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Molecular characterization of extensively drug resistant (XDR), extended spectrum beta-lactamases (ESBL) and New Delhi Metallo beta-lactamase-1 (*bla*NDM1) producing *Escherichia coli* isolated from a male dog - a case report

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ABSTRACT

In this article, we report the molecular characterization of extensively drug resistant (XDR), extended spectrum, class C beta-lactamases and NDM-1 carbapenemase producing *E. coli,* isolated from the scrotal fluid of a 3-year-old male dog. In an antibiotic susceptibility test the *E. coli* isolate was susceptible only to tigecycline and resistant to all clinically applicable antibiotics tested in the study. The minimum inhibitory concentration (MIC) of meropenem, cefotaxime and cefepime was 256, 128 and 64 µg/mL, respectively. On genotypic screening by PCR, the isolate was positive for *bla*NDM, *bla*CTX-M, *bla*AmpC, *bla*TEM and *sul1* genes. The isolate was a ESBL, AmpC and metalo beta-lactamase producer. On molecular pathotyping, the isolate harbored the Shiga toxin producing gene (*Stx*2). The extensively drug resistant, carbapenem resistant and ESBL producing *E. coli* constitutes a major public health concern, since there is a great chance of dissemination of resistance genes to humans due to the close association of humans and companion animals. To the best of our knowledge, this is the first report of *bla*NDM1 isolated from a dog in India.

Key words: dog; NDM; ESBL; carbapenemase; beta-lactamase

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Introduction

Nowadays keeping companion animals, especially dogs and cats, is gaining popularity due to the changes in lifestyle of human beings and also the growing attention given to pet care. As a result of increased pet care, antimicrobial agents are commonly used in companion animal practice, often including antimicrobial preparations used in human medicine. At present, antimicrobial drug resistance is a problem all over the world. The blanket use of antimicrobials has led to the emergence and spread of antimicrobial drug resistance in microbes, including pathogens, leading to imbalance in the microbiota of our systems and environment. The occurrence of antimicrobial resistant strains in food producing and companion animals is increasing and leading to alarm that the cross species spread of these resistant bacteria could be of great public health significance (STOLLE et al., 2013; GHATAK et al., 2013; PRUTHVISHREE et al., 2017). The term "extensive drug resistance" (XDR) is used to signify gram negative bacteria resistant to all potentially effective antibiotics, except one to two antibiotics left as treatment options (FALAGAS and KARAGEORGOPOULOS, 2008). The antibacterial resistant bacteria harboring virulence markers are hazardous, since they cause infections which are difficult to treat. Shiga toxin (SETC) producing *E. coli* is of public health concern, and animals may be reservoirs, and they produce two potent cytotoxins (*Stx*1, *Stx*2), which inhibit protein synthesis (BENTANCOR et al., 2012).

Rising numbers of carbapenem resistant and ESBL-producing *E. coli* in food-producing and companion animals (CARATTOLI, 2008; SMET et al., 2010; GHATAK et al., 2013; PRUTHVISHREE et al., 2017) may spread into the environment and also to humans. Companion animals have become more similar to family members and they share intimate contact with their owners, which can result in transmission cycles of multi-drug resistant bacteria (MDR), as it is known that the microbiota of pets and their owners can be very similar (EWERS et al., 2011; WALTHER et al., 2012; JOHNSON et al., 2008). Increasing infections with antibacterial resistant bacteria are also accompanied by a decrease in the efficacy of treatment (ALANIS, 2005; STOYCHEVA and MURDJEVA, 2006; WALSH and FANNING, 2008; AJIBOYE et al., 2009). The present study reports the molecular characterization of a XDR *E. coli* harboring *bla*NDM1 gene.

Materials and methods

Sample collection and Isolation of E. coli. A male Labrador dog aged 3 years was presented to the referral veterinary hospital, Indian Veterinary Research Institute, Izatnagar, Bareilly, India with loss of appetite, scrotal edema and fever. The scrotal fluid was collected aseptically and processed for bacteriological analysis and biochemical characterization (QUINN et al., 1994).

Phenotypic screening for carbapenem resistance and metallo betalactamase and extended spectrum and class C beta-lactamase production. The strains (Accession No: KT853018, KR296661, KX090926 and KT593874) were collected from the repository maintained by the Division of Epidemiology, Indian Veterinary Research Institute, Izatnagar, to serve as reference strains. The biochemically confirmed *E. coli* isolate was screened for antibiotic susceptibility patterns with amikacin (30 µg), amoxicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), aztreonam (30 µg), cefoxitin (30 µg), cefixime (5 µg), cefpodoxime (10 µg), cefotaxime/clavulanic acid (30/10 µg), cephalexine (30 µg), ceftrioxone (30 µg), ceftazidime (30 µg) ceftazidime/clavulanic acid (30/10 µg), cefipime (30 µg), cefadroxil, (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), cefoperazone (75 µg), cefepime-tazobactam (80/10 µg), colisitin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), co-trimoxazole (1.25/23.75 µg), ertapenem (10 µg), fosfomycin (200 µg), gatifloxacin (5 µg), imipenem (10 µg), meropenem (10 µg), pipercillin (100 µg), tetracycline (30 µg), nitrofurantoin (300 µg), gentamicin (10 µg), triple sulpha(0.25 µg), ofloxacin (5 µg), norfloxacin (10 µg), piperacillin-tazobactam (100/10 µg), polymyxin B (300 U), tobramycin (10 µg), vancomycin (5 µg), tigecycline (15 µg),) and moxalactam (30 µg). The Clinical and Laboratory Standard Institute (CLSI, 2015) breakpoints were used for inference of antibacterial susceptibility pattern.

The isolate was also assayed for carbapenem susceptibility and ESBL production. Metallo beta-lactamase production (MBL) detection was performed using meropenem (10 µg), imipenem (10 µg) and ertapenem (10 µg) along with metal ion chelator Ethylene diamine tetra acetic acid (EDTA) (1900 mg) disks for the Double disk synergy test (DDST) as mentioned by GALANI et al. (2008). A keyhole reaction, i.e. any synergistic inhibition between the carbapenem drug and EDTA was tentatively assumed for the metallo-beta lactamase producer. The minimum inhibitory concentration (MIC) for meropenem and colistin was determined using an Etest strip (HiMedia, India). For phenotypic identification of ESBL producers the combination disc method was used (ANDREWS, 2012) with cefotaxime, ceftazidime, with and without clavulanic acid. The triple ESBL, ESBL & AmpC detection Ezy MIC Strip (#cat EM079, EM081; HiMedia, India) was used to phenotypically confirm the extended spectrum and class C beta-lactamase production. The minimum inhibitory concentration (MIC) of cefotaxime, cefepime, ceftazidime, imipenem, meropenem, ertapenem and colistin was determined by Estrips (HiMedia, India).

Molecular detection of antimicrobial resistance and virulence genes. The genomic DNA was extracted by QIAamp DNA Mini Kit (Qiagen, India). All the PCR reactions were carried out in 96 well thermal cyclers (MJ Research PTC-200, Peltier Thermal Cycler, USA). The details of primers and PCR conditions used for major carbapenemase genes, ESBL producing genes, AmpC and virulence genes for Shiga toxin producing *E. coli* (STEC; *Stx*1 and *Stx*2), enteropathogenic *E. coli* (EPEC: *eae*A), enterohemolysin (*hly*A)

Table 1. Details of the primers and PCR cycling conditions

No	Gene	Primers sequence	Cyclical condition	Product size (bp)	Reference
1	*bla*IMP	F- GGAATAGAGTGGCTTAAYTCTC R- GGTTTAAYAAAACAACCAC	95 °C x 5 m/95 °C x1m -55 °C x1m - 72 °C x1m (35 Cycles)/72 °C x10 m	232	Poirel et al. (2011)
2	*bla*VIM	F-GATGGTGTTTGGTCGCATA R-CGAATGCGCAGCACCAG	95 °C x 5 m/95 °C x1m - 55 °C x1m - 72 °C x1m (35 Cycles)/72 °C x10 m	390	Poirel et al. (2011)
3	*bla*OXA	F-GCGTGGTTAAGGATGAACAC R- CATCAAGTTCAACCCAACCG	95 °C x 5 m/95 °C x1m - 55 °C x1m - 72 °C x1m (35 Cycles)/72 °C x10 m	438	Poirel et al. (2011)
4	*bla*NDM	F-GGTTTGGCGATCTGGTTTTC R-CGGAATGGCTCATCACGATC	95 °C x 5 m/95 °C x1m - 55 °C x1m - 72 °C x1m (35 Cycles)/72 °C x10 m	621	Poirel et al. (2011)
5	*bla*KPC	F-CGTCTAGTTCTGCTGTCTTG R- CTTGTCATCCTTGTTAGGCG	95 °C x 5 m/95 °C x1m - 55 °C x1m - 72 °C x1m (35 Cycles)/72 °C x10 m	798	Poirel et al. (2011)
6	*Stx1*	F- TAAATCGCCATTCGTTGACTAC R- AGAACGCCCACTGAGATCATC	95 °C x 5 m/95 °C x45 s - 59 °C x45 s - 72 °C x1m (30 Cycles)/72 °C x6 m	180	Paton and Paton (1998)
7	*Stx2*	F- GGCACTGTCTGAAACTGCTCC R- TCGCCAGTTATCTGACATTCTG	95 °C x 5 m/95 °C x45 s - 59 °C x45 s - 72 °C x1m (30 Cycles)/72 °C x6 m	255	Paton and Paton (1998)
8	*eae*	F-GACCCGGCACAAGCATAAGC R- CCACCTGCAGCAACAAGAGG	95 °C x 5 m/95 °C x45 s - 59 °C x45 s - 72 °C x1m (30 Cycles)/72 °C x6 m	384	Paton and Paton (1998)
9	*hlyA*	F-GCATCATCAAGCGTACGTTCC R- AATGAGCCAAGCTGGTTAAGCT	95 °C x 5 m/95 °C x45 s - 59 °C x45 s - 72 °C x1m (30 Cycles)/72 °C x6 m	534	Paton and Paton (1998)
15	*bla*TEM	F- ATGAGTATTCAACATTTCCG R- CTGACAGTTACCAATGCTTA	95 °C X 5 m / 95 °C X 1 m - 55 °C X 1 m - 72 °C X 1 m (35 Cycles)/72 °C X 7 m	867	Bhattacharjee et al. (2007)
16	*bla*CTXM	F- CAATGTGCAGCACCAAGTAA R- CGCGATATCGTTGGTGGTG	95 °C X 5 m/95 °C X 30 s - 65 °C X 30 s - 72 °C X 30 s (5Cycles) 95 °C X 30 s - 62 °C X 30 s - 72 °C X 30 s (10 Cycles)/95 °C X 30 s - 60 °C X 30 s - 72 °C X 30 s (15 Cycles)/95 °C X 30 s - 58 °C X 30 s - 72 °C X 30 s (15 Cycles)/72 °C X 7 m	540	Dutta et al. (2013)
17	*bla*AmpC	F- CCCCGCTTATAGAGCAACAA R- TCAATGGTCGACTTCACACC	94 °C X 5 m/94 °C X 30 s - 57 °C X 30s - 72 °C X 30 s (30 Cycles)/72 °C X 7 m	631	Shahid et al. (2012)
18	*bla*SHV	F- AGGATTGACTGCCTTTTTG R- ATTTGCTGATTTCGCTCG	94 °C X 5 m/94 °C X 30 s - 52 °C X 30s - 72 °C X 30 s (30 Cycles)/72 °C X 7 m	393	Bhattacharjee et al. (2007)
19	*Sul1*	F- CGGCGTGGGCTACCTGAACG R- GCCGATCGCGTGAAGTTCCG	95 °C X 5 m/ 95 °C X 30 s - 65 °C X 1 m - 72 °C X 1 m (35 Cycles)/72 °C X 7 m	433	Kerrn et al. (2002)

are listed in Table 1. The amplified products were visualized by a gel documentation system (UVP, UK) after electrophoresis in 1.5% (w/v) agarose gel containing ethidium bromide (0.5 µg/mL, Loba Chemie, India).

Cloning and sequencing. Amplified positive PCR products were purified using a gel purification kit (Qiagen, India) and cloned in a p-Drive cloning vector (Qiagen, India). The plasmids containing the expected insert were sequenced using commercial sequencing services (Eurofins Ltd, Bangalore) for further purification and sequencing by the Sanger dideoxy method. After sequencing, homology searches were made using the BLAST algorithm available at http://blast.ncbi.nlm.nih. gov/Blast.cgi.

Results

The bacteriological analysis of scrotal fluid yielded an *E. coli* isolate and on phenotypic antimicrobial screening the isolate was found to be extensively drug resistant, of extended spectrum, class C and a metallo betalactamase (MBL) producer. The isolate was resistant to all the antimicrobial agents screened except tigecycline. The MIC of the different antibiotics are given in Table 2.

Table 2. Minimum inhibitory concentration, virulence and antibiotic resistance markers of *E. coli* isolate

Isolate	Minimum Inhibitory Concentration (µg/mL)							Virulence marker	Antibiotic resistance markers	GenBank Accession number
	CTX	CPM	CAZ	IMP	MRP	ETP	CL			
7897P *E. coli*	>128	>64	>64	>256	>256	>32	>8	*Stx*2	*bla*NDM, *bla*CTX-M, *bla*AmpC, *bla*TEM, *sul1*	KX158451 KX090926 KX158450

(CTX-cefotaxime; CPM- cefepime; CAZ-ceftazidime; IMP-imipenem; MRP- meropenem; ETP-ertapenem; CL-colistin)

On phenotypic MBL screening, the isolate showed a keyhole reaction between EDTA and meropenem. On MBL Estrip the ratio between meropenem MIC and meropenem with EDTA MIC was more than 8, which indicated the isolate as a potential MBL producer. The meropenem MIC of the isolate was more than 256 µg/mL and meropenem with EDTA MIC was 64 µg/mL. The combined disc method showed the difference in zone of inhibition between cefotaxime, ceftazidime, with and without clavulanic acid, was more than 8 mm, indicating ESBL producing *E. coli*. The ESBL & AmpC detection Ezy MIC Strip showed the ratio between MIX+ (ceftazidime, cefotaxime & cefepime with clavulanic acid) and MIX- (ceftazidime, cefotaxime & cefepime) was more than 8, indicating the isolate as a potential ESBL and AmpC producer.

Molecular detection and sequence analysis revealed the isolate was positive for beta-lactamase genes *bla*TEM (Accession No: KX158451) *bla*CTX-M, *bla*AmpC (Accession No: KX158450), the carbapenemases gene *bla*NDM1 (Accession No: KX090926) and the gene for sulphonamide resistance *sul*1. On blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) the dog *E. coli* isolate *bla*TEM, *bla*AmpC and *bla*NDM1 sequences had 100 per cent homology with *bla*TEM, *bla*Ampc, *bla*NDM1 sequences of bacterial isolates from humans and food animals.

Discussion

In general, ESBL producing and carbapenem resistant bacteria screening is limited to humans and the hospital environment, although recent reports suggest the emergence of such resistant pathogens in livestock, and companion and food animals (GHATAK et al., 2013; PRUTHVISHREE et al., 2017). There is an urgent need for routine screening for extended spectrum beta-lactamase producing and carbapenemase resistant bacteria in animals, since these mechanisms of resistance are transferable and are of public health significance. The companion animals' role has become more similar to that of family members, and they share intimate contact with their owners, which can result in transmission cycles of multi-drug resistant bacteria, as it is known that the microbiota of pets and their owners can be very similar (JOHNSON et al., 2008; EWERS et al*.,* 2011; SONG et al., 2013; WALTHER et al., 2012).

STOLLE et al. (2013) studied carbapenemase producing *Escherichia coli* in dogs harbouring *bla*CTXM-1, *bla*OXA-2 and *bla*TEM-1 genes. YOUSFI et al. (2015) reported NDM-5 producing *E. coli* in a three-year-old German shepherd dog. HARADA et al. (2012) screened fecal samples of apparently healthy pups below two months of age from Japanese kennels, with no history of antimicrobial use, and they were found to harbor MDR *E. coli* isolates, including ESBL producers. The transmission of MDR pathogenic *E. coli* between companion animals and humans has been documented by a few studies (EWERS et al., 2010; PLATELL, et al. 2011).

RZEWUSKA et al. (2015) studied the frequency of MDR *E. coli* in dogs and cats in Poland and the prevalence of MDR was 66.8% in the isolates studied. Cats and dogs represent potential sources of the spread of antimicrobial resistance, due to the extensive use of antimicrobial agents in these animals and their close contact with humans, and some studies indicate a possible association between antimicrobial use and the emergence of antimicrobial resistance in pets (GUARDABASSI et al., 2004). In the present study, the SETC harboured *bla*CTXM, *bla*TEM, *bla*AmpC, *bla*NDM and *sul*1 genes. BOERLIN et al. (2005) observed a high correlation of antimicrobial resistance and virulence genes of *E. coli* isolates from swine.

The emergence of XDR, carbapenem resistant and ESBL producing *E. coli* reflects the abundant use of broad-spectrum antimicrobials in veterinary care and human health. Household pets can be reservoirs of bacterial species and resistance genes of clinical importance in humans (SANCHEZ et al., 2002; GUARDABASSI et al., 2004). Carbapenem antibiotics are not licensed for veterinary use anywhere in world but, in contrast, the usage of carbapenems is increasing in humans (ASHIRU-OREDOPE et al., 2012). The presence of carbapenem resistant *E. coli* in the dog of the present study might be from humans or the contaminated environment. Carbapenem resistant, ESBL producing and MDR *E. coli* in companion animals is an important cause of concern in human medicine due to the fact that carbapenems are vital for the effective therapy of community-acquired and healthcare-associated infections caused by ESBL producing bacteria. Resistance to this class of antibiotic compromises the therapeutic options for patients by leaving only a few or in some cases no other antimicrobials available for effective treatment. To the best of our knowledge, this is the first report of *bla*NDM1 isolated from a dog in India.

Conclusion

The isolation of extensively drug resistant, extended spectrum, class C beta-lactamases and *bla*NDM-1 carbapenemase producing *E. coli* from a dog is of great public health concern and there is a great possibility of spread of these genes to other pathogens. Therefore preventive measures are needed to combat the rise of antimicrobial resistant pathogens in human and animal populations.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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SAŽETAK

U radu se izvješćuje o molekularnoj karakterizaciji bakterije *E. coli* izdvojene iz skrotalne tekućine psa, iznimno otporne na antibiotike širokog spektra, koja proizvodi klasu C beta-laktamaza i NDM-1 karbapenemazu. Pas je bio u dobi od od tri godine. Izolat *E. coli* bio je osjetljiv samo na tigeciklin, a otporan na sve antibiotike primjenjivane u kliničkoj praksi. Minimalna inhibicijska koncentracija (MIC) za meropenem iznosila je 256, cefotaksim 128 i cefepim 64 µg/mL. Pretragom genotipa lančanom reakcijom polimerazom izolat je bio pozitivan na gene *bla*NDM, *bla*CTX-M, *bla*AmpC, *bla*TEM i *sul1*. Proizvodio je ESBL, AmpC i metalo-beta-laktamazu. Molekularnom patotipizacijom dokazano je da posjeduje gen za *shiga*-toksin (*Stx*2). *E. coli* otporna na karbapenem, koja proizvodi beta-laktamaze širokog spektra, velika je prijetnja za javno zdravstvo s obzirom na to da postoji velika mogućnost prijenosa *E. coli* s genom za rezistenciju na ljude u bliskom dodiru s kućnim ljubimcima. Ovo je prvo izvješće o *bla*NDM1 dokazanom u psa u Indiji.

Ključne riječi: pas; NDM; ESBL; karbapenemaza; beta-laktamaza

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