Explor Anim Med Res,
Vol.12, Issue - 1, 2022, p. 91-98
DOI : 10.52635/eamr/12.1.91-98

ISSN 2277- 470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

SURVEILLANCE OF CARBAPENEM-RESISTANT GRAM-NEGATIVE BACTERIA FROM ANIMAL SOURCES IN MATHURA REGION, UTTAR PRADESH, INDIA

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Received 05 February 2022, revised 11 June 2022

ABSTRACT: A cross-sectional study was conducted to determine the prevalence of Carbapenem-resistant Gram-negative bacteria (CR-GNB) in animals. The study involves one hundred eighty-four GNB isolates from 214 samples (faeces, milk, pus, and uterine discharge) from Buffalo (N=112), Cattle (N=50) and, Dog (N=52). Healthy and diseased animals reported to Veterinary Clinical Complex were sampled. Carbapenemase production was evaluated by phenotypic methods and presence of metallo β-lactamase genes was assayed by PCR. We observed a 9.78% overall prevalence of CR-GNB in animal sources. CR-GNB was more frequently recovered from companion animals (19.23%) when compared to livestock (4.93%). IMP (44.4%), VIM (38.8%), and OXA-48 (16.66 %) were the main MBLs observed in the study.

Key words: Gram-negative bacteria, Carbapenem, Antibiotic resistance, Animals.

INTRODUCTION

Carbapenems are a beta-lactam class of antibiotics. Carbapenems are stable to most beta-lactamase enzymes mediated inactivation unlike other beta-lactam antibiotics (Perrott *et al.* 2010). Carbapenems are clinicians' preferred choice for the therapeutic management of serious infections caused by MDR pathogens (Falagas and Karageorgopoulos 2009). The ever-growing dependence has led to the recent emergence of carbapenem-resistant bacterial strains. Genes encoding carbapenem-resistance are often associated with mobile genetic elements leading to their spread across a variety of carbapenem-resistant Gram-negative bacteria (CR-GNB) (Schwaber *et al.* 2011).

Most clinically relevant carbapenem resistance appears to have arisen and propagated because of its therapeutic uses in humans (Poirel *et al.* 2014). CR-GNB has been isolated predominantly from humans and environmental samples. Presently, carbapenems are not authorized for use in veterinary medicine in most parts of the world; hence carbapenem resistance is not common in GNB isolated from animals. Notwithstanding, CR-GNB has been detected in livestock, companion animals, and their environment by several workers across the globe in the recent past (Wang *et al.* 2012, Woodford *et al.* 2014). The overall carriage rate of CR-GNB has been on the rise in food-producing animals and their environment in India (Ghatak *et al.* 2013, Pruthvishree *et al.* 2017, Nirupama *et al.* 2018). The colonization of CR-GNB in livestock and companion animals has a potential multiplier effect on rapid dissemination to humans through close contact, environmental and, food-borne transmission. The prevalence of CR-GNB has risen significantly in animal healthcare settings over the past few years, but the data on the population prevalence among livestock and pet animals are scanty in India. Therefore, it is necessary to include CR-GNB for routine epidemiological investigations in animal population. The present study aimed to predict the population prevalence of CR-GNB from various animal sources and their characterization.

MATERIALS AND METHODS

Bacterial Isolates

The carbapenem resistance surveillance includes 214 GNB samples (faces, milk, pus, and uterine discharge)

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collected between during May 2018 and April 2021 from separate animals in and around the Mathura region, India, without any inclusion or exclusion criteria. Sample details given in Table 1. A total of 184 GNB were isolated, and further identified by the standard microbiological procedure (Barrow and Feltham 2004). Control strain includes *Klebsiella pneumoniae* ATCC BAA 1705^{+KPC} and *Klebsiella pneumoniae* ATCC BAA 1706.

Antibiotic susceptibility test

Antimicrobial susceptibility analysis was carried out by standard Kirby-Bauer disk diffusion method using Mueller-Hinton agar (Sigma-Aldrich) following the CLSI (2017) guidelines and interpretative criteria (Table S2).

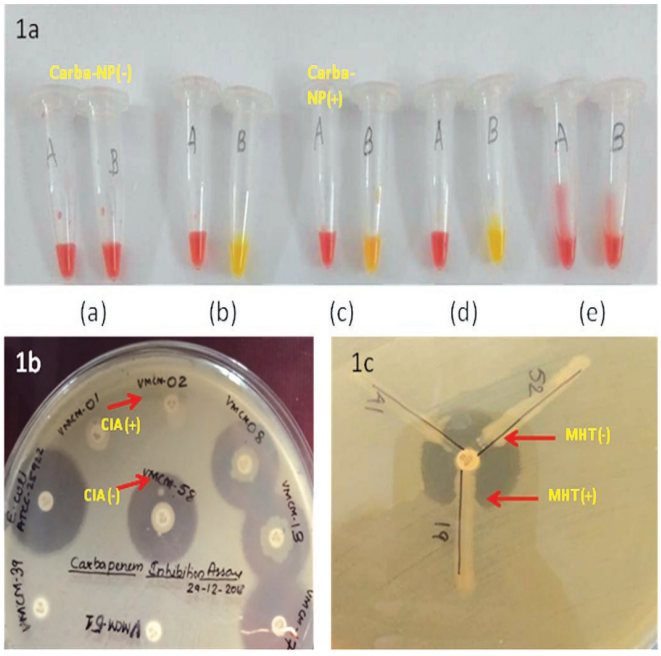

Fig. 1. Representative results obtained by Modified Carba NP test. [b,c,d] represents results obtained by imipenem- non susceptible strain where 'A' tube (red colour/control tube) is a negative and tube 'B' (yellow colour) is a positive result *i.e.*, carbapenemase-producing isolate. [a & e] represents results obtained by imipenem-susceptible strain where both tubes (A,B) are negative results *i.e.*, carbapenemase-non producing isolate. (a) Negative Control *E. coli* ATCC 25922 (b) Positive Control *Klebsiella pneumoniae* ATCC BAA 1705 [1b] Representative results obtained by mCIM: CIA (-) represents negative result of ertapenem susceptible strain *i.e.*, zone = 20 mm and CIA (-) represents positive result of ertapenem non-susceptible strain *i.e.*, no zone of inhibition around the carbapenem disk. *E. coli* ATCC 25922 has been used as reporter strain. [1c] Representative results obtained by Modified hodge test: Isolates VA-41 and VA-52 were Modified Hodge Test Negative, while isolates VA-19 was Modified Hodge Test Positive.

Table 1. Details of samples collected.

Species	Sample type			
	Pus	**Uterine discharge**	**Mastitis Milk**	**Fecal sample**
Buffalo (N=112)	1	52	33	26
Cattle (N=50)	1	41	-	8
Dog (N=52)	-	-	-	52
Total (N=214)	2	93	33	86

The panel of antimicrobial agents consisted of 10 different antimicrobial-impregnated disks: namely, ertapenem (10 µG), cefotaxime (30 µG), ceftazidime (30 µG), gentamicin (10 µG), ampicillin (10 µG), amoxicillin-clavulanate (10µg), ciprofloxacin (5 µg), cefoxitin (30 µg), ceftriaxone (30 µg) and cefpodoxime (10 µg). The zone of inhibition was measured in mm and interpreted as sensitive, intermediate, or resistant.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) of carbepenems (imipenem, ertapenem and meropenem) were tested by the broth microdilution method (Wiegand *et al.* 2008). Standardized bacterial inoculums were prepared for each isolate to give a turbidity equivalent to that of a 0.5 McFarland standard corresponding to 1 X10^8 cfu/ml. The final test concentration of the bacteria was achieved by further diluting the adjusted suspension by a factor of 1:100 to achieve approximately 5 × 10^5 cfu/ml. The working antibiotic stock solution was prepared by 1:10 dilution of antibiotic stock solution (potency adjusted 1.28 mg/ml) in Muller Hinton Broth (MHB). The plates were covered by sterile covers and incubated at 37ºC for 18-24 h. The lowest concentration of the antibiotics that did not have visible bacterial growth was defined as the MIC.

Phenotypic and genotypic carbapenemase identification

Carbapenemase activity was assessed by modified Carba NP test (Rudresh *et al.* 2017), Carbapenemase Inactivation Assay (Zwaluw *et al.* 2015) and Modified Hodge test (Amjad *et al.* 2011). For genotypic detection, DNA isolated by the snap chill method was subjected to a target amplification of β-Lactamase genes using a panel

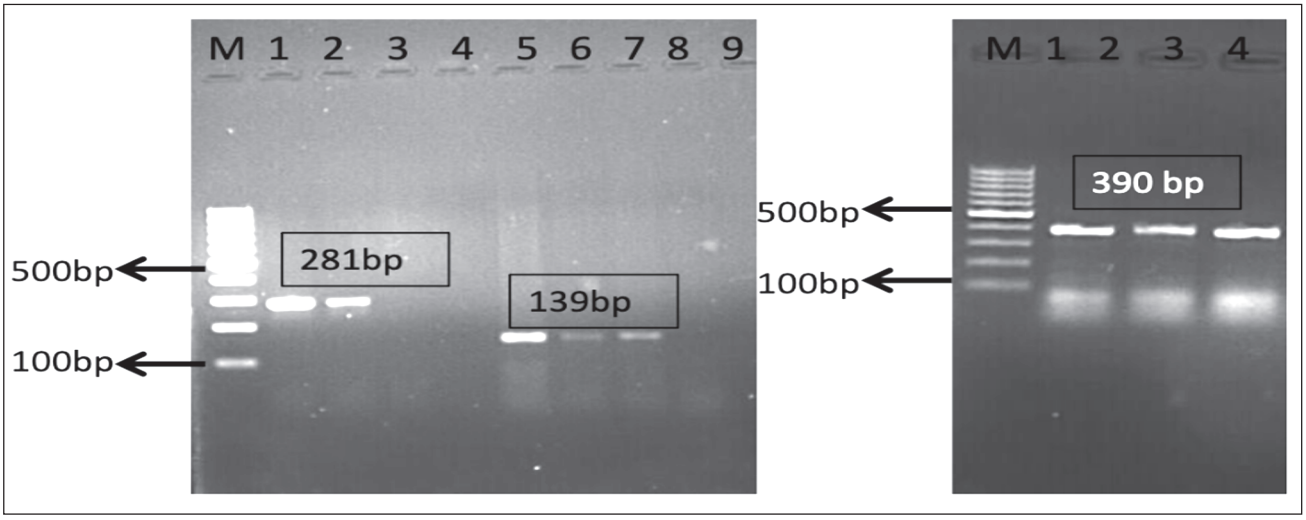

Fig. 2. [a] PCR amplification of blaOXA48: 281 bp, blaIMP: 139bp, Lane M: 100 bp DNA Ladder, Lane 1-9: Test Isolates [b] PCR amplification of blaVIM : 390 bp , Lane M: 100 bp DNA Ladder, Lane 1-8: Test Isolates.

of primers for detection of OXA-48, KPC, VIM, IMP, and NDM genes by multiplex PCR (Dallenne *et al.* 2010). A 25 µl reaction mixture containing 12.5 µl Dream Taq Master Mix, variable number of primers and 2 µl of isolated DNA template was used. Amplification was carried involving initial denaturation at 94^{0}C for 10 min and 30 cycles of denaturation at 94^{0}C for the 40s, annealing at 55^{0}C for 40s, and extension at 72^{0}C for 1 min followed by final elongation step at 72^{0}C for 7 min. A. The primer concentration and amplification conditioned for the PCR reactions were used as per the Table 1.

RESULTS AND DISCUSSION

Between May 2018 and April 2021, we processed 214 non-repeated samples comprised of faces, milk, uterine swab, and pus. A total of 184 GNB isolates were obtained on a MacConkey agar plate. A total of 18 CR-GNB isolates including *Escherichia coli* (n=12), *Klebsiella pneumoniae* (n=2), *Citrobacter freundii* (n=2), *Enterobacter cloacae* (n=1), and *Pseudomonas aeruginosa* (n=1) showed reduced susceptibility to ertapenem, based on zone interpretative criteria. The carbapenem resistance has been reported in *E. coli* (Zhang *et al.* 2013), *Klebsiella pneumonia* (Diab *et al.* 2017), *Enterobacter*, *Citrobacter* (Mollenkopf *et al.* 2017). MIC of 18 CR-GNB isolates for imipenem, meropenem, and ertapenem was shown in Table 2. MIC of 18 CR-GNB isolates for imipenem, meropenem, and ertapenem were detected in the range of 0.625 µg/ml to 64 µg/ml, 0.0625 µg/ml to 1 µg/ml, and 0.312 to 16 µg/ml, respectively (Table 2). None of the CR-GNB isolates were resistant to meropenem. All the isolates exhibited resistance to amoxicillin-clavulanate, while various resistance rates were observed for ceftazidime (83.3 %), cefotaxime (75 %), ceftriaxone (88.8 %), cefpodoxime (94.44 %), and ciprofloxacin (91.6 %). The least frequent resistances were against gentamicin (33.3%).

Out of eighteen carbapenem non-susceptible isolates, thirteen (72.22 %) showed a positive reaction in the carbapenemase biochemical test (Fig. 1) while PCR-based identification revealed the presence of one or more carbapenemase (IMP, VIM, and Oxa-48) in 12 isolates (66.66%). Molecular testing showed the presence of IMP, VIM, and OXA-48 MBLs in eight (44.44%), seven (38.88%), and three isolates (16.66%), respectively (Fig. 2, Table 2). All three OXA-48 bearing isolates were

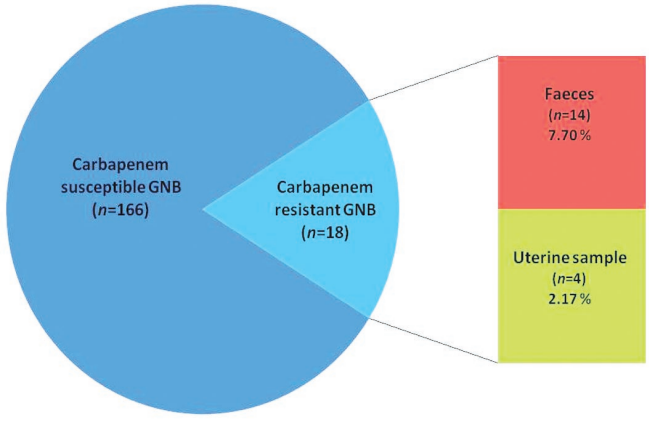

Fig. 3. Distribution of CRE-GNB isolates from various sample sources.

Table 2. Details on the tests performed using different carbapenam suspected isolates.

Isolate No	Source	Sample Origin	Isolate	Antibiotic resistant profile	MIC			MBL gene
					Ertapenem	Imipenem	Meropenem	
VS-01	Cow	Uterine discharge	*Pseudomonas aeruginosa*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	8 µg	32 µg	*0.5* µg	*bla*IMP, *bla*VIM
VS-02	Cow	Uterine discharge	*E. coli*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	1 µg	32 µg	0.5 µg	*bla*IMP
VS-39	Cow	Uterine discharge	*Enterobacter cloacae*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	16 µg	4 µg	0.0625 µg	*bla*IMP, *bla*VIM
VS-51	Cow	Uterine discharge	*E. coli*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	4 µg	64 µg	0.5 µg	*bla*IMP, *bla*VIM
VA-19	Dog	Faecal	*E. coli*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, GM, FOX, ETP	6 µg	1 µg	1 µg	*bla*Oxa48
VA-52	Dog	Faecal	*E. coli*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, GM, FOX, ETP	2 µg	0.125 µg	0.0625 µg	*bla*IMP
VA-53	Buffalo	Milk	*Citrobacter freundii*	AMC, CPD, CRO,CTX, AM, CIP, GM, ETP	4 µg	0.0625 µg	0.125µg	*bla*IMP
VA-55	Dog	Faecal	*E.coli*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	8 µg	0.125 µg	0.0625 µg	*bla*Oxa48
VA-66	Dog	Faecal	*Klebsiella pneumoniae*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, GM, FOX, ETP	6 µg	1 µg	0.25 µg	-
VA-77	Buffalo	Faecal	*Klebsiella pneumonia*	AMC, AM, FOX, ETP	0.25 µg	2 µg	0.125µg	*bla*VIM
VA-99	Buffalo	Faecal	*E. coli*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	4 µg	1µg	0.25 µg	-
VA-100	Buffalo	Faecal	*E. coli*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	16 µg	8 µg	0.125 µg	*bla*IMP
VU-02	Dog	Faecal	*E. coli*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	2 µg	32 µg	0.0625 µg	*bla*IMP *bla*Oxa48
VU-03	Dog	Faecal	*Citrobacter freundii*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	4 µg	32 µg	0.0625 µg	*bla*VIM
VU-08	Dog	Faecal	*E. coli*	AMC, CPD, CRO,CTX, CAZ, AM, FOX, ETP	2 µg	64 µg	0.0625 µg	-
VU-14	Dog	Faecal	*E.coli*	AMC, CPD, CRO,CTX, CAZ, AM, FOX, ETP	6 µg	1 µg	0.125 µg	-
VU-16	Dog	Faecal	*E.coli*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	1 µg	4 µg	0.125 µg	-
VU-17	Dog	Faecal	*E.coli*	AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP	1 µg	16 µg	0.125 µg	-

(AMC: Amoxicillin–Clavulanic acid, CPD: Cefpodoxime; AMP**:** Ampicillin, CAZ**:** Ceftazidime, CRO: Ceftriaxone, CIP: Ciprofloxacin, CTX: Cefotaxime, FOX: Cefoxitin, GM: Gentamicin, ETP: Ertapenem).

Table 3. Number (%) of Carbapenem resistant isolates from animal source.

Total GNB isolates	Livestock		Companion Animal
	Buffalo (n =112)	Cattle (n=50)	Dog (n =52)
Carbapenem resistant GNB	4 (3.57%)	4 (8.00%)	10 (19.23%)

recovered from dogs. Three out of 18 isolates carried both VIM and IMP, while the co-existence of IMP and OXA-48 was found in one isolate. Previous reports suggest rare prevalence of VIM and Oxa-48 carbapenemase genes from bacterial strains of animal sources in India, however, recent report indicate more frequent occurrence of OXA-48, VIM and IMP genotype from fecal sample of the piglets, calves and dogs (Nirupama *et al.* 2018, Murugan *et al.* 2019, Sankar *et al.* 2021). The carbapenemase genes variants (KPC and

Table S1. Primers used in PCR analysis.

β-lactamase (s) targeted	Primer name	Sequence (5'-3')	Primer concentration (20 picomol)	Reference
OXA-48-like	OXA-48_for	GCTTGATCGCCCTCGAT	20	
	OXA-48_rev	GATTTGCTCCGTGGCCGAAA	20	
New Delhi metallo-beta lactamase (NDM)	NDM_F	GGTTTGGCGATCTGGTTTTC	20	
	NDM_R	CGGAATGGCTCATCACGATC	20	
IMP	Multi IMP-F	TTGACACTCCATTTACDGA	25	Dallenne *et al.* 2010
	Multi IMP-R	GATYGAGAATTAAGCCACYCTA	25	
VIM	Multi VIM-F	GATGGTGTTTGGTCGCATA	25	
	Multi VIM-R	GATGGTGTTTGGTCGCATA	25	
KPC	Multi KPC-F	CATTCAAGGGCTTTCTTGCTGC	10	
	Multi KPC-R	ACGACGGCATAGTCATTTGC	10	

Table S2. AST Zone diameters for control strain *Escherichia coli* (ATCC®25922™) and the test isolates used in this assay (PSAST 2017).

Antibiotic	Disc code	Antibiotic concentration (μg)	Control strain zone diameter (mm)	Control diameter observed (mm)	Test zone diameters (mm)		
					Resistant	Intermediate	Susceptible
Amoxicillin & Clavulanic acid	AMC-30	20/10	18-24	22	<=13	14-17	>=18
Ampicillin	AM	10	16-22	20	<=13	14-16	>=17
Cefotaxime	CTX-30	30	29-35	34	<=14	15-22	>=23
Ceftazidime	CAZ-30	30	25-32	29	<=14	15-17	>=18
Cefpodoxime	CPD-10	10	23-28	25	<=17	18-20	>=21
Ceftriazone	CRO-30	30	29-35	29	<=13	14-20	>=21
Cefoxitin	FOX-30	30	23-29	24	<=14	15-17	>=18
Ciprofloxacin	CIP-5	5	30-40	30	<=15	16-20	>=21
Gentamicin	GM-10	10	19-26	17	<=12	13-14	>=15
Ertapenem	ETP-10	10	29-36	32	<=15	16-18	>=19

NDM) widely known for their rapid acquisition and dissemination, were not found.

The prevalence of CRE in different species of animals was recorded. Based on the results described herein, CR-GNB appears to be having a significant prevalence (9.78 %) in cattle and dogs. The earlier studies showed the varied incidence of CR-GNB ranging from 0.5 % to 25 % in different parts of the world. In the current study, we found slightly higher prevalence rates of CR-GNB in animals, than the rates reported by other researchers (Stolle *et al.* 2013, Saheen *et al.* 2013, Reynolds *et al.* 2019). In absence of strict regulatory framework governing the use of antimicrobials in animal production system and irrational therapeutic usage of antibiotics in veterinary practices in India, may have contributed for higher prevalence of CR-GNB in animals. The recovery of CR-GNB from cattle and dogs indicates a potential future public health crisis (Abraham *et al.* 2014). We recorded a higher prevalence of CR-GNB in dogs (19.23%) in comparison to bovine (4.93%) (Table 3). A significantly higher prevalence of CRE among companion animals observed in our study is in agreement with previous findings of Kock *et al* (2018) who inferred higher prevalence rate (1-15%) among livestock and companion animals in Asia.

We observed faecal samples 7.70% (14/184) were the major source of CRE isolates followed by uterine samples 2.17% (4/184) (Fig. 3). Traditionally, bovine excrements are used for mud-flooring, as manure in agricultural farmland, and dung cake preparation in villages in India. Human exposure to antibiotic-resistant bacteria present in bovine excrements poses a health risk. The colonization of CR-GNB in the animal gut microbiome is a concern since it could be readily transmitted to pet owners, veterinarians, farmers through close physical contact and may result in community spread. The frequent use of beta-lactams selects and maintains CR-GNB within the animal population. The prevalence of CR-GNB in faecal samples of dogs has been widely reported (González-Torralba *et al.* 2016, Gentilini *et al.* 2018). The faecal carriage of CR-GNB in dogs indicates the possible occurrence of interspecies transmission between humans and companion animals within the same household. Industrialization and urban expansion of Indian cities have resulted in an exponential rise in the stray dog population in urban and peri-urban areas. Humans can be exposed to CR-GNB through soil contaminated with stray-dog faeces in densely populated urban neighbourhoods inhabited by the low socio-income group.

CONCLUSION

The recovery of CR-GNB from livestock and companion animals has significant public health ramifications and this may be related to illegal carbapenem use in veterinary practice. The dissemination of carbapenem-resistant bacteria in livestock and the environment potentially has a far-reaching effect. Evidence of such transmission is the cause of concern for public health experts and warrants strict vigil to limit the species spillover cross-species transmission. Hence continuous surveillance for antimicrobial-resistant must include screening for CR-GNB in livestock and companion animals.

ACKNOWLEDGMENT

The authors are also thankful to Vice-chancellor, U.P. Pandit Deen Dayal Upadhyayay Pashu Chikitsa Vigyan Vishwavidhyalaya Ewam Gau Anusandhan Sansthan Mathura, 281001 (U.P.), for providing infrastructural facility to review this study.

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***Cite this article as:** Arun A, Jaiswal U, Tripathi S, Singh AP, Choudhury S, Prabhu SN (2022) Surveillance of carbapenem-resistant Gram-negative bacteria from animal sources in Mathura region, Uttar Pradesh, India. Explor Anim Med Res 12(1): 91-98. DOI: 10.52635/eamr/ 12.1.91-98.