

Carbapenem resistant *Escherichia coli* and *Pseudomonas aeruginosa* in captive blackbucks (*Antilope cervicapra*) and leopards (*Panthera pardus*) from India

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ABSTRACT

The study aimed to investigate the occurrence of carbapenem resistant *E. coli* and *P. aeruginosa* in apparently healthy, captive blackbucks and leopards of India. Faecal samples of blackbucks (n = 7) and leopards (n = 7) were processed to isolate carbapenem resistant *E. coli* (CRE) and *P. aeruginosa* (CRP). Forty (leopards n = 26; blackbuck n = 14) *E. coli* and two *P. aeruginosa* (blackbuck n = 2) samples were isolated from the faecal samples (n = 14). Eleven carbapenem resistant isolates were recovered, of which 10 were CRE and one was CRP. The minimum inhibitory concentration (MIC) was determined for meropenem for carbapenem resistant isolates and was between 8 and 64 µg/mL. All the CRE and CRP were phenotypically multidrug resistant, and six CRE were extended-spectrum beta-lactamases (ESBL) producers. On genotypic screening, seven CRE and one CRP were positive for the *bla*NDM carbapenemase gene. Efflux pump-mediated carbapenem resistance was noticed in four CRE isolates (36.4%, 4/11). Of the six ESBL producing CRE, four isolates carried *bla*CTX-M-1 genes. The CRE isolates also harbored *bla*TEM-1, *bla*AmpC, *qnrA*, *qnrB*, *qnrS*, *tetA*, *tetB* and *sul1* resistance genes. On Shiga toxin virulence screening, *Stx1*, *Stx2* genes were detected in two and one isolates, respectively. Plasmid typing of CRE revealed that the *bla*NDM genes were carried on an Inc11 plasmid. The plasmid multilocus sequence typing (pMLST) of the isolates showed the Sequence Type (ST) 297. The occurrence of carbapenem resistance bacteria in captive wildlife should be a major public health priority.

Key words: ESBL; *E. coli*; Shiga toxin; wildlife; Carbapenem-resistance; NDM

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Introduction

Antimicrobial resistance is an emerging global threat and needs immediate attention. It is a complex and multidimensional problem involving humans, animals, and the environment. The intensive and indiscriminate use of antimicrobials in humans and animals has resulted in the emergence of antimicrobial resistance (AMR) in humans, animals, and the environment (BERENDONK et al., 2015). Transmission of carbapenem resistant Enterobacteriales between animals and exposed humans has been reported (KOCK et al., 2018).

Carbapenems are one of the most important groups of antimicrobials, and are considered as the last line of drugs for the treatment of severe infections. Carbapenem resistance in animals appears to have developed and disseminated as a consequence of clinical carbapenem use in human medicine (MURUGAN et al., 2019). The present study was planned to investigate the occurrence and to characterize carbapenem resistant *E. coli* and *P. aeruginosa* in captive blackbucks and leopards of India.

Materials and methods

Sample collection. A cross-sectional study was conducted during 2015-16 in a zoo for screening antibacterial resistance in apparently healthy captive blackbucks and leopards. The representative faecal samples from blackbucks (n = 7) and leopards (n = 7) were collected in sterile transport swabs.

Isolation and identification of *E. coli* and *P. aeruginosa*. Each faecal swab was pre-enriched in buffered peptone water (incubation at 37 °C for 2-4h) 37 °C, the inoculums were streaked on Hektoen enteric agar (HEA) plates, and on HEA yellow colour colonies were considered to be presumptive *E. coli*, while the transparent non-fermenting colonies were considered to be presumptive *Pseudomonas*. From each plate, 3-4 presumptive *E. coli* and *Pseudomonas* colonies were taken out and the respective colonies were streaked on MacConkey agar plates with imipenem (0.5 mg/L) added, and incubated at 37 °C overnight (PRUTHVISHREE et al., 2017). Subsequently, the characteristic isolated colonies were extracted for identification by biochemical tests.

Antibacterial screening of *E. coli* and *P. aeruginosa* isolates. Reference strains (GenBank accession number KT853018, KT867018, KT867020 and KT867021) obtained from the Division of Epidemiology, Indian Veterinary Research Institute, Izatnagar repository, were used in this study as positive controls. The recovered *E. coli* (n = 40) and *P. aeruginosa* (n = 2) isolates were tested for antibacterial susceptibility patterns against 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), to screen for metallo beta-lactamase (MBL) production (PRUTHVISHREE et al., 2017). A keyhole reaction between the carbapenem drug and EDTA was considered positive for MBL.

For phenotypic identification of ESBL producers, the combination disc method was used (ANDREWS, 2003) by cefotaxime and ceftazidime, with and without clavulanic acid. Once the zone of inhibition between the combination disc and the corresponding single antibacterial disc differed by ≥ 5 mm, the isolate was identified as an ESBL producer. The isolates were further confirmed as ESBL producers using the E-test. The isolates were also screened for aztreonam (ATZ, 30 µg), chloramphenicol (C, 30 µg), ceftriaxone (CTR, 30 µg), cefpodoxime (CPD, 10 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), cefepime (CPM, 30 µg), cefixime (CFM, 5 µg), ceftiofuran (FOX, 30 µg), piperacillin - tazobactam (TZP, 100/10 µg), cefotaxime/clavulanic acid (30/10 µg), tetracycline (TE, 30 µg), nitrofurantoin (F/M, 300 µg), gentamicin (GEN, 10 µg), co-trimoxazole (COT, 25 µg), ciprofloxacin (CIP, 5 µg) and norfloxacin (NX, 10 µg) by the disc diffusion method. The CLSI, 2014 breakpoints were used for interpretation of the susceptibility pattern. The MIC against meropenem, ceftriaxone, cefotaxime was determined using E-test strips (HiMedia, Mumbai, India) for the carbapenem resistant isolates (n = 11).

PCR for carbapenemase producing genes and sequencing. PCR was carried out targeting beta-lactamase (PRUTHVISHREE et al., 2017), major

carbapenemase (POIREL et al., 2011), Shiga toxin virulence markers (PATON and PATON, 1998), sulphonamide resistance (KERRN et al., 2002), and plasmid mediated quinolone resistance (PMQR) determinants (CIESIELCZUK et al., 2013) and tetracycline resistance (MAYNARD et al., 2004) genes. The reactions were optimized individually in 25 µL volumes with 10 picomoles of each primer. 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). The PCR amplicons (*bla*NDM and *bla*AmpC) 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 the accession numbers were obtained.

Plasmid replicon and multilocus sequence (MLST) typing of E. coli isolates. The plasmid DNA was extracted from *E. coli* by a Qiagen Miniprep kit (Qiagen, India) from the *bla*NDM positive *E. coli*. Plasmids were assigned to incompatibility groups by multiplex PCR (JOHNSON et al., 2007). After assigning the incompatibility groups, the corresponding plasmid MLST was performed using specific primers (GARCIA-FERNANDEZ et al., 2018) and sequenced by the Sanger method (Eurofins India Ltd, Bengaluru). The sequences were edited using BioEdit v7.0.5 and examined on the plasmid MLST website (https://pubmlst.org/bigsub?db=pubmlst_plasmid_seqdef) for the allelic profile, sequence type, and clonal complex.

Assay for overexpressed efflux pumps. Isolates not showing carbapenemase mediated resistance were screened for the presence of an active efflux pump by the ethidium bromide agar cartwheel method (PRUTHVISHREE et al., 2017).

Results and discussion

From the faecal samples, forty (leopards n = 26; blackbucks n = 14) *E. coli* and two *P. aeruginosa* (blackbucks n = 2) were isolated. From each animal swab, 3-4 presumptive *E. coli* and *Pseudomonas*

colonies were picked up and characterized. The *E. coli* isolates from leopards (n = 26) and blackbucks (n = 14) screened for carbapenem resistance revealed that nine *E. coli* from leopards and one *E. coli* from blackbucks were carbapenem resistant. *Pseudomonas aeruginosa* could only be isolated from blackbuck faecal samples. The CRE (n = 9) from leopards were resistant to MEM (9/9, 100%) followed by IMP (8/9, 88.9%) and ETP (6/9, 66.7%). Similarly the CRE (n = 1) and CRP (n = 1) from blackbucks were resistant to MRP.

In addition, the CRP (n = 1) was also resistant to IMP and ETP. The reason for the higher MEM resistance might be associated with the increased clinical use of meropenem in India (GANDRA et al., 2016). Of the carbapenem resistant isolates (n = 11), seven CRE (n = 6 from leopards and n = 1 from blackbucks) and one CRP from blackbucks showed the keyhole reaction with EDTA, indicating metallo beta-lactamase (MBL) production. The minimum inhibitory concentration (MIC) for meropenem of the carbapenem resistant isolates ranged from 8 to 64 µg/mL (Table 1). All the carbapenem resistant isolates (n = 11) were phenotypically multidrug resistant (i.e. strains showing resistance to at least two groups of antibacterials), and six were ESBL producers. Extended spectrum beta-lactamase and carbapenemase producing Gram-negative bacteria have been reported in neonates of India (DEVI et al., 2018). The increase in infections associated with multi-drug resistant and ESBL producing pathogens demands the use of carbapenems. This might have led to the emergence of carbapenem resistance among organisms, and causes a challenge for treatment (GANDRA et al., 2016).

The antibiogram of the CRE (n = 10) and CRP (n = 1) showed that the isolates were 100% resistant to ATZ, CTR, CAZ, CTX, CPD, CFM, and GEN. The isolates were also resistant to TE (9/11, 81.8%), CIP (9/11, 81.8%), COT (7/11, 63.6%), C (5/11, 45.5%), F/M (5/11, 45.5%) and TZP (5/11, 45.5%) (Table 1). Resistance to sulfonamides, aminoglycosides, trimethoprim, tetracycline, and fluoroquinolones was detected in migratory avian species in Pakistan (MOHSIN et al., 2017).

Table 1. Details of carbapenem resistant isolates (n = 11) from the leopards and blackbucks

S. No	Isolates	Host	Sex	Antibacterial susceptibility pattern																				
				CIP	ATZ	COT	CX	CTX	CFM	CPD	CPM	CAZ	CTR	C	GEN	TE	FOX	F/M	TZP	NX	IMP	MEM	ETP	
1	L2 - <i>E. coli</i>	leopard	F	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	
2	PL2 - <i>E. coli</i>	leopard	F	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	
3	L3 - <i>E. coli</i>	leopard	F	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4	L3a - <i>E. coli</i>	leopard	F	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R
5	L4 - <i>E. coli</i>	leopard	F	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R
6	L4a - <i>E. coli</i>	leopard	F	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
7	PL6a - <i>E. coli</i>	leopard	M	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S
8	L7 - <i>E. coli</i>	leopard	F	I	R	I	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
9	L5 - <i>E. coli</i>	leopard	F	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
10	9B - <i>P. aeruginosa</i>	black-buck	F	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I
11	2B - <i>E. coli</i>	black-buck	M	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	I

R - resistant; I - intermediately resistant; S - sensitive; ATZ - aztreonam; C - chloramphenicol; CTR - ceftriaxone; CPD - cefpodoxime; CAZ - ceftazidime; CFM - ceftixime; CTX - cefotaxime; CPM - cefepime; CIP - ciprofloxacin; COT - cotrimoxazole; ETP - Ertapenem; F/M - Nitrofurantoin; FOX - ceftiofur; GEN - Gentamicin; IMP - imipenem; NX - norfloxacin; MEM- meropenem; TE - tetracycline; TZP - piperacillin-tazobactam; ND - not done; NT - Nontypeable

Table 1. Details of carbapenem resistant isolates (n = 11) from the leopards and blackbucks (continued)

S. N ^o	Minimum inhibitory concentration (µg/mL)			Efflux pump	Virulence and antibacterial resistance determinants	Accession number	Plasmid type	Sequence type
	CTX	CTR	MRP					
1	8	16	16	Active	blaNDM-7, tetA, qnrS	KU318701	Inc1	ST-297
2	16	32	32	Not- active	blaNDM-7, blaCTX-M-1, sul1, tetA, qnrS, stx2	KU318696	Inc1	ST-297
3	16	8	8	Not- active	blaNDM-5, blaAmpC, blaCTX-M-1, tetA, stx2	KU318691, KU382501	Inc1	ST-297
4	8	32	16	Not- active	blaNDM-5, blaTEM-1, sul1, qnrA	KU318700	Inc1	ST-297
5	16	16	32	Not- active	blaNDM-5, sul1, tetA, qnrS	KU318698	Inc1	ST-297
6	16	32	64	Not- active	blaNDM-7, sul1, tetB, qnrB	KU318702	Inc1	ST-297
7	8	8	16	Not- active	blaNDM-5, sul1, tetA, sxt1	KU318699	NT	NT
8	32	32	16	Active	blaCTX-M-1, tetB, sul1	-	ND	ND
9	16	16	8	Active	blaCTX-M-1, sul1	-	ND	ND
10	32	64	8	Not- active	blaNDM-1, blaAmpc, blaTEM-1	KU523520, KU382500	ND	ND
11	16	16	16	Active	tetA, qnrS	-	NT	NT

R - resistant; I - intermediately resistant; S - sensitive; ATZ - aztreonam; C - chloramphenicol; CTR - ceftriaxone; CPD - cefpodoxime; CAZ - ceftazidime; CFM - cefixime; CTX - cefotaxime; CPM - cefepime; CIP - ciprofloxacin; COT - co-trimoxazole; ETP - Ertapenem; F/M - Nitrofurantoin; FOX - cefoxitin; GEN - Gentamicin; IMP - imipenem; NX - norfloxacin; MEM- meropenem; TE - tetracycline; TZP - piperacillin-tazobactam; ND - not done; NT - Nontypeable

The genotypic screening, targeting five major carbapenemase genes, showed that seven CRE and one CRP (blackbuck, 9B) isolates were positive for the *bla*NDM carbapenemase gene. Of the eight NDM positives, four CRE isolated from leopards were NDM-5, three CRE from leopards were NDM-7, and one CRE from a blackbuck was a NDM-1 variant. The *bla*NDM and *bla*AmpC gene sequences were submitted to GenBank, and the accession numbers were obtained (Table 1). Carbapenemase producing *P. aeruginosa* was reported in livestock animals in Lebanon (Al BAYSSARI et al., 2014). Carbapenem resistant *bla*NDM positive *E. coli* were detected in food animals and pet animals in India (PRUTHVISHREE et al., 2017; PRUTHVISHREE et al., 2018; MURUGAN et al., 2019) and wild black kites (FISCHER et al., 2013). Efflux pump mediated carbapenem resistance was noticed in three leopard and one blackbuck CRE isolates (L2, L5, L7, 2B). One CRE (L2) isolate of a leopard harboured both *bla*NDM -7 genes and an active efflux pump. Earlier, efflux pump mediated carbapenem resistance was observed in *E. coli*

isolated from piglets (PRUTHVISHREE et al., 2017). The carbapenem resistance of the isolates (n = 11; CRE = 10; CRP = 1) was either by means of carbapenem genes or active efflux pumps.

All the carbapenem resistant and MBL positive isolates (n = 8) carried *bla*NDM genes. In the ESBL genotypic screening (n = 6), four isolates carried *bla*CTX-M-1 genes. In addition, *bla*TEM-1, *bla*AmpC, *qnrA*, *qnrB*, *qnrS*, *tetA*, *tetB* and *sul1* resistance genes were detected in CRE isolates. Carbapenem and extended spectrum beta-lactam co-resistance in *E. coli* have been reported in critical care human patients (JOHNSON et al., 2017) and *bla*CTX-M-1 has been reported in *E. coli* isolated from healthy animals and humans (BEN SALLEM et al., 2014). On Shiga toxin virulence screening, *Stx1*, *Stx2* genes were detected in two and one isolates, respectively. *E. coli* isolates carrying both carbapenem resistance and virulence factors have been reported in humans, dogs and food animals (CANDAN and AKSOZ, 2017; PRUTHVISHREE et al., 2018; MURUGAN et al., 2019; VINODH KUMAR et al., 2019; TAMTA et al., 2020). Shiga

toxigenic markers were detected in 16.0% of the ESBL producing *E. coli* of piglets (NIRUPAMA et al., 2018). The presence of virulence markers and carbapenem resistance genes in *E. coli* isolates from captive wildlife is of critical importance. Plasmid replicon typing of CRE revealed that the *bla*NDM5 and *bla*NDM7 genes were harboured on Inc11 plasmids. The plasmid multilocus sequence typing (pMLST) of the *bla*NDM isolates displayed ST 297, which was also reported in the ESBL producing *E. coli* isolated from wild birds (YAHIA et al., 2018). Interestingly, the CRE from leopards harboured Inc11 plasmids carrying NDM genes and also a similar sequence type (ST- 297), indicating the transmission of resistance. Inc11 type plasmids were reported in ESBL producing *E. coli* from migratory avian species in Pakistan (MOHSIN et al., 2017), and most of the ESBL genes were carried on Inc11 plasmids (GARCIA-FERNANDEZ et al., 2008).

Conclusion

This is a *prima facie* report on carbapenem resistance in *E. coli* and *pseudomonas* in captive wildlife. Despite the small sample size, the study highlighted the emergence of carbapenem resistance in *E. coli* and *P.aeruginosa* isolated from apparently healthy leopards and blackbucks. The occurrence and spread of carbapenem resistance bacteria in captive wildlife should be a major public health priority. Thus, surveillance and appropriate measures for the control of their spread are extremely important.

Conflict of interest

There is no conflict of interest among the authors.

Funding and Ethical Approval

The authors thank the Director, IVRI, for providing the necessary funds to carry out the research work. The research meets all applicable standards with regard to the ethics of experimentation and research integrity, and the following is certified/declared to be true.

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SAŽETAK

Cilj rada bio je istražiti slučajeve otpornosti bakterija *E. coli* i *P. aeruginosa* na karbapenem u zdravih antilopa i leoparda iz zatočeništva u Indiji. Uzorci izmeta antilopa (n = 7) i leoparda (n = 7) obrađeni su kako bi se izolirale bakterije *E. coli* (CRE) i *P. aeruginosa* (CRP) otporne na karbapenem. Iz uzoraka izmeta (n = 14) dobiveno je 40 izolata (leopard n = 26, antilopa n = 14) *E. coli* i 2 izolata *P. aeruginosa* (antilopa n = 2). Pronađeno je 11 izolata otpornih na karbapenem, od kojih je 10 *E. coli* i 1 *P. aeruginosa*. Određena je minimalna inhibicijska koncentracija (MIK) za meropenem za izolate otporne na karbapenem, od 8 za *E. coli* i 64 µg/mL za *P. aeruginosa*. Svi izolati *E. coli* i *P. aeruginosa* fenotipski su bili otporni na širok spektar lijekova, a 6 izolata *E. coli* proizvodilo je beta-laktamaze širokog spektra (ESBL). Genotipskim probirom 7 izolata *E. coli* i 1 izolat *P. aeruginosa* bili su pozitivni na karbapenemaza gen *bla*NDM. Otpornost na karbapenem putem efluks pumpe zabilježena je u 4 izolata *E. coli* (36,4 %, 4/11). Od 6 ESBL producirajućih CRE, 4 izolata nosila su gen *bla*CTX-M-1. Izolati *E. coli* također su sadržavali *bla*TEM-1, *bla*AmpC, *qnrA*, *qnrB*, *qnrS*, *tetA*, *tetB* i *sul1* gene otpornosti. Pretragom na šiga-toksin, *Stx1* i *Stx2* geni utvrđeni su u dva odnosno jednom izolatu. Tipiziranje plazmida CRE otkrilo je prisutnost *bla*NDM gena na Inc11 plazmidu. Multilokusno tipiziranje sekvencija plazmida (pMLST) izolata otkrilo je sekvenciju tipa (ST) 297. Pojava otpornosti bakterija na karbapenem u divljih životinja iz zatočeništva trebala bi biti javnozdravstveni prioritet.

Ključne riječi: ESBL; *E. coli*; šiga-toksin; divlje životinje; otpornost na karbapenem; NDM
