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

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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 (faeces, milk, pus, and uterine discharge)
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<sup>KPC</sup> 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).

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 carbapenems (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 X10⁸ 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 × 10⁷ 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 37°C 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.
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°C for 10 min and 30 cycles of denaturation at 94°C for the 40s, annealing at 55°C for 40s, and extension at 72°C for 1 min followed by final elongation step at 72°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 %), ceftiraxone (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
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 | gene  |
| 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, *
| VS-02      | Cow    | Uterine discharge | *E. coli*        | AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP | 1 µg | 32 µg | 0.5 µg | *
| VS-39      | Cow    | Uterine discharge | *Enterobacter cloacae* | AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP | 16 µg | 4 µg | 0.0625 µg | *
| VS-51      | Cow    | Uterine discharge | *E. coli*        | AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP | 4 µg | 64 µg | 0.5 µg | *
| VA-19      | Dog    | Faecal        | *E. coli*        | AMC, CPD, CRO,CTX, CAZ, AM, CIP, GM, FOX, ETP | 6 µg | 1 µg | 1 µg | *
| VA-52      | Dog    | Faecal        | *E. coli*        | AMC, CPD, CRO,CTX, CAZ, AM, CIP, GM, FOX, ETP | 2 µg | 0.125 µg | 0.0625 µg | *
| VA-53      | Buffalo Milk | Citrobacter freundii | *Citrobacter freundii* | AMC, CPD, CRO,CTX, AM, CIP, GM, ETP | 4 µg | 0.0625 µg | 0.125 µg | *
| VA-55      | Dog    | Faecal        | *E. coli*        | AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP | 8 µg | 0.125 µg | 0.0625 µg | *
| 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 | *Klebsiella pneumonia* | AMC, AM, FOX, ETP | 0.25 µg | 2 µg | 0.125 µg | *
| VA-99      | Buffalo Faecal | E. coli | *E. coli*        | AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP | 4 µg | 1µg | 0.25 µg | *
| VA-100     | Buffalo Faecal | E. coli | *E. coli*        | AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP | 16 µg | 8 µg | 0.125 µg | *
| VU-02      | Dog    | Faecal        | *E. coli*        | AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP | 2 µg | 32 µg | 0.0625 µg | *
| VU-03      | Dog    | Faecal        | *Citrobacter freundii* | AMC, CPD, CRO,CTX, CAZ, AM, CIP, FOX, ETP | 4 µg | 32 µg | 0.0625 µg | *
| 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).
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 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%) |

Table S1. Primers used in PCR analysis.

| β-lactamase (s) targeted | Primer name | Sequence (5’-3’) target | Primer concentration (20 picomol) | Reference |
|--------------------------|-------------|--------------------------|----------------------------------|-----------|
| OXA-48-like              | OXA-48_for  | GCTTGATCGCCCTCGAT        | 20                               |           |
|                          | OXA-48_rev  | GATTTGCTCCGTCGGCCGAA     | 20                               |           |
| New Delhi metallo-beta lactamase (NDM) | NDM_F      | GGTGGTGCCGATCCTGAATTC    | 20                               |           |
|                          | NDM_R      | CGGAATGGCTCATAGGCAGATT   | 20                               |           |
| IMP                      | Multi IMP-F | TTGACACTCCATTTACGTA      | 25                               | Dallenne et al. 2010 |
|                          | Multi IMP-R | GATYGAGAATTAAGCCAYCTA    | 25                               |           |
| VIM                      | Multi VIM-F | GATGGTGTTTGGTGCATA       | 25                               |           |
|                          | Multi VIM-R | GATGGTGTTTGGTGCATA       | 25                               |           |
| KPC                      | Multi KPC-F | CATTCAAGGGCTTTCTGCTGC    | 10                               |           |
|                          | Multi KPC-R | ACGACGCGCATAGTCATTGC     | 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) |
|------------|-----------|-------------------------------|-----------------------------------|--------------------------------|--------------------------|
| 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.

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