowest resistance to imipenem (0.22%). Further, on screening by DDST

method, a total of 654 (28.55%) isolates were suspected for ESBL producers of which 136 (5.94%), 65 (2.84%), 49 (2.14%) and 23 (1.00%) were found to be positive for *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{CMY} and *bla*_{SHV} gene respectively in specific PCR assay. With the present data it may not be possible to conclude with a statement on the prevalence of ESBL genes in the *E. coli* isolates from pigs in this region. Based on the published evidences, we have targeted only 4 major ESBL genes out of estimated genes of more than 500 for detection, which are associated with resistance against beta lactam antibiotics applied for treatment in men and animals. Previously, several workers from India and abroad have also reported the prevalence of ESBLs producers varying from 6.6% to 91% from time to time (Jain *et al.*, 2003; Wattal *et al.*, 2005; Bhattacharjee *et al.*, 2008; Basavaraj *et al.*, 2011). In India, Basavaraj *et al.*, (2011) reported 27.9% *Enterobacteriaceae* organism as ESBLs producer by DDST, in which *E. coli* and *K.*

pneumoniae were the major ESBLs producers. Interestingly, in the present study imipenem underperformed against *E. coli* isolates. Earlier, Patricia *et al.*, (2010) and Aly *et al.*, (2012) reported no resistance against imipenem.

The class 1 and 2 integrons gene in ESBL-producing *E. coli* isolates from NE states of India was screened by PCR assay. We found, a total of 366 (15.98%) *E. coli* isolates were positive for Class1 integrons. However, Class 2 integrons (*IntI2*) were not detected in any of the isolates. Prevalence of class 1 integron gene in ESBL-producing *E. coli* isolated from NE states of India is depicted in Table 3. Altogether 80 (3.49%) of *E. coli* isolates were positive for both ESBLs genes and class 1 integrons. Previously, it was reported that class 1 integrons are the most common antibiotic resistant genes found in the clinical isolates of Gram-negative bacteria (Betteridge *et al.*, 2011; Ribeiro *et al.*, 2011).

Table.1 Details of the oligonucleotide Primers used in the present study

Primer name	Sequence (5'→3')	Expected amplicon size (bp)	Annealing temperature (°C)	Reference
<i>bla</i> _{TEM}	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	1080	53	Weill Francois-Xavier <i>et al.</i> , (2004)
<i>bla</i> _{SHV}	CTTTCCCATGATGAGCACCT CGCTGTTATCGCTCATGGTA	206	60	This study
<i>bla</i> _{CTX-M}	CAATGTGCAGCACCAGTAA CGCGATATCGTTGGTGGTG	540	58	Perez and Hanson,2002
<i>bla</i> _{CMY}	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	60	Perez and Hanson,2002
<i>IntI1</i>	GGGTCAAGGATCTGGATTTTCG ACATGGGTGTAAATCATCGTC	483	60	Mazel <i>et al.</i> , (2000)
<i>IntI2</i>	CACGGATATGCGACAAAAGGT GTAGCAAACGAGTGACGAAATG	788	60	Mazel <i>et al.</i> , (2000)
5'-CS 3'-CS	GGCATAACAAGCAGCAAGC AAGCAGACTTGACCTGAT	variable	52	Zhang <i>et al.</i> , (2004)
Ti-F Ti-B	ACCTTTTTGTGCGCATATCCGTG CTAACGCTTGAGTTAAGCC	variable	55	Su <i>et al.</i> , (2006)

Table.2 Antimicrobial resistance pattern of *E. coli* isolated from faecal samples of pig of NE states of India

Antimicrobial agents	No. of isolates			
	S	%	R	%
Amoxicillin (AMX)	348	15.19	1943	84.81
Ampicillin (AMP)	1686	73.91	595	26.09
Aztreonam (AT)	1862	81.63	419	18.37
Cefalexin(CN)	523	22.83	1768	77.17
Cefexime (CFM)	1474	64.34	817	35.66
Cefotaxime (CTX)	2102	91.75	189	8.25
Ceftazidime (CAZ)	1767	77.13	524	22.87
Ceftriaxone(CTR)	2119	92.49	172	7.51
Ciprofloxacin (CIP)	2151	93.89	140	6.11
Co-Trimoxazole(COT)	1651	72.06	640	27.94
Gentamicin (GEN)	1940	83.73	377	16.27
Imipenem (IPM)	2286	99.78	5	0.22
Nalidixic acid (NA)	1791	78.18	500	21.82
Piperacillin (PI)	1228	53.60	1063	46.40
Streptomycin (S)	2082	90.88	209	9.12
Sulphafurazole (sulfoxazole)(SF)	990	43.21	1301	56.79
Tetracycline (TE)	1415	61.71	878	38.29
Trimethoprim(TR)	1688	73.68	603	26.32

*S- sensitive, R-resistant

Table.3 Prevalence of class 1 integron gene in ESBL-producing *E. coli* isolated from NE states of India

Sl. No.	ESBL gene(s)	No. of class1 integron positive strains
1.	<i>bla</i> _{TEM}	44 (1.92%)
2.	<i>bla</i> _{SHV}	4 (0.17%)
3.	<i>bla</i> _{CTX-M}	4 (0.17%)
4.	<i>bla</i> _{CMY}	4 (0.17%)
5.	<i>bla</i> _{TEM} + <i>bla</i> _{CMY}	7 (0.30%)
6.	<i>bla</i> _{TEM} + <i>bla</i> _{CTX-M}	12 (0.52%)
7.	<i>bla</i> _{TEM} + <i>bla</i> _{CTX-M} + <i>bla</i> _{CMY}	3 (0.13%)
8.	<i>bla</i> _{TEM} + <i>bla</i> _{CMY} + <i>bla</i> _{SHV}	1(0.04%)
9.	<i>bla</i> _{TEM} + <i>bla</i> _{CTX-M} + <i>bla</i> _{SHV}	1(0.04%)
Total numbers of <i>E. coli</i> isolates = 2291		80 (3.49%)

The class1 integron was observed in 43% of the strains isolated from animals and humans,

while the class 2 integrons was observed in only 1% (van Essen-Zandbergen *et al.*, 2007).

In another study conducted by Zeeshan Khan *et al.*, (2018), 79% of MDR *E. coli* isolates was recorded with class 1 integrons. Integrons have been identified as a primary source of resistance genes and are claimed to be reservoirs of antimicrobial resistance genes within microbial populations (Nijssen *et al.*, 2005). As far as pig is concerned, Gebreyes and Thakur (2005) reported that of the 28 isolates, 21 were multidrug resistant and all of them harboured the class 1 integron. However, Martin *et al.*, (2008) could detect both class 1 and class 2 integrons in nearly similar proportions in *Salmonella* spp. isolated from healthy swine from 126 different farms of Chile. Integron gene sequences contribute to the spread of antimicrobial resistance alleles by lateral gene transfer of gene cassettes in a variety of enteric bacteria including *Campylobacter* spp., *Escherichia coli* and *Salmonella enterica* subsp. *enterica* serotype Typhimurium (Roe *et al.*, 2003). As indicated above, in the present study, class 1 integron was detected in 15.98% of *E. coli* isolates of swine, which was comparatively lower than the reports by other workers (Phongpaichit *et al.*, 2011; Pongpech *et al.*, 2008). This may be an indication that there is comparatively less selection pressure on integron-positive *E. coli* isolates in NE states of India. Various workers in different countries also mentioned that the accumulation of resistance genes by integrons is an important factor in the development of multi-drug-resistant *E. coli* strains. Phongpaichit *et al.*, (2011) reported that 74.7% of ESBL-producing *E. coli* was integron-positive isolates. Similarly, Chen *et al.*, (2013) also found that 69% of clinical ESBL-producing isolates were carrying class 1 integron.

Analysis on correlation between integrons and ESBL genes (Table 3) indicated that 24 [$bla_{TEM+CMY}$ (12), $bla_{TEM} + bla_{CTX-M} + bla_{CMY}$ (3), $bla_{TEM} + bla_{CMY} + bla_{SHV}(1)$, $bla_{TEM} + bla_{CTX-M}$

$+$ $bla_{SHV}(1)$] isolates were positive for multiple ESBL genes and class 1 integron. At the same time, individually, 1.92%, 0.17%, 0.17% and 0.17% of the isolates positive for class 1 integrons were also positive for bla_{TEM} , bla_{CTX-M} , bla_{CMY} and bla_{SHV} genes, respectively. Our result indicated that class 1 integron were more commonly associated with the bla_{TEM} gene than with the other three genes, suggesting that in ESBL-producing isolates, bla_{TEM} carriers were more closely related to class 1 integron, which may be due to genetic linkage between them. Chen *et al.*, (2013) also reported that class 1 integron was more commonly associated with the bla_{TEM} gene than bla_{CTX-M} , bla_{CMY} or bla_{SHV} genes. Association between antibiotic resistance integrons and bla_{SHV-5} as well as co-location of bla_{SHV-12} and a class 1 integron on the same plasmid have been reported by Jones *et al.*, (2005) and Gruteke *et al.*, (2003). However, other investigators reported a low rate of association between integrons and ESBL genes with the exception of $bla_{CTX-M-9}$ (Machado *et al.*, 2007). In this study, the rates of combination of at least two different ESBL genotypes along with class 1 integron were variable, in which the combination of $bla_{TEM} + bla_{CTX-M}$ (0.79%) was highest.

The present study demonstrated the *E. coli* isolates from pigs of NE states are a major carrier of class I integrons and ESBL genes. In addition, multiple ESBL genotypes have a greater opportunity to carry class 1 integron. Therefore, bacteria carrying both integrons and ESBL genes have stronger MDR potential.

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