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RESEARCH ARTICLE
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Detection of extended-spectrum β-lactamases (*bla*$_{CTX-M-1}$ and *bla*$_{TEM}$) in *Escherichia coli*, *Salmonella* spp., and *Klebsiella pneumoniae* isolated from poultry in North Eastern India

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Abstract

Aim: The present study was conducted to record the association of extended spectrum β-lactamases (ESBLs) producing enteric bacteria with diarrhea of poultry birds in Mizoram, India.

Materials and Methods: Fecal samples were collected from poultry birds with the history of diarrhea from different parts of Mizoram. Samples were processed for isolation and identification of *Escherichia coli, Salmonella,* and *Klebsiella pneumoniae*. All the isolates were subjected to antibiotic sensitivity assays. Phenotypically, ESBLs production ability was determined by double discs synergy test (DDST) method. ESBLs producing isolates were subjected to polymerase chain reaction (PCR) for detection of ESBLs genes. Plasmids were cured by acridine orange. Transfer of resistance from donor to recipient strains was done by *in vitro* horizontal method.

Results: A total of 134 enteric bacteria was isolated, of which 102 (76.12%), 21 (15.67%) and 11 (8.21%) were *E. coli, Salmonella* spp. and *K. pneumoniae*, respectively. By DDST 7 (5.22%) isolates (6 *E. coli* and 1 *K. pneumoniae*) were ESBLs producer. PCR analysis confirmed 5 (3.73%) (4 *E. coli and* 1 *K. pneumoniae*) isolates harboured *bla*$_{CTX-M-1}$ gene and/or *bla*$_{TEM}$ gene. All the isolates were carrying plasmids ranging between 0.9 kb and ~30 kb. Of the 4 isolates positive for *bla*$_{CTX-M-1}$ and/or *bla*$_{TEM}$, 2 (1.84%) were confirmed for *bla*$_{CTX-M-1}$ gene in their plasmid. No *bla*$_{TEM}$ gene was detected from plasmid. The resistance plasmid could not be transferred to the recipient by *in vitro* horizontal gene transfer method.

Conclusion: ESBLs producing enteric bacteria are circulating in poultry in North Eastern Region of India. As poultry is one of the most common food animals in this region, these organisms may enter in human population through them.

Keywords: *bla*$_{CTX-M-1}$, *bla*$_{TEM}$, extended spectrum β-Lactamases, North East India, poultry.

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 poultry 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 feces. The MDR bacteria may re-enter the human and animal populations through various routes including natural water, irrigation water, drinking water, vegetables, and foods.

Till date, no authentic data are published regarding the association of ESBLs producing enteric bacteria in poultry in North Eastern Region (NER) of India. Hence, the present study is conducted to detect and characterize the ESBLs producing enteric bacteria in poultry in NER of India.

Materials and Methods

Ethical approval

The work has been carried out after obtaining the approval from the Institutional Animal Ethical Committee, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram.

Bacterial isolates

A total of 51 fecal samples from poultry birds of either sex, irrespective of the age was collected from various districts of Mizoram between September 2011 and March 2012. The birds were reared under different housing system and belonged to organized as well as unorganized farms. Samples were collected using sterilized dry absorbent cotton swab. However, for collection of samples from distant locations, a sterilized swab dipped in nutrient broth was used as a transport medium. Each swab containing the sample was then inserted into separate sterilized test tube, plugged with non-absorbent cotton and carried to the laboratory under cold chain for further processing.

For the isolation of *Escherichia coli* and *Klebsiella pneumoniae*, the collected fecal samples were inoculated on MacConkey's agar and single colonies were selected and confirmed by standard bacteriological technique. For the isolation of *Salmonella* spp., 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 experiment were ceftriaxone, cefotaxime, cefixime, cefazolin, cephalexin, ampicillin, erythromycin, chlortetracycline, streptomycin, enrofloxacin, oxytetracycline, and lincomycin. The isolates conferring resistance to the extended spectrum cephalosporin group of antibiotics were selected for confirmation of ESBLs production, which was done by placing cefotaxime and cefotaxime/clavulanate discs on the inoculated Muller Hinton Agar plate at a distance of 30 cm apart. It was incubated overnight, and the increase 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 polymerase chain reaction (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 1× 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 harboring 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 overnight. Next day tubes containing the highest concentration of acridine orange showing growth were selected, and loop-full was streaked on MacConkey'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 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 [21,22]. 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

Sample collection and bacterial isolates

In the present study, a total of 51 fecal samples was collected from various districts of Mizoram, of which 30 (58.82%) were collected from organized farms and 21 (41.18%) were from local backyard farms.

A total of 134 bacteria was isolated, of which 102 (76.12%) were *E. coli*, 21 (15.67%) were *Salmonella* spp. and 11 (8.21%) were *K. pneumoniae* as confirmed by standard bacteriological techniques.

Phenotypic detection of ESBLs production

Of total 134 isolates, 7 (5.22%) isolates (6 *E. coli* and 1 *K. pneumoniae*) showed resistance to cephalosporin group of antibiotics, whereas no *Salmonella* spp. isolates showed resistance against extended spectrum cephalosporins. Out of 7 *E. coli* isolates, 7 (100%), 7 (100%), 4 (57.14%), 2 (28.57%), 2 (28.57%), 1 (14.28%), 5 (71.43%), 2 (28.57%), 6 (85.71%), 5 (71.43%), 6 (85.71%) and 7 (100%)

showed 100% resistance to cefixime, cefazolin, cephalexin, ceftriaxone, cefotaxime, enrofloxacin, oxytetracycline, streptomycin, ampicillin, chlortetracycline, erythromycin, and lincomycin, respectively (Table-2).

All the 7 (5.22%) isolates suspected for the ESBLs production by disc diffusion method were confirmed to be an ESBLs producer, based on the double discs synergy test.

Genotypic characterization of β-lactamase genes

Of the 7 phenotypically positive isolates screened for the presence of *bla* genes by PCR using bacterial lysate as template DNA, 5 (3.73%) were found to be positive for $bla_{CTX\text{-}M\text{-}1}$ gene (950 bp) and/or bla_{TEM} gene (1080 bp). Four isolates (3 *E. coli* and 1 *K. pneumoniae*) were positive for $bla_{CTX\text{-}M\text{-}1}$ gene, of which 3 were having an additional bla_{TEM} gene. Only 1 *E. coli* isolate was found to be positive for bla_{TEM} gene alone (Figure-1 and Table-3).

Plasmid profiling and curing

Agarose gel electrophoresis of the extracted plasmids showed that all the isolates were carrying plasmids ranging between 0.9 kb and ~30 kb (Figure-2). Of 7 isolates positive for $bla_{CTX\text{-}M\text{-}1}$ and/or bla_{TEM} genes, 2 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* isolates were 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 any of the isolates could not be transferred to the recipient host. The transconjugants remained sensitive to cefixime, cefazolin, cephalexin, ceftriaxone, cefotaxime, enrofloxacin, oxytetracycline, streptomycin, ampicillin, chlortetracycline, erythromycin, and lincomycin. Transconjugants were 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 investigators from other places [1,3,23]. Furthermore, the presence of more than one *bla* genes is often reported worldwide [1,23]. *TEM-1*, the most prevalent bla encoded enzyme in human clinical isolates worldwide [1,24,25], is not classified as an ESBL. However, several *TEM-1* derivatives confer ESBL properties [26].

Prevalence of *CTX-M* genes along with *TEM* gene has been often reported by various workers [5,27,28].

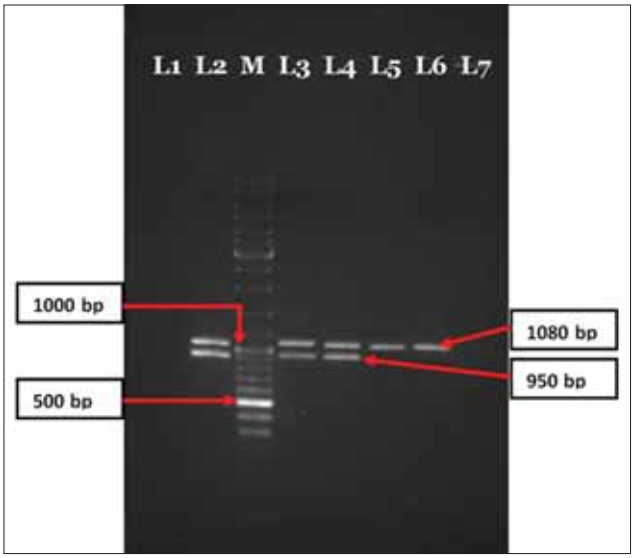

Figure-1: Multiplex polymerase chain reaction assay for detection of $bla_{CTX\text{-}M\text{-}1}$ and bla_{TEM} genes from phenotypically positive isolates using chromosomal DNA as template. Lane 1: Negative control, Lane 2: Positive control, Lane M: 100 bp DNA ladder, Lane 3: Sample no. AE3, Lane 4: sample no. AE9, Lane 5: Sample no. AE8, Lane 6: Sample no. AK4, Lane 7: Sample no. AE45.

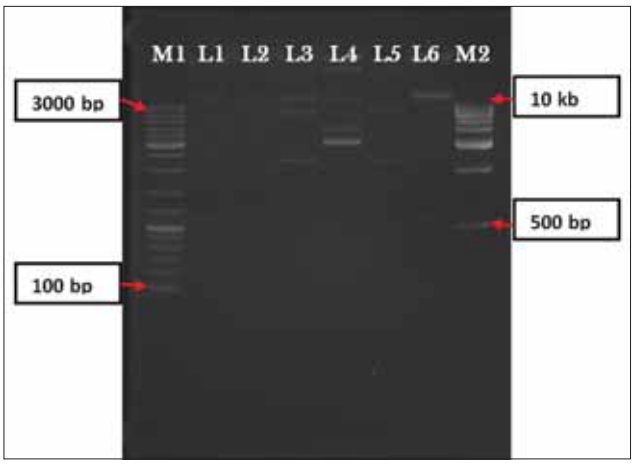

Figure-2: Agarose gel electrophoresis for demonstration of plasmids extracted from the isolates positive for $bla_{CTX\text{-}M\text{-}1}$ and/or bla_{TEM} genes by polymerase chain reaction assay. Lane M1: 100 bp DNA ladder, Lane 1: Sample no. AE3, Lane 2: Sample no. AE8, Lane 3: Sample no. AE9, Lane 4: Sample no. AK4, Lane 5: Sample no. AE45, Lane 6: Sample no. AE5, Lane M2: 1 kb DNA ladder.

Table-1: Details of the oligonucleotide primers used in the present study.

Gene	Primer sequence	Expected amplicon size (bp)	Reference
$bla_{CTX\text{-}M\text{-}1}$	Forward: 5`-CCATGGTTAAAAAACACTGC-3` Reverse: 5`-CAGCGCTTTTGCCGTCTAAG-3`	950	3
bla_{TEM}	Forward: 5`-ATAAAATTCTTGAAGACGAAA-3` Reverse: 5`-GACAGTTACCAATGCTTAATC-3`	1080	22

Table-2: Antimicrobial drug resistance pattern of selected bacterial isolates obtained from poultry in NER of India.

Isolates	Zone of inhibition (in mm)											
	CZ	CTX	CTR	CFM	CN	EX	O	S	L	A	CT	E
AE3	0	18	19	0	0	13	0	12	0	0	0	0
AE5	0	19	19	0	13	14	0	14	0	0	0	0
AE6	0	19	22	0	15	20	0	14	0	14	0	0
AE8	0	17	19	0	13	15	0	12	0	0	0	0
AE9	0	19	19	0	0	16	13	12	0	0	15	0
AK4	0	0	0	0	0	0	0	0	0	0	0	0
AE45	0	0	0	0	0	16	15	0	0	0	16	15

A=Avian, E=*Escherichia coli*, NER=North-Eastern Region

The prevalence of *CTX-M* may be due to wide use of third generation cephalosporins especially ceftriaxone and cefotaxime or may be associated with high mobilization of the encoding genes [29]. Barlow *et al*. [30] reported that the bla_{CTX-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 may be an indication of this being common in North East Region of India. Muzaheed *et al*. [31] also reported high prevalence of *CTX-M* genes in *K. pneumoniae* and *E. coli* from Southern India.

Of the 5 ESBLs positive isolates, 4 isolates were from organized farm. Only 1 positive isolate (AE45) was recorded from the sample collected from local backyard farm. The predominance of ESBLs positive isolates from organized farms may be due to the frequent and routine use of third-generation antibiotics. The prevalence of ESBLs in the farm animals is also reported by other workers from various parts of the world [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 is in accordance with the study done by Denholm *et al.* [32] and Randall *et al*. [33]. Perez *et al*. [9] also reported similar results, where the ESBLs producing enteric bacteria were 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, loss of growth on antibiotic containing plates 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) [34]. It is, however, important to note that all antibiotic resistance genes are not plasmid-mediated [35]. The 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 [36].

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 [19,37]. Franiczek *et al*. [38] also reported that none of the 4 *E. coli* isolates could transfer their resistance gene to other recipient strains. Yuan *et al.* [39] 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. Involvement of insertion sequence *ISEcp1* in the mobility of bla_{CTX-M} has also been confirmed by studies of plasmids [40,41]. Hypothetically, the plasmid may be of different incompatibility group other than *IncI1* incompatibility group because most of the conjugative plasmids generally belonged to *IncI1* incompatibility group [42,43].

Conclusion

ESBLs producing *E. coli, Salmonella* spp., and *K. pneumoniae*, were recorded from poultry birds in Mizoram. PCR analysis confirmed 3.73% isolates harboring $bla_{CTX-M-1}$ gene and/or bla_{TEM} gene. $bla_{CTX-M-1}$ gene was detected in the plasmid. Transmission of such organisms to humans via the food chain cannot be dismissed. More strict veterinary antibiotic policies are needed in order to prevent emergence and dissemination of these strains among animals and humans.

Authors' Contributions

HL collected the samples, extracted nucleic acids, detected virulence genes and ESBLs genes. TKD designed the program, analyzed the data, prepared the manuscript. IW isolated the organisms, conducted antibiotic assays. RC monitored the program, prepared and edited the manuscript. All authors read and approved the final manuscript.

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Table-3: PCR-based detection of $bla_{CTX-M-1}$ and bla_{TEM} genes in *E. coli* and *K. pneumoniae* isolates obtained from poultry in NER of India.

Isolates	$bla_{CTX-M-1}$ (950 bp)			bla_{TEM} (1080 bp)		
	Lysate	Plasmid	Chromosomal	Lysate	Plasmid	Chromosomal
AE3	+		+	+		+
AE8				+		+
AE9	+		+	+		+
AK4	+	+		+		+
AE45	+	+				

A=Avian, E=*Escherichia coli* (none of the *Salmonella* spp. was found positive), *K. pneumonia=Klebsiella pneumonia*, NER=North-Eastern Region, PCR=Polymerase chain reaction

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Competing Interests

The authors declare that they have no competing interests.

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