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Phenotypic Characterisation of *Klebseilla pneumoniae* Carbapenemase and Metallo Beta-lactamase in Carbapenem Resistant Gram-negative Bacteria

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ABSTRACT

Background: Antibiotic resistance is an emerging concern in the therapy of clinical infections worldwide. Previous studies conducted in our laboratory have confirmed an increase in the prevalence of extended spectrum beta-lactamase (ESBL) among the Gram-negative bacterial pathogens associated with dogs, which could act as a potential source for the transfer of these resistant pathogens or their genetic determinants to human. Since carbapenems are the last resort drugs against these resistant pathogens, the study was aimed to isolate and characterise carbapenem resistance among *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) associated with common clinical infections in dogs.

Methods: A total of 100 samples were collected from lesional skin, urine and anterior vagina of dogs presented to the Veterinary Hospitals of Kerala Veterinary and Animal Sciences University at Mannuthy and Thrissur. The samples were cultured onto Brain Heart Infusion Agar (BHIA), Eosin Methylene Blue (EMB) and Mac Conkey (MAC) for isolation of bacteria. Identification of the isolates was performed based on cultural, morphological and biochemical characteristics. The isolates were subjected to antimicrobial susceptibility test (ABST) against the 12 commonly used beta-lactam and non-beta-lactam group of antibiotics by disc diffusion method and further subjected to screening for ESBL double disc diffusion method. Carbapenem-resistant isolates were subjected to phenotypic confirmatory test for carbapenemase production employing Imipenem-EDTA and Ertapenem-boronic acid minimum inhibitory concentration (MIC) strip method.

Result: Forty four Gram-negative bacterial isolates obtained were *viz*., *E. coli* (30), *K. pneumonia* (11) and *P. aeruginosa* (3) from the 100 samples. Apart from these, other isolates obtained were *Staphylococcus* spp. (53) and *Bacillus* spp. (2). All the Gram-negative isolates were subjected to ABST employing 12 common antibiotics belonging to beta-lactam and non-beta-lactam groups. Multidrug resistance (MDR) could be observed in 28 *E. coli*, 11 *K. pneumoniae* and three *P. aeruginosa* isolates. All the 42 MDR isolates showed positive results for ESBL production. A total of 14 isolates out of the 44 Gram-negative bacilli were found to be resistant to carbapenem either to imipenem, meropenem or ertapenem. Among the 14 Gram-negative isolates, nine turned out to be positive for metallo-beta-lactamase (MBL) and none for *K. penumoniae carbapenemase* (KPC) on phenotypic confirmatory test for detecting major carbapenemase enzymes. The present study documented that Gram- negative bacteria like *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolated from dogs are showing an increase rate of resistance against carbapenems which are the last resort drugs against ESBL producers. Hence, there is an urgent need to curb the irrational and excessive use of antibiotics in veterinary sector.

Key words: Carbapenem, Dog, KPC, MBL, Resistance.

INTRODUCTION

Antibiotic resistance particularly among Gram-negative bacterial pathogens is an emerging concern all over the world. Many guidelines and recommendations both at international and national level have been published to tackle the threats posed by antibiotic resistance. Despite this awareness and attention by mass media, the problem continues to increase throughout the world. The escalating burden of Gram-negative bacterial resistance is largely due to beta-lactamases, which are the enzymes that hydrolyse beta-lactam antibiotics, rendering them ineffective (Morrill *et al*., 2015). The most significant mechanism behind the beta-lactam resistance is the production of beta-lactamases, extrusion by efflux pumps, permeability alterations and to a lesser extent alteration in penicillin binding proteins. The constant exposure of bacterial organisms to a wide range

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of beta-lactam antibiotics has induced robust and incessant production and mutation of beta-lactamases, expanding their activity against the recently developed drugs. These enzymes are known as extended spectrum beta-lactamases (ESBL). Extended spectrum beta-lactamase producing Gram-negative pathogens is rising at an alarming rate worldwide (Paterson and Bonomo, 2005; Pitout and Laupland, 2008).

Carbapenems are beta-lactam antibiotics (includes imipenem, meropenem, ertapenem and doripenem) which are considered as a last line of therapy for the treatment against bacteria producing ESBL. However, carbapenem resistant bacteria having the ability to hydrolyse nearly all beta-lactam and several non beta-lactam antibiotics have emerged in different parts of the world causing a grave concern. The most frequently observed mechanism of carbapenem resistance among Gram-negative pathogens is the production of carbapenem inactivating enzymes, called carbapenemases (Swathi *et al*., 2016). Several chromosomal and plasmid encoded carbapenemases such as KPC, MBL, Verona integron-encoded MBL (VIM), Imipenem-resistant *Pseudomonas* (IMP), New Delhi MBL (NDM) and Oxacillinase (OXA) - 48, have been documented throughout the world, that could easily be transferred between different enterobacterial species (Stolle *et al*., 2013; Abraham *et al*., 2014; Netikul and Kiratisin, 2015). Today, the population of companion animals such as dog and cat is substantially increasing in modern society and the major mechanism of antibiotic resistance among bacterial agents from pets and humans is the exchange of resistance genes (Pruthvishree *et al*., 2018). Therefore, firm control on infection and surveillance measures combined with prudent use of antibiotics in both human and veterinary practice is essential to reduce the spread of resistance against carbapenems.

Keeping in view all these, the current research was undertaken to study the antibiogram profile of the major Gram-negative bacteria *viz*., *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolated from dermatological and urogenital tract infections in dogs followed by their phenotypic confirmation for carbapenem resistance. This will give an overview of the current situation regarding carbapenem resistance among these pathogens associated with clinical infections in dogs with a focus on antibiotic resistance mechanism and subsequent management of such infections.

MATERIALS AND METHODS

Collection of samples

A total of 100 samples (skin swabs, urine and vaginal swabs) were collected from dogs with skin and urogenital tract infection brought to the Teaching Veterinary Clinical Complex, CVAS, Mannuthy and University Veterinary Hospital, Kokkalai. Those samples brought to the Department of Veterinary Microbiology, CVAS, Mannuthy were also utilized.

Isolation and characterisation of *E. coli, K. pneumoniae* and *P. aeruginosa*

The collected samples were immediately plated onto Brain heart infusion agar (BHIA) under aseptic conditions and were incubated at 37°C for 24 h. The isolates were analysed for its colony morphology and subjected to Gram's staining. Those colonies, which revealed Gram negative bacilli were sub cultured onto MacConkey agar (MCA) and Eosin methylene blue agar (EMB). The bacterial isolates were identified up to species level employing various biochemical tests *viz*., Catalase, Oxidase, Methyl Red, Indole production, Voges-Proskauer, Citrate utilization, Urease and Triple sugar iron agar test (Quinn *et al*., 1994).

Antimicrobial susceptibility test

Kirby-Bauer disc diffusion method was performed on MHA plates to determine the susceptibilities of different beta-lactam and non beta-lactam antibiotics. Pure culture was used as inoculum. Three to four similar colonies were selected and transferred into 3 ml of MHA broth and incubated at 37°C for 2-8 h for enrichment. The following antibiotics were used for the test; amikacin (30 µg), amoxycillin-clavulanic acid (30 µg), cefotaxime (30 µg), cefuroxime (30 µg), ceftriaxone (30 µg), co-trimoxazole (30 µg), ceftazidime (30 µg), ciprofloxacin (30 µg), cefepime (30 µg), cefoxitin (30 µg), gentamicin (30 µg), piperacillin/ tazobactam (30 µg) on Mueller Hinton agar plate. The zones were compared with the standard inhibition zone chart to find out sensitive and resistant antibiotics (CLSI, 2018).

Phenotypic confirmation for extended spectrum beta lactamases

The MDR isolates were tested for ESBL production and double disc diffusion method was used for detection of ESBL. The 0.5 Macfarland suspensions of the isolates were streaked onto MHA. A disc of either cefotaxime alone and with clavulanic acid (CTX 30 mcg + CEC 10 mcg) and ceftazidime alone and with clavulanic acid (CAZ 30 mcg + CAC 10 mcg) was placed at distance of 20 mm. After incubation overnight at 37°C, a positive test result was considered as a 5 mm increase in inhibition zone compared with a disc without clavulanic acid (Drieux *et al*., 2008).

Screening of Carbapenem

The Gram-negative isolates were tested for susceptibility to carbapenem *viz*., imipenem, meropenem and ertapenem by disc diffusion method according to CLSI criteria (CLSI, 2018). The resistant isolates were screened for carbapenemase production by combined disc test and are presented in Table 1.

Phenotypic detection of *Klebseilla pneumoniae* carbpenemase employing Ertapenem-boronic acid MIC strips

The phenotypic confirmatory test for the production of KPC was done using Ertapenem/Ertapenem + Boronic Acid Ezy MIC™ Strips EM141 (ETP/ETP + For KPC detection) with

the following concentration, ETP: 0.125 - 8 µg/mL, ETP+: 0.032 - 2 µg/mL (Himedia, Mumbai) and the procedure was carried out as per the manufacturer's recommendations and the results were interpreted as per Table 2.

Combined Imipenem-EDTA disc test for detection of MBL

All the isolates were tested with combined imipenem-EDTA test as per Yong *et al.* (2002) employing appropriate positive and negative controls. Test organisms were inoculated on to Mueller Hinton agar plates as per CLSI guidelines. A 10 µg imipenem disc and imipenem (10 µg) - EDTA (750 µg) combined disc were placed on the plate (Hi-Media, Mumbai) and incubated for 16-18 h at 35°C. The increase in inhibition zone with the imipenem and EDTA disc 7 mm than the imipenem disc alone was considered as a MBL positive strain.

RESULTS AND DISCUSSION

Isolation and identification

In the present study, 44 Gram- negative bacteria were isolated *viz*., 30 *E. coli*, 11 *K. pneumoniae* and three *P. aeruginosa*, from a total of 100 samples collected from skin and urogenital tract infections in dogs. Apart from these, other isolates obtained were *Staphylococcus* spp. (53) and *Bacillus* spp. (2). The isolates which produced irregular, cream, shiny and smooth colonies in BHIA, lactose fermenting colonies on Mac Conkey agar, distinctive metallic green sheen on eosin methylene blue agar, positive for catalase, indole production, methyl red test and negative for VP, citrate utilization, oxidase test urease test and TSI test respectively were confirmed as *E. coli*. The isolates which produced, large mucoid viscous colonies in BHIA, lactose fermenting colonies on Mac Conkey agar, positive for catalase, VP, citrate utilization, urease test and TSI test and are negative for oxidase, indole production and MR test were confirmed as *K. pneumonia*. The isolates which produced large, opaque, irregular colonies with fruity odour and fluorescent greenish colour on BHIA agar with positive results for catalase test, oxidase test, citrate utilization test and negative results for indole production test, methyl red test and VP test were confirmed as *P. aeruginosa.* Similar results were obtained by Shakya *et al*. (2017) and Shetty (2017) who documented these pathogens predominantly from dogs with skin and urogenital tract infections.

All the Gram-negative isolates were subjected to ABST employing 12 common antibiotics belonging to beta-lactam and non-beta lactam groups. Multidrug resistance could be observed among 28 *E. coli*, 11 *K. pnuemoniae* and three *P. aeruginosa* isolates.

This is in accordance with Magiorakos *et al*. (2017) and Koulenti *et al*. (2018) who reported that the spread of MDR bacteria is an ever-growing concern, particularly among Gram- negative bacterial spp. because of their intrinsic resistance and the various mechanisms through which they acquire and spread resistance characteristics. They pointed that the most common mechanism for the spread of MDR in Gram-negative bacilli could be attributed to the presence of plasmids harbouring antibiotic resistance genes.

Table 1: Screening for carbapenem detection employing disc diffusion method.

No.	Antibiotic disc concentration		Interpretative criteria
1.	Ertapenem	ETP 10 mcg	Inhibition zone ≥ 22 mm
2.	Imipenem	IPM 10 mcg	Inhibition zone ≥ 23 mm
3.	Meropenem	MRP 10 mcg	Inhibition zone ≥ 23 mm

Phenotypic confirmation for ESBL production

Among the 44 Gram- negative bacilli isolated, 30 *E. coli*, 11 *K. pneumoniae* and three *P. aeruginosa* showed positive results for ESBL production. The results are similar to the findings of Bradford (2001) and Paterson *et al*. (2003) who documented an alarming increase in ESBL producers among Gram-negative bacteria, showing resistance to a wide range of cephalosporins, the mainstay drug in the therapy of clinical infections in both human and veterinary sector.

Screening for carbapenem resistance

On screening the ESBL producers for carbapenem resistance, ten *E. coli* isolates, three *K. pneumoniae* and one *P. aeruginosa* showed resistance to at least one of the three carbapenems employed in the study. Similar observations were made by Datta and Wattal (2010), Livermore (2012) and Meletis (2016). Above results indicated a rise in ESBL producers among companion animals like dogs with activity against carbapenems also, the last sort

Table 2: Phenotypic confirmation for *Klebseilla pneumoniae* carbpenemase employing Ertapenem-boronic acid MIC strips.

Result	(Ertapenem alone and with phenyl boronic acid for detection of KPC (Ertapenem: 0.125-8, boronic acid: 0.032-2)	Interpretative criteria
KPC positive strain	ETP/ETP+ =>8	When the ratio of the value obtained for ETP: The value of ETP+ boronic acid is more than 8
		Or
	ETP/ETP+ =>8/<2	If the zone is observed on the side coated with ETP+ boronic acid and no zone is observed on the side coated with ETP
KPC negative strain	ETP/ETP+ =≤8	When the ratio of the value obtained for ETP: The value of ETP+ boronic acid is less than or equal to 8
Non-conclusive	ETP/ETP+ =>8/>2	When no zone of inhibition is obtained on either side

drugs in treatment of infections against ESBL producing Gram- negative bacilli. This creates an impending crisis in the treatment of clinical infections in human since these MDR, ESBL producing bacterial spp. showing resistance to carbapenem could transfer their resistance genes to other sensitive bacterial species causing infections in animals/ humans and even to commensals.

Confirmatory test for carbapenemase

The most predominant mechanism of carabpenem resistance among Gram- negative bacilli is the presence of carbpenemase enzymes which are divided into three classes which includes Class A enzymes (*eg*: KPC types), class B enzymes or MBL (*eg*: VIM, IPM, NDM types) and class D enzymes or oxacillinases (*eg*: OXA types) (Ahmed-Bentley *et al*., 2013). The predominant carbapenemase among them were reported to be KPC and NDM (Cadjoe and Donkor, 2018). Hence, the phenotypic confirmation tests to detect the presence of these enzymes were also carried out.

On phenotypic confirmation for KPC employing ertapenem-boronic acid MIC strip method, all the isolates showed negative results. This indicated that their resistance might be attributed to the presence of MBL or oxacillinase enzymes or mechanisms other than the presence of carbapenemase like the extrusion by efflux pumps, permeability alterations and to a lesser extent alteration in penicillin binding proteins. On confirmation for MBL using imipenam-EDTA combined disc test, eight *E. coli* isolates and one *P. aeruginosa* showed positive results and two *E. coli* and three *K. pneumoniae* isolates didn't reveal the presence of MBL. Bartolini *et al*. (2014) reported that NDM and KPC were the predominant cause for carbepenem resistance among Gram-negative bacteria and reported it as a worldwide emerging crisis (Diene and Rolain, 2014). In the present study, among the fourteen isolates, nine turned out to be positive for MBL. Any isolate positive for KPC couldn't be observed and no tests have been conducted to detect the presence of oxacillinase enzymes. The phenotypic confirmatory tests should always be reinforced by carrying out genotypic characterisation specific for KPC and NDM genes.

CONCLUSION

Carbapenems are resorted as the last choice drugs against multidrug resistant ESBL possessing bacterial species. The results of the present study documented that Gram-negative bacterial species like *E. coli*, *K. pneumoniae* and *P. aeruginosa* are showing an increased rate of resistance against carbapenems also. Hence, there is an urgent need to curb the irrational and excessive use of antibiotics in both human and veterinary sector. The already established national policies and guidelines might be the beginning to combat this problem. There is a need for regular area wise monitoring of the antibiogram profile of various bacterial organisms associated with clinical infections in animals which could provide an adequate data for the implementation of these.

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