Multi-drug Resistant (MDR), Extended Spectrum Beta-lactamase (ESBL) and New Delhi Metallo Beta-lactamase (NDM) Producing Escherichia Coli in Rescued Sloth Bears (*Melursus Ursinus*), India

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Abstract

The study reports the MDR, ESBL, and NDM producing *Escherichia coli* (CRE) isolated from the rescued sloth bear (*Melursus ursinus*), India. The faecal samples of adult rescued sloth bear (n=21) were collected from a rescue center located in India during 2015-2016 and processed for isolation and antibacterial susceptibility pattern of *E. coli*. 45 *E. coli* isolates were recovered, and on phenotypic screening, 23 were MDR, 17 were ESBL producers, and five were carbapenem-resistant (CR). The MDR isolates carried beta-lactamase, chloramphenicol, aminoglycosides, tetracycline, fluoroquinolone, and sulphadimidine resistance genes. All the phenotypic ESBL producing isolates had *bla*CTX-M genes. On genotypic screening, three CRE (60.0 %, 3/5) were positive for *bla*NDM carbapenemase gene. Efflux pump-mediated carbapenem resistance was noticed in two CRE isolates (40.0 %, 2/5). The CRE also isolates co-harbored AMR genes like *bla*TEM-1, *bla*AmpC, *qnrA*, *qnrB*, *qnrS*, *tetA*, *tetB* and *sulI*. Virulence screening of the resistant isolates revealed the presence of *Stx1*, *Stx2*, *eae*, *hlyA* genes. Plasmid incompatibility (Inc) types of three NDM positive isolates revealed that two isolates *bla*NDM-5 gene on IncI1 and one isolate on IncF plasmid. Apart from NDM genes, the plasmids also carried tetracycline, beta-lactamase and quinolone resistance genes. The plasmid multilocus sequence typing (pMLST) of the *E. coli* IncI1 plasmid showed the Sequence Type (ST) 297. This appears to be the first report of multi-drug resistant, extended spectrum beta-lactamase (ESBL) producing and *bla*NDM-5 genes on IncI1 and IncF plasmids in rescued sloth bear.

Introduction

Antimicrobial resistance is an emerging global threat and needs immediate attention. Extended spectrum beta-lactamases (ESBL) and/or carbapenemases producing *Escherichia coli* are of great concern worldwide and is a complex and multidimensional problem involving humans, animals, and the environment (ATTERBY et al. 2017). Carbapenems are one of the most important groups of antimicrobials and are considered as a last line of drugs for the treatment of severe infections. Carbapenem resistance in animals appear to have developed and disseminated because of clinical carbapenem use in human medicine and presence of carbapenem resistance in food animals have been reported (Pruthivishree et al. 2017; Nirupama et al. 2018). Genes conferring AMR can spread quickly, being passed on to bacteria that had never been exposed to the initial selective pressure and thereby disseminating throughout microbial communities (Allen et al. 2010). Wild birds and mammals acts as important reservoirs and potential vectors for the spread of resistant bacteria and genetic determinants of AMR (Caroll et al. 2015). Wild animals are not naturally exposed to antimicrobials; though, they may be infected with antibiotic-resistant bacteria via the acquisition of resistant bacteria from human sources, agricultural facilities, and associated contaminated environments (Dolejska et al. 2015). Antimicrobial agents, such as antibiotics, are used extensively in human and veterinary medicine and often as part of routine agricultural production (Kummerer 2009). The prevalence level of resistant bacteria is linked with human activity (Bonnedahl et al. 2009; Alroy and Ellis 2011). Hence, the study was carried out to identify the molecular epidemiology of multi-drug resistant, extended spectrum beta-lactamase (ESBL) producing and carbapenem resistant *Escherichia coli* isolated from rescued sloth bear.

Materials And Methods

A cross-sectional study was conducted in a bear rescue centre for screening of antimicrobial resistance in rescued sloth bears of India during 2015-16. The representative faecal samples from apparently healthy adult sloth bears (n = 21) were collected in sterile transport swabs (HiCulture, HiMedia, India). Following enrichment in MacConkey broth at 37°C for 6–10 h, the faecal swabs were streaked on MacConkey agar and incubated at 37°C for 12–18 h. Two to three lactose-fermenting colonies were streaked on eosin methylene blue (EMB) agar medium and incubated at 37°C overnight for preliminary characterization. Then, the colonies showing characteristic metallic sheen on EMB agar were picked up for biochemical tests. The reference strains were retrieved from the Division of Epidemiology, ICAR-Indian Veterinary Research Institute, Izatnagar repository, and used in this study (GenBank accession number: KU318701, KU318691, KU382501).

The biochemically confirmed *E. coli* (n = 45) isolates were tested for antibiotic susceptibility pattern with amoxicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), aztreonam (30 µg), chloramphenicol (30 µg), ceftriaxone (30 µg), cefazolin (30 µg), cefpodoxime (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), cefixime (5 µg), ceftiofur (30 µg), piperacillin - tazobactam (100/10 µg), cefotaxime/clavulanic acid (30/10 µg), tetracycline (30 µg), nitrofurantoin (300 µg), gentamicin (10 µg), kanamycin (30 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg) and norfloxacin (10 µg) by disc diffusion method. For phenotypic identification of ESBL producers, the combination disc method by cefotaxime, ceftazidime with and without clavulanic acid was used (Andrews 2003). The isolates were further confirmed by ESBL E-test (HiMedia, Mumbai, India). Screening for carbapenem resistance was carried out with meropenem (MEM, 10 µg), imipenem (IMP, 10 µg), and ertapenem (ETP, 10 µg) disks (BD, Sparks, MD, USA) along with metal ion chelator ethylene diamine tetra acetic acid (EDTA; 1900 mg) for metallo beta-lactamase (MBL) production (Pruthivishree et al. 2017). The keyhole reaction between carbapenem drug and EDTA was considered positive for MBL. The MIC for meropenem was determined using E-test strip (EZY MRP, HiMedia, Mumbai, India). The CLSI, 2014 (CLSI 2014) breakpoints were used for interpretation of susceptibility patterns.

A conservative approach was utilized, and only isolates classified as resistant were further analysed for AMR encoding genes. Total DNA was isolated from *E. coli* isolates using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). The isolates were screened for the presence of antimicrobial resistance genes (ARG), including beta-lactamase, major carbapenemase, sulphonamide, plasmid-mediated quinolone (PMQR), and tetracycline resistance genes. The isolates were also screened for putative virulence genes (Paton and Paton 1998). The reactions were optimized individually in 25 µL volumes, with 10 picomole of each individual primer (supplementary table 1). The PCR amplicon from all reactions was electrophoresed in 0.5 X TBE buffer using 1.5% agarose gel and visualized under UV illumination (Syngene, USA). Representative PCR amplicons were purified and sequenced by commercial sequencing services (Eurofins, Bengaluru, India). The sequencing results were obtained, and homology searches were performed using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequence data were submitted to GenBank, and accession numbers were obtained.

Plasmid DNA was isolated using a QIA prep Spin miniprep kit (QIAGEN, Helsinki, Finland) from the *bla*NDM positive *E. coli*. PCR-based replicon typing (PBRT) was used to determine the plasmid incompatibility groups (Johnson et al. 2007). Plasmid MLST was performed using specific primers

(<https://pubmlst.org/plasmid/primers/>) and sequenced by the Sanger method (Eurofins India Ltd, Bengaluru). The sequences were edited using BioEdit v7.0.5 and queried in the plasmid MLST website (https://pubmlst.org/bigssdb?db=pubmlst_plasmid_seqdef) for an allelic profile, sequence type, and clonal complex. The isolates not showing carbapenemase mediated resistance were screened for the presence of active efflux pump by the ethidium bromide cartwheel method.

Statistical analysis

Antibiotic susceptibility phenotypic profiles and gene presence was converted into binary code. For each antibiotic 0 denoted susceptibility and 1 denoted represented resistance similarly the presence of a gene was designated as 1 and absence as 0. Principal component analysis (PCA) was performed using the package Factoextra from the open-source statistical program R.

Results And Discussion

A total of 45 *E. coli* isolates were isolated from 21 sloth bear faecal

samples and the antibiogram showed that the isolates were resistant to gentamicin (62.2%, 28/45), cefotaxime (60.0%, 27/45), ceftazidime (60.0 %, 27/45), cefotaxime/clavulanic acid (57.8%, 26/45), ceftriaxone (55.6 %, 25/45), ceftazidime /clavulanic acid (55.6%, 25/45), norfloxacin (55.6%, 25/45), cefpodoxime (53.3%, 24/45), ceftazidime (53.3%, 24/45), piperacillin/tazobactam (53.3%, 24/45), cefexime (51.1%, 23/45), ciprofloxacin (51.1%, 23/45), tetracycline (51.1%, 23/45), sulphadimidine/trimethoprim combination (46.7%, 21/45), cefepime (46.7%, 21/45), chloramphenicol (42.2%, 19/45), nitrofurantoin (40.0%, 18/45), aztreonam (35.6%, 16/45), meropenem (11.1%, 5/45), imipenem (8.9%, 4/45) and ertapenem (8.9%, 4/45). Of the 45 isolates, 51.1 % (23/45) were resistant to more than two classes of screened antibiotics and classified as MDR. The ESBL screening by combined disc method revealed 37.8% (17/45) isolates as ESBL producers (Table 1).

Table 1
Antibiotic susceptibility pattern, virulence and antibiotic resistance determinants of *E. coli* is

S. no	Isolates	Antibacterial resistance pattern																			
		AMX	AMC	CZ	ATZ	COT	CX	CTX	CFM	CPD	CIP	CAZ	CTR	K	C	GEN	TE	FOX	F/M	TZP	ND
1	SB-1	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
2	SB-2 ESBL	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
3	SB-3 ESBL	R	R	R	S	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R	R
4	SB-4	R	S	R	S	R	R	S	S	R	S	S	S	R	S	S	S	S	S	S	R
5	SB-5	R	S	R	S	S	R	S	S	S	S	S	S	R	R	R	S	S	S	S	S
6	SB-6	R	R	R	S	R	R	S	S	S	S	S	S	R	S	S	S	R	S	S	S
7	SB-7	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
8	SB-8 ESBL	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	R	S	R	S
9	SB-9	R	S	S	S	R	R	S	R	S	R	S	S	R	R	S	S	S	S	R	R
10	SB-10	R	S	S	S	R	S	R	S	S	R	S	S	R	R	R	R	S	S	S	R
11	SB-11	R	S	S	S	S	S	R	S	S	R	S	S	R	R	S	S	S	S	S	S
12	SB-12 ESBL	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	S
13	SB-13 ESBL	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
14	SB-14	R	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	R
15	SB-15 ESBL	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
16	SB-16 ESBL	R	R	R	R	S	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R
17	SB-17 ESBL	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R
18	SB-18	R	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	S
19	SB-19 ESBL	R	R	R	R	S	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R
20	SB-20 ESBL	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	S	R	R
21	SB-22 ESBL	R	R	R	S	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R	S
22	SB-23 ESBL	R	R	R	S	R	R	R	R	R	S	R	R	S	R	R	R	R	R	R	R
23	SB-24	R	S	S	S	S	S	S	S	S	S	R	S	R	S	S	S	S	S	S	S
24	SB-25	S	R	S	S	S	S	R	S	R	R	S	S	R	S	R	R	R	S	S	S
25	SB-26	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S
26	SB27/ 17-CRE	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
27	SB28/ 12-CRE	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
28	SB28/ 16-CRE	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R

R: resistant ; S: sensitive; AMX: amoxicillin; AMC: amoxicillin–clavulanic acid; ATZ: aztreonam; C: chloramphenicol; CTR: ceftriaxone; CPD: cefpodoxime; CAZ co-trimoxazole; ERP: ertapenem; F/M: nitrofurantoin; FOX: ceftiofur; GEN : gentamicin; IMP: imipenem; K-kanamycin, NX: norfloxacin; MRP- meropenem; TE: tetracycline

S. no	Isolates	Antibacterial resistance pattern																			
		AMX	AMC	CZ	ATZ	COT	CX	CTX	CFM	CPD	CIP	CAZ	CTR	K	C	GEN	TE	FOX	F/M	TZP	ND
29	SB-29/18-CRE	R	R	R	S	S	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R
30	SB-30 CRE	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
31	SB-31 ESBL	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R
32	SB-32 ESBL	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
33	SB-33 ESBL	R	R	R	S	S	R	R	R	R	S	R	R	R	S	R	R	R	S	R	R
34	SB-34 ESBL	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
35	SB-35	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S	S
36	SB-36	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
37	SB-37	S	S	S	S	S	S	R	S	S	S	R	S	S	S	S	S	S	S	R	R
38	SB-38	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	S	S	S	S	S
39	SB-39	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
40	SB-40	S	S	S	S	S	S	R	S	S	S	R	S	S	S	R	S	S	S	S	R
41	SB-41	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
42	SB-42	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S	S
43	SB-43	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S
44	SB-44	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
45	SB-45	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

R: resistant ; S: sensitive; AMX: amoxicillin; AMC: amoxicillin–clavulanic acid; ATZ: aztreonam; C: chloramphenicol; CTR: ceftriaxone; CPD: cefpodoxime; CAZ co-trimoxazole; ERP: ertapenem; F/M: nitrofurantoin; FOX: ceftiofur; GEN : gentamicin; IMP: imipenem; K-kanamycin, NX: norfloxacin; MRP- meropenem; TE: t

Citations are available as wild birds as reservoirs of extended-spectrum β -lactamase (ESBL) and AmpC β -lactamase producing *E. coli* (Alcala et al. 2016). Interestingly, a significant percentage of MDR *E. coli* isolates detected in black-headed gulls (3.3%), starlings (19.2%), herring gulls (3.3%), deer (3.3%) of Ireland (Carroll et al. 2015). Screening for carbapenem resistance showed that 11.11% (5/45) isolates were resistant to any carbapenem drugs. Three carbapenem-resistant isolates showed keyhole between EDTA and carbapenem drugs, indicating metallo beta-lactamase production (MBL). A conservative approach was utilized for genotypic screening, and only isolated classified as resistant were analysed for AMR encoding genes. All the ESBL producing isolates were MDR type and genotypic screening of the ESBL positive isolates (n = 17) revealed *bla*CTX-M-1 (n = 10), *bla*CTX-M-15 (n = 7) and other antibiotic resistance genes like *bla*TEM (n = 6), *bla*AmpC (n = 4), *qnrS* (n = 6), *qnrA* (n = 3), *qnrB* (n = 3), *sulI* (n = 7), *sulII* (n = 3), *tetA* (n = 4), *tetB* (n = 5). Similarly, most of the ESBL *E. coli* isolates in wild birds were multi-drug resistant, and the most common resistant phenotype are beta-lactams, quinolones, tetracycline, and sulfamethoxazole/trimethoprim (Alcala et al. 2016). The dominant extended-spectrum cephalosporin resistance genes in animals were *bla*CTX-M-1, while in humans were *bla*CTX-M-15 and *bla*CTX-M-14. However, recent studies showed that the prevalence of CTX-M-1 genes in humans (Madec et al. 2015) and rare identification of CTX-M-15 in animal isolates (Dahmen et al. 2013). In the present study, *E. coli* isolated from sloth bear harbored both CTX-M-1 and CTX-M-15 variants indicated sharing of gene pool. Screens for carbapenemase genes revealed three isolates were positive for the *bla*NDM-5 gene. All five carbapenem resistant isolates were MDR ESBL producers. The *bla* NDM positive isolates also co-harboured *bla*CTX-M1/15, *bla*TEM-1, *qnrS*, *qnrA*, *qnrB*, *sulI*, *tetA* and *tetB* genes.

The efflux pump-mediated carbapenem resistance was noticed in two (40 %, 2/5) isolates. Efflux pump mediated carbapenem resistance was noticed in *E. coli* isolated from piglets and dairy calves (Pruthivishree et al. 2017; Murugan et al. 2019). Chloramphenicol (catI, floR), aminoglycoside (ac (3)-IV, aph (3')-IIa), quinolone (*qnrS*, *qnrA*, *qnrB*), trimethoprim (*dhfrI*), sulphadiazine (*sulI*, *sulII*) and tetracycline resistance genes (*tetA*, *tetB*) were commonly detected in the phenotypic resistance isolates. Furness et al. (2017) reported *E. coli* from the faeces of small free-living mammals were resistant to trimethoprim, ampicillin, ciprofloxacin, and cefotaxime. Screens for virulence genes revealed that five ESBL producing and one carbapenem-resistant isolates harbored any of the virulence genes screened (Table 1). Reports were available in India on ESBL producing and carbapenem resistant *E. coli* harboring virulence genes in animals (Pruthivishree et al. 2017; Nirupama et al. 2018; Pruthivishree et al. 2018). Plasmid replicon typing of the three *bla*NDM-5 gene-positive *E. coli* revealed that the NDM gene was on IncI1 plasmid in two isolates (SB27/ 17-CRE, SB28/ 12-CRE) and IncF plasmid was on one isolate (SB28/ 16-CRE). The IncI1 and IncF plasmids also co-harbored PMQR, extended-spectrum cephalosporin, sulphonamide, and tetracycline resistance genes. The IncF plasmids have been reported worldwide among *Enterobacteriaceae* and are one of the most prevalent incompatibility types involved in the transfer of resistance determinants (Yang et al.

2015). Recently, IncF plasmid carrying multi-drug resistant genes were reported in *Shigella flexneri* isolates from India (Sethuvel et al. 2019). In our study, the IncI1 and IncF plasmids also co-harbored *bla*CTX-M-15, *bla*TEM-1, *tetA*, and *sulI* AMR genes. Similarly, IncF plasmid encoding beta-lactamase (NDM-1, OXA-1), aminoglycoside (*aacA4*, *aadA2*, and *aacC2*), and extended-spectrum cephalosporin resistance (CTX-M-15) genes from *E.coli* ST131 was reported (Bonnin et al. 2012). The plasmid multilocus sequence typing (pMLST) of the *bla*NDM isolates showed ST 297. Recently we reported *bla*VIM positive *E.coli* to isolate with ST 297 (Murugan et al. 2019).

The ESBL producing, carbapenem and multidrug resistant *E. coli* were divided into three groups in the principal component analysis (PCA). The first two dimensions which included showed the most variance of data (first: 62.5%; second: 7.3%). The third and fourth dimensions, despite being significant, were not plotted due to the complexity of the corresponding output (Fig. 1). Similar PCA analysis of ESBL producing *E. coli* isolates were carried out in piglets and pig farm workers (Tamta et al. 2020).

In conclusion, the study documented the presence of multi-drug resistant (MDR), ESBL producing, and carbapenem resistant *E. coli* carrying virulence genes. The isolates co-harboring antibiotic resistant with virulence genes are critically important in public health point of view. To the best of our knowledge, this is the first report of IncF and IncI1 type plasmids carrying multidrug-resistant and NDM carbapenemase producing genes in sloth bear.

Declarations

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Ethical Approval

Not applicable

Consent to Participate

Not applicable

Consent to Publish

We give consent to publish

Authors Contributions

Vinodh Kumar O. R - Conceptualization, Data curation, Formal analysis, Writing - original draft

M.Karikalan - Data curation, Formal analysis, Writing - original draft.

S. Ilayaraja – Methodology, Data curation

B.R Singh - Validation

Arun A Sha– Methodology, Resources, Software

D.K.Sinha– Validation

S. Chandra Mohan - review & editing

A.M.Pawde - review & editing

AK Sharma- Validation

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Competing Interests

The authors declare no conflict of interest.

Availability of data and materials

All data generated or analysed during this study are included in this published article

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Figures