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ORIGINAL ARTICLE

PCR-Based Detection of Extended-Spectrum β-Lactamases ($bla_{CTX-M-1}$ and bla_{TEM}) in *Escherichia coli*, *Salmonella* spp. and *Klebsiella pneumoniae* Isolated from Pigs in North Eastern India (Mizoram)

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Abstract Cephalosporins are major antimicrobials used to treat serious infections. However, their effectiveness is being compromised by the emergence of extended-spectrum β-lactamases (ESBLs). A total of 138 enteric bacteria were isolated from 53 faecal samples of pigs collected from different districts of Mizoram, of which 102 (73.91 %) were *Escherichia coli*, 26 (18.84 %) were *Salmonella* spp. and 10 (7.25 %) were *Klebsiella pneumoniae*. Phenotypic confirmatory test (Double Discs Synergy Test) showed that 8 (5.80 %) *E. coli* isolates were ESBLs producer. PCR analysis confirmed that out of the eight isolate, 7 (5.07 %) harboured $bla_{CTX-M-1}$ gene and/or bla_{TEM} gene. Of the eight positive isolates, 7 (5.07 %) and 3 (2.17 %) were found to be positive for $bla_{CTX-M-1}$ gene and bla_{TEM} gene, respectively, of which 3 (2.17 %) isolates were positive for both the genes. Only 4 (2.90 %) *E. coli* isolates carried $bla_{CTX-M-1}$ gene alone. Agarose gel electrophoresis showed that all the isolates were carrying plasmids ranging between 0.9 and ~30 kb. Out of the seven isolates positive for $bla_{CTX-M-1}$ and/or bla_{TEM}, 2 (1.84 %) isolates were confirmed for $bla_{CTX-M-1}$ gene in their plasmid. Only one *E. coli* isolate was found to be positive for both the genes in its plasmid. The resistance plasmid could not be transferred to a recipient by in vitro horizontal gene transfer method.

Keywords ESBLs · $Bla_{CTX-M-1}$ · Bla_{TEM} · Pigs · North East India

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Introduction

The rapid emergence of extended-spectrum β-lactamases (ESBLs) in the food producing animals has been recorded and published worldwide [1–5]. It is important to note its impact on the treatment and therapeutic strategy of serious infections [6–10]. Food animals, including pigs are one of the most important sources of development of multi-drug resistant (MDR) bacteria because of continuous use of antibiotics as feed additives and growth promoting factors in a sub-therapeutic level [11–13]. This practice may lead to selection of a resistant population in the native microbiota of the animal and the local environment due to shedding through faeces. The MDR bacteria may re-enter the human and animal populations through various routes including natural water, irrigation water, drinking water, vegetables and foods. The present study is carried out to record the prevalence of ESBLs producing *Escherichia coli*, *Salmonella* spp. and *Klebsiella pneumoniae* in pig population in North Eastern India.

Materials and Methods

Bacterial Isolates

A total of 53 faecal samples from pigs were collected from different districts of Mizoram between September 2011 and March 2012. Samples were collected from animal of either sex, irrespective of their age, sex or breed. Animals under the study were reared under different housing system and belonged to organized as well as unorganized farms. Samples were collected by dry rectal swabbing. However, for collection of samples from distant locations, a sterilized swab dipped in nutrient broth was used as transport

medium. Each swab containing the collected sample was then inserted into separate sterilized test tube and carried to the laboratory under cold chain for further processing.

For isolation of *E. coli* and *K. pneumoniae*, the collected faecal samples were inoculated on MLA and single colonies were selected and confirmed by standard bacteriological technique. For isolation of *Salmonellae*, samples were first enriched in Selenite F broth, and streaked on *Salmonella Shigella* agar plate. Pure colonies were then selected and identified as per standard bacteriological technique [14].

Phenotypic Detection of ESBLs

All the isolates were subjected to in vitro antibiotic sensitivity test by disc diffusion method against commonly used antibiotics as per the recommendation of Clinical Laboratory Standard Institute [15]. The antibiotics used for the study were ceftriaxone, cephotaxime, cefixime, cefazolin, cephalexin, ampicillin, erythromycin, chlortetracycline, streptomycin, enrofloxacin, oxytetracycline and lincomycin. The isolates exhibiting resistance to the extended-spectrum cephalosporin group of antibiotics were selected for confirmation of ESBLs production by placing cefotaxime and cefotaxime/clavulanate discs on the inoculated Mueller–Hinton agar plate at a distance of 30 cm apart. It was incubated overnight and the increase in zone size of more than 5 mm was considered as positive for ESBLs production.

Characterization of ESBLs Producing Isolates

Bacterial lysate was prepared from all the isolates found to be positive for ESBLs production phenotypically, and were tested for the presence of $bla_{CTX\text{-}M\text{-}1}$ and bla_{TEM} genes by PCR assay using specific primers (Table 1). PCR was carried out in a 0.2 ml thin wall PCR tubes using the bacterial lysate as template DNA with a final volume of 25 µl containing 10× buffer, 1.5 mM $MgCl_2$, 200 pM of each oligonucleotide primers, 200 µM of each dNTPS, 1 U of *Taq* polymerase and 4.0 µl DNA lysate. PCR was carried out in a thermal cycler and the cycling condition for $bla_{CTX\text{-}M\text{-}1}$ was: initial denaturation at 94 °C for 7 min followed by 30 cycles of amplification with denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s, ending with a final extension at 72 °C for 5 min. For bla_{TEM} gene, the annealing temperature was 53 °C.

Multiplex PCR was carried out using the same composition of PCR reaction mixture mentioned above. However, the annealing temperature was set to 54 °C.

Extraction of Plasmid and Genomic DNA

Plasmid DNA was extracted as per the method described by Sambrook and Russel [16] and the chromosomal DNA was extracted as per the method of Nazik et al. [17] from the isolates harbouring the ESBLs genes. PCR was performed using the Plasmid and chromosomal DNA separately following the above mentioned settings to find out the location of the target genes.

Curing of Plasmid

All the isolates, carrying $bla_{CTX\text{-}M\text{-}1}$ and/or bla_{TEM} genes in their plasmid were subjected to curing using acridine orange as curing agent following the method described by Silhavy et al. [18] with suitable modifications. In brief, 0.2 ml of overnight culture was inoculated in 5 ml LB broth containing different concentrations (2.5, 1.25, 1.0, 0.7, 0.5, 0.25 and 0.1 mg/ml) of acridine orange. Positive control contained only cells without acridine orange, while negative control contained only acridine orange without cells. All the tubes were incubated (in dark) at 37 °C for overnight. Next day tubes containing the highest concentration of acridine orange showing growth were selected and loopful was streaked on Mac Conkey's agar plates and incubated overnight.

Horizontal Gene Transfer

The ability of transfer of antibiotic resistance genes within *Enterobacteriaceae* group of bacteria was recorded by in vitro conjugation study. *E. coli* isolates harboring the ESBLs gene were used as donor and *Salmonella enteritidis* (ATCC 13076), which was made resistant to nalidixic acid was used as recipient strain. The recipient strain was sensitive to cefazolin, cephalexin, ceftriaxone and cefotaxime

Table 1 Details of the oligonucleotide primers used in the present study

Genes	Primer sequences	Expected amplicon size (bp)	References
$bla_{CTX\text{-}M\text{-}1}$	Forward: 5′-CCCATGGTTAAAAAACACTGC-3′	950	Horton et al. [3]
	Reverse: 5′-CAGCGCTTTTGCCGTCTAAG-3′		
bla_{TEM}	Forward: 5′-ATAAAATTCTTGAAGACGAAA-3′	1080	Weill et al. [22]
	Reverse: 5′-GACAGTTACCAATGCTTAATC-3′		

and was not carrying $bla_{CTX\text{-}M\text{-}1}$ and/or bla_{TEM} genes in its plasmid as confirmed by PCR analysis. In vitro mating experiments were performed by broth mating [19], filter paper mating [20] and plate mating [21]. Transconjugants were selected on Mac Conkey's agar containing ceftriaxone (50 μg/ml) and nalidixic acid (100 μg/ml). Donor and recipient strains were grown separately in antibiotic free medium as well as antibiotic medium as control. Selected transconjugants were further characterized for their antimicrobial susceptibility, ESBLs phenotype and presence of $bla_{CTX\text{-}M\text{-}1}$ and/or bla_{TEM} genes by PCR.

Results

Bacterial Isolates

Out of the 53 faecal samples collected in this study, 27 (50.94 %) were collected from organized farms, while the remaining 26 (49.06 %) were from local backyard farms. A total of 138 bacteria were isolated, of which 102 (73.91 %) were *E. coli*, 26 (18.84 %) were *Salmonella* spp. and 10 (7.25 %) were *K. pneumoniae* as confirmed by standard bacteriological techniques.

Phenotypic Detection of ESBLs Production

Of the 138 isolates, 8 (5.8 %) *E. coli* isolates showed resistance to cephalosporin group of antibiotics, while no *Salmonella* spp. and *K. pneumoniae* isolates showed resistance against extended-spectrum cephalosporins. Of the eight *E. coli* isolates, 8 (100 %), 7 (87.5 %), 8 (100 %), 6 (75 %), 6 (75 %), 6 (75 %), 7 (87.5 %), 4 (50 %), 6 (75 %), 7 (87.5 %), 4 (50 %) and 5 (62.5 %) showed 100 % resistance to cefixime, cefazolin, cephalexin, ceftriaxone, cefotaxime, enrofloxacin, oxytetracycline, streptomycin, ampicillin, chlortetracycline, erythromycin and lincomycin, respectively (Table 2). All the 8 (5.8 %) *E. coli* isolates suspected for the ESBLs production by disc diffusion method were confirmed to be a ESBLs producer, based on the Double Discs Synergy Test.

Genotypic Characterization of β-Lactamase Genes

Out of the eight phenotypically positive isolates screened for the presence of *bla* genes by PCR using bacterial lysate as template DNA, 7 (5.07 %) were found to be positive for $bla_{CTX\text{-}M\text{-}1}$ gene (950 bp) and/or bla_{TEM} gene (1,080 bp). All the seven isolates were positive for $bla_{CTX\text{-}M\text{-}1}$ gene and

Table 2 Antimicrobial drug resistance pattern of selected bacterial isolates obtained from pigs of different districts of Mizoram

Isolates	Zone of inhibition (in mm)											
	CZ	CTX	CTR	CFM	CN	EX	O	S	L	A	CT	E
PE4	0	0	0	0	0	0	0	0	12	0	0	13
PE5	0	0	0	0	0	0	0	0	13	0	0	14
PE6	0	15	17	0	0	12	0	14	12	13	0	13
PE29	18	23	23	0	0	20	14	12	0	12	15	13
PE76	0	0	0	0	0	0	0	0	0	0	0	0
PE98	0	0	0	0	0	0	0	0	0	0	0	0
PE99	0	0	0	0	0	0	0	12	0	0	0	0
PE100	0	0	0	0	0	0	0	12	0	0	0	0

P pig, *E E. coli*

Table 3 PCR-based detection of $bla_{CTX\text{-}M\text{-}1}$ and bla_{TEM} genes in *E. coli* isolates obtained from pigs of different districts of Mizoram

Isolates	$bla_{CTX\text{-}M\text{-}1}$ (950 bp)			bla_{TEM} (1080 bp)		
	Lysate	Plasmid	Chromosomal	Lysate	Plasmid	Chromosomal
PE4	+	+		+	+	
PE5	+	+				
PE29	+		+			
PE76	+		+	+		+
PE98	+		+	+		+
PE99	+		+			
PE100	+		+			

P pig, *E E. coli* (None of the *K. pneumoniae* and *Salmonella* spp. isolates was found positive)

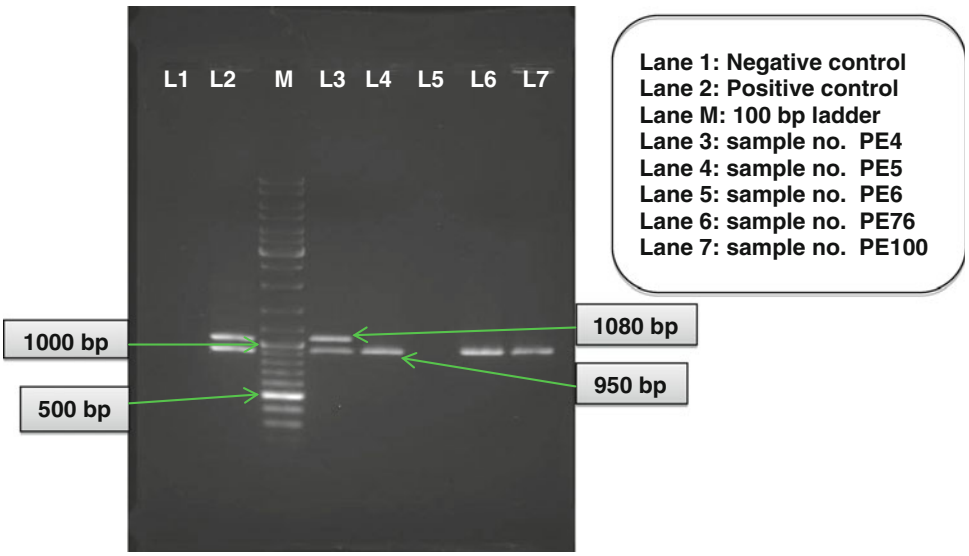

Fig. 1 Multiplex PCR assay for detection of $bla_{CTX\text{-}M\text{-}1}$ and bla_{TEM} genes from phenotypically positive isolates

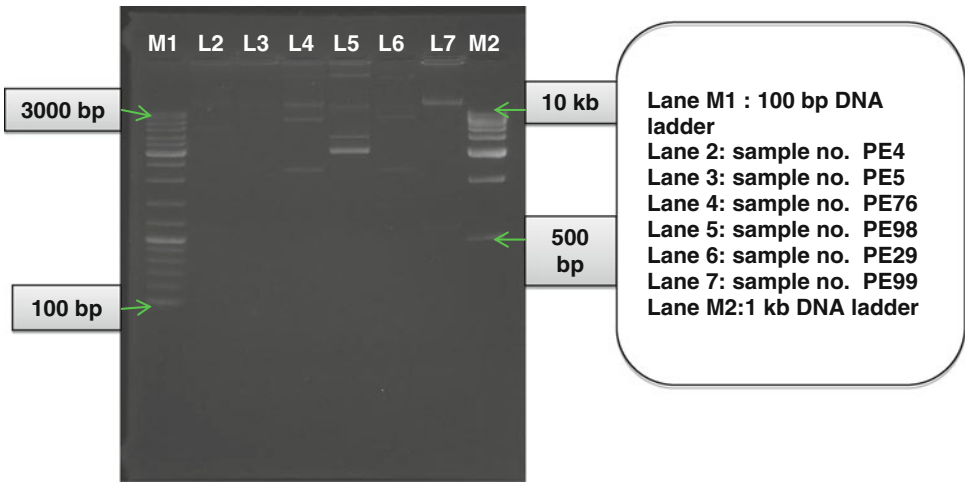

Fig. 2 Agarose gel electrophoresis for demonstration of plasmids extracted from the isolates positive for $bla_{CTX\text{-}M\text{-}1}$ and/or bla_{TEM} genes by PCR assay

three of the isolates have an additional bla_{TEM} gene (Table 3; Fig. 1).

Plasmid Profiling and Curing

Agarose gel electrophoresis of the extracted plasmids showed that all the isolates were carrying plasmids ranging between 0.9 and ~30 kb (Fig. 2). Out of the seven isolates positive for $bla_{CTX\text{-}M\text{-}1}$ and/or bla_{TEM} genes, two isolates were confirmed to harbor the $bla_{CTX\text{-}M\text{-}1}$ gene in their plasmid. Only one bla_{TEM} gene was detected in plasmid.

Using acridine orange (1.25–1.5 mg/ml), the *E. coli* isolate (PE4) was successfully cured. Confirmation of curing was done by disc diffusion assay, where the organism showed 100 % sensitivity against all the antibiotics; plasmid extraction could not trace any plasmids and by PCR assay, no ESBLs genes could be detected.

In Vitro Horizontal Gene Transfer

The resistance trait from the donor isolates could not be transferred to the recipient isolate. There is no growth of transconjugants on the Mac Conkey's agar plate. The recipient strain, after conjugation experiments remained susceptible to cefixime, cefazolin, cephalexin, ceftriaxone, cefotaxime, enrofloxacin, oxytetracycline, streptomycin, ampicillin, chlortetracycline, erythromycin and lincomycin and was also found negative for $bla_{CTX\text{-}M\text{-}1}$ and bla_{TEM} genes by PCR assay.

Discussion

The present study revealed that $bla_{CTX\text{-}M\text{-}1}$ is the most abundant ESBLs type in this region, with *E. coli* being the major ESBLs producer, which is in accordance with the reports of other workers from different places of the world [1, 3, 23]. And also the presence of more than one *bla* genes is often reported worldwide [1, 23].

Prevalence of higher rate of *CTX-M* over *TEM* gene was recorded in this study, which is in agreement with the studies conducted by Ensor et al. [24] as well as Jones et al. [25]. *CTX-M* may be increased due to wide use of third generation cephalosporins, especially ceftriaxone and cefotaxime or may be associated with high mobilization of the encoding genes [26]. Barlow et al. [27] reported that the $bla_{CTX\text{-}M}$ genes have been mobilized to plasmid almost 10 times more frequently than other class A β-lactamases. The predominance of *CTX-M* type of ESBLs gene is may be an indication that this allele would now be common in North East Region of India. Muzaheed et al. [28] also reported high prevalence of *CTX-M* genes in *K. pneumoniae* and *E. coli* from Southern India.

Out of the seven ESBLs positive isolates, six were isolated from organized farm. Only one positive isolate (PE29) was observed from the sample collected from local backyard farm. It may be due to the frequent and routine use of third generation antibiotics in the organized farm. The prevalence of ESBLs in the farm animals is also reported by other workers from India and abroad [1–3].

Most of the ESBLs producing organisms under this study were also found to be co-resistant to fluoroquinolones, aminoglycosides as well as co-trimoxazole, which corroborate with the study done by Denholm et al. [29] and Jabeen et al. [30]. Perez et al. [9] also reported similar kind of information, where the ESBLs producing enteric bacteria are also resistant to other group of antibiotics including aminoglycosides, tetracycline, sulfonamides, trimethoprim and chloramphenicol. Development of co-resistance against other antibiotics along with β-lactam antibiotics by the ESBLs producing organisms generally appeared in the large plasmids, where most of the resistant genes may co-exist.

In the present study, the resistant plasmids could be successfully cured by acridine orange. Although curing provides only the preliminary evidence that genetic traits are of extra-chromosomal nature but loss of growth on antibiotic containing plates also shows that the MDR genes may be plasmid borne. The resistance determining traits are often transposable, which exist in both plasmid and chromosomal locations (flip–flop mechanism) [31]. It is however, important to note that not all antibiotic resistance genes are plasmid mediated [32] and copies of the plasmid lying closer to the membranes are readily eliminated by chemical agents, while those lying closer to the nucleus may escape the curing effect, thereby; one may observe partial curing [33].

During conjugation study neither of the plasmids carrying any one of the target gene could be transferred horizontally to the recipient isolate. Similarly, low transconjugation success was also reported by other workers in Switzerland and Germany [19, 34]. Franiczek et al. [35] also reported that none of the four *E. coli* isolates could transfer their resistance gene to other recipient strains. Yuan et al. [36] reported that plasmids are transferred under the influence of environmental condition (in vitro vs in vivo). In vitro experiment showed transfer of the plasmids ranging from 108 to 157 kb, while in vivo conjugation experiment showed a transfer of smaller sized plasmids. Failure of conjugation in the present study may be because of the small size plasmids carried by the donors. It is suggestive to study the involvements of the insertion sequence *ISEcp1* [37, 38], as well as assessment of the incompatibility group [39, 40] of the plasmid may help to understand the failure.

In summary, we reported the presence of ESBLs in *Enterobacteriaceae* from pigs in NER India. The findings are worrisome as transmission to human via the food chain of bacteria resistant to practically all antimicrobial classes cannot be dismissed. More strict veterinary antibiotic policies are needed in order to prevent emergence and dissemination of these strains among animals and humans, limiting future problems of therapy failure.

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