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Genetic Characterization of Extended-Spectrum Beta- Lactamase (ESBL) and Metallo-Beta-Lactamase (MBL) Producing *Klebsiellapneumoniae* from Diabetic Foot Ulcer

Dipak S. Kale[1], Kailas D. Datkhile[2]*, Geeta S. Karande[1], Kalpana D. Kale[3], Satish R. Patil[1]

1. Department of Microbiology, Krishna Vishwa Vidyapeeth "Deemed to be University", Taluka-Karad, Dist-Satara, Maharashtra, India

[2]Department of Molecular Biology and Genetics, Krishna Vishwa Vidyapeeth "Deemed to be University", Taluka-Karad, Dist-Satara, Maharashtra, India

Kale Pathology Laboratory, Karad, Taluka-Karad, Dist-Satara, Maharashtra, India

*Corresponding Author:

Kailas D. Datkhile, Ph.D., Department of Molecular Biology and Genetics, Krishna Vishwa Vidyapeeth 'Deemed to be University', Karad, Maharashtra, India

Email- hodgeneticslab@kimskarad.in

ORCID ID: https://orcod.org/0000-0001-9222-8579

Abstract

Background: Antibiotic resistance in common pathogenic bacteria is linked with the genetic makeup. The genetic basis of the antibiotic resistance may vary in different species or pathophysiological conditions.

Objectives: We studied the antibiotic resistance in *Klebsiellapneumoniae* isolates from diabetic foot ulcer (DFU) in western Indian population. We also studied the presence of extended-spectrum beta- lactamase (ESBL) and metallo-beta-lactamase (MBL) mechanism of antibiotic resistance along with the prevalence of the genes involved in ESBL (TEM_{ESBL}, SHV_{ESBL}, and $CTX\text{-}M_{ESBL}$) and MBL ($NDM\text{-}1_{bla}$, KPC_{bla}, $OXA\text{-}48_{bla}$, and VIM_{bla}) production.

Results:A total 161 *K.pneumoniae* isolates were analysed; among which 50.93% were positive for ESBL and 45.96% were positive for MBL production. Most of the isolates were resistant to antibiotics used in the present study and partially resistant to Imipenem and Amikacin. There was no relation between the antibiotic resistance of the isolates and the production of ESBL or MBL mechanism of antibiotic resistance. Further, TEM_{ESBL} was the most prevalent gene in *K.pneumoniae*isolates followed by $CTX\text{-}M_{ESBL}$, $NDM\text{-}1_{bla}$, SHV_{ESBL}, and KPC_{bla}. VIM_{bla} was the least prevalent gene found in *K.pneumoniae* isolates. There was no difference in the prevalence of the genes with respect to presence or absence of ESBL and MBL mechanism of resistance. Further, there was no relation between the prevalence of the genes and antibiotic resistance in *K.pneumoniae* isolates.

Conclusion: These results along with literature review suggest that the prevalence of the genes involved in antibiotic resistance mechanisms are widespread in India and their distribution vary in different studies.

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Keywords: Antibiotic resistance, Beta-lactamase, Diabetic foot ulcer, *Klebsiellapneumoniae*

Introduction

The antibiotic resistance in pathogenic bacteria inhabiting lesions is a major concern for medical microbiologists worldwide. Especially, the Gram negative bacteria belonging to the family Enterobacteriaceae are common nosocomial pathogens responsible for the deterioration of wounds in various medical conditions. For example, *Klebsiellapneumoniae*, an opportunistic pathogen able to express multidrug resistant phenotypes that can hinder treatment strategies (Hu et al., 2021; Li et al., 2014). Pathogenic bacteria are generally equipped with several antibiotic resistance mechanisms including beta-lactam andCarbapenem resistance, biofilm formation, etc. (Doorduijn et al., 2016; Lee et al., 2017; Wang et al., 2020). Recent literature provides sufficient evidences for the genetic linkage of the antibiotic resistance in bacteria. For example, Extended-spectrum beta-lactamases (ESBL) and Metallo beta-lactamases (MBL) involved in beta lactam antibiotic resistance are encoded by the genes located on plasmids (Gomez-Simmonds and Uhlemann, 2017; Hussain et al., 2021; Zhang et al., 2016). Wound inhabiting bacteria may carry different sets of antibiotic resistance genes (Blair et al., 2015; Wright, 2007). These co-existing bacteria cooperate with each other and develop resistance against antibiotics (Noor et al., 2015; Wall et al., 2002; Wright, 2007). Moreover, the genetic background of antibiotic resistant pathogenic bacteria may vary in different pathophysiological conditions(Ferreira et al., 2019; Shahi and Kumar, 2016). Therefore, the genetic background of the pathogenic bacteria inhabiting chronic wounds such as diabetic foot ulcer (DFU) need to be investigated to understand the exact mechanism of antibiotic resistance.

Diabetic foot ulcer (DFU) is one of the most extreme and complicated pathophysiological conditions in diabetic patients(Noor et al., 2015). India is a leading country with significant proportion of diabetic patients with high risk of developing DFU (Noor et al., 2015; Pradeepa and Mohan, 2021; Sultana et al., 2023; Viswanathan, 2010). Resent literature suggest that most of the DFUs were infected by multiple bacteria suggesting the high risk of developing chronic ulcers which may lead to amputation (Noor et al., 2015; Sultana et al., 2023). As bacteria are known to cooperate and cumulatively develop resistance toward antibiotics, chronic DFUs are difficult to treat(Jneid et al., 2017; Noor et al., 2015). Recent review summarising the etiology of DFU in India showed that the DFUs in Indian are generally inhabited by Gram negative bacteria and the isolates belonging to the genus Klebsiella were one of the most common bacteria reported in DFUs (Kale et al., 2023). Moreover, the studies focusing on the genetic basis of antibiotic resistance in targeted pathogens are limited (Bajpai et al., 2017; Govindaswamy et al., 2019; Nagaraj et al., 2012; Shahi et al., 2013).

In the present study, we isolated *K. pneumoniae*bacteria from the patients with DFUs from Maharashtra and studied the presence of ESBL and MBL mechanisms of antibiotic resistance and tested their antibiotic resistance to common antibiotics. Additionally, we also studied the presence of the genes involve ESBL and MBL production in all *K. pneumoniae* isolates.

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Material and methods

The study was conducted on the diabetic patients visited to Krushna Medical Institute, Karad, Maharashtra, India. A total of 252 diabetic patients were screened for the bacterial infection in DFU. The data on age, sex, socio-economic status of the patients were recorded. Diabetic history and physiological conditions of each patient were recorded. Blood tests including sugar (glucose) level and HBA1C were performed before bacterial sampling. In addition, urea, creatinine, potassium, and sodium levels were determined following standard procedures.

Microbiological procedure

Bacterial infections were identified in the pus samples from the ulcer. Two cotton sterile swabs were used to collect the pus samples. Of which, one samples was processed for the bacterial culture on blood and MacConkey's agar and another samples was processed for Gram staining. The culture plates were incubated at 37^{o}C and on next day, colony morphology was observed and Gram staining was performed. A total of 161 *K. species* isolates were identified from 152 patients.

Biochemical characterization

The tests for indol production, glucose and citrate utilization, and urea hydrolization by *E. coli* isolates were confirmed following the standard protocols described by CLSI. ESBl production was confirmed (reduced susceptibility to) by double disc synergy method using Ceftazidime and Calvulanic acid (30/10) mcg on Muller-Hinton agar following CLSI guidelines. ESBL producer was determined based on ≥5 mm increase in the zone diameter of ceftazidime/clavulanic acid disc and ceftazidime disc alone and ≥5 mm increase in the zone diameter of cefotaxime/clavulanic acid disc and cefotaxime disc alone. Imipenem-EDTA disc method was used for the detection of Metallo-beta-lactamase (MBL) producing isolates.[23,24]

Antibiotic susceptibility testing

Antibiotic susceptibility of the isolates (*K. species*) to Ampicillin, Amoxicillin, Piperacillin, Cefuroxime, Ceftriaxone, Cefoperazone, Cefepime, Imipenem, Meropenem, Amikacin, Gentamicine, and Ciprofloxacin was studied by Kirby-Bauer disc diffusion following guidelines established by CLSI.

Genetic characterization of beta lactamase production

The genomic and plsmid DNA from the isolates were extractedby using the HipurA bacterial genomic DNA purification Kit and HipurA Plasmid DNA miniprep purification kit (HiMedia), respectively following the manufacturer's instructions. Beta lactamase producing genes (TEM_{ESBL}, SHV_{ESBL}, CTX-M_{ESBL},NDM-1_{bla}, KPC_{bla}, OXA-48_{bla}, and VIM_{bla}) were amplified by Polymerase chain reaction using primers given in Table 1. PCR amplification was carried out in 20µL reaction mixture containing 1X PCR assay buffer (10 mMTrisHCl (pH 8.3), 1.5 mM MgCl2, 50 mM of KCl, 200 µM each dNTP, and 1U of Taq DNA polymerase; Merk Millipore), 0.2 nmole of each primer, and 200 ng of purified DNA

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template of each sample. The PCR reaction for amplification was carried out in a Master Cycler gradient PCR machine (Eppendorf). Detailed PCR programme is given in Table 2. Amplified products were analysed using 2.0% agarose gel electrophoresis in 1X TAE buffer. The gel was stained with ethidium bromide (10mg/ml), visualized under UV transilluminator, and photographed in gel documentation system (Bio- Rad Laboratories). *Klebsiellapneumoniae* ATCC 700603 was used as positive control strain for TEM_{ESBL}, SHV_{ESBL}, and CTX-M_{ESBL}. *Klebsiellapneumoniae*ATCC BAA-2146 and *K.pneumoniae*ATCC BAA-1705 strains ware used as positive controls for NDM-1_{bla} and KPC_{bla}, respectively. The PCR product of OXA-48_{bla} positive isolate (307 bp size) was confirmed by DNA sequencing and further used as positive control.

Results

Age of the patients (with DFU) involved in the study ranged between 40 to 82 years (average 61.26 years). A total 161 patients were analysed for biochemical tests including HBA1C and blood sugar. All the patients were diabetic having higher values of blood sugar and HBA1C. Supporting biochemical data of the patients is summarized in Table 3.

A total of 161 *K. species* isolates were isolated from 110 male and 51 female patients. All isolates were negative for indole production and used citrate for energy production. All *K. species* isolates were tested negative for glucose utilization (Methyl red) for energy production and were capable for hydrolysing urea for nitrogen utilization.Among 161 isolates, 50.93% isolates were positive for ESBL production and 45.96% isolates were positive for MBL production.

MDR analysis revealed that more than 85% isolates were resistant Ampicillin, Amoxicillin, Cefuroxime, Ceftriaxone, and Ciprofloxacin while Imipenemand Amikacinis the most potent antibiotic against *K. species* inhibiting 67.28% isolates (Figure 3A). The isolates from male and female patients showed small difference in the resistance to Cefepime and Ciprofloxacin (Figure 3B). The isolates obtained from the male patients were more resistant to Ciprofloxacin than those from the female patients while the isolates from the female patients were more resistant to Cefepime as compared to those obtained from male patients (Figure 3B).

K. species isolates tested positive for ESBL production were more resistant to Cefoperazone, Imipenem,Gentamicine, and Ciprofloxacin as compared to those without ESBL production (Table 4). Contrary, the isolates positive for MBL production were vulnerable to Cefoperazone, Imipenem, Gentamicine, and Ciprofloxacin as compared to the isolates capable of MBL production (Table 4).

Genetic analysis revealed that TEM_{ESBL}(72.04%) was the most prevalent gene present in *K. species* isolates followed by CTX-M_{ESBL} (48.44%),NDM-1_{bla}(48.44%), and SHV_{ESBL}(42.23%; Table 4). VIM_{bla}(11.18%) was the least prevalent gene in *K. species* isolates (Table 5).CTX-M_{ESBL}(51.81%) gene was more prevalent in the isolates obtained from male patients as compared to those from the female patients (41.17%; Table 5). Irrespective of the ability of *K. species* isolates to produce ESBL and MBL, the prevalence of the genes involved in their (ESBL and MBL) production did not differ from the general trend (Table 5).

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Antibiotic resistance in the *K. species* isolates could not be correlated with the presence of a particular gene (Figure 4). Irrespective of the presence of a particular gene, most of the isolates were vulnerable to Imipenem and Amikacin belonging to Carbapenem and Aminoglycoside antibiotics, respectively (Figure 4). More than 80% of the isolates were resistant to Ampicillin, Amoxicillin, Cefuroxime, Ceftriaxome, and Ciprofloxacin (Figure 4).

Discussion

The evaluation of the antibiotic resistance mechanism in bacteria is the preliminary step toward understanding the genetic basis of antibiotic resistance. In the present study, we tested the ability of *Klebsiellapneumoniae* isolates to produce beta-lactamases and found that 50.93% and 45.96% isolates tested positive for ESBL and MBL production, respectively. Previous studies reported the variations in the proportions of ESBL and MBL producing bacteria isolated in different pathophysiological conditions. (Datta et al., 2019; Gupta et al., 2020; Saseedharan et al., 2018) reported 25%,69%, and 16.5% MBLproducers among Enterobacteriaceae isolates. (Sood, 2014) reported 88.33% MBL producers *K. pneumoniae* while (Patil et al., 2022) reported 42.5% MBL producers *K. pneumoniae*. (Govindaswamy et al., 2019) reported 85.43% MBL producing *E. coli* while (Zubair et al., 2011)reported 81.1% MBL producing *Pseudomonas aeruginosa* isolates. Similarly, (Babypadmini and Appalaraju, 2004; Gupta et al., 2018; Singh et al., 2015; Varaiya et al., 2008) and(Sahoo et al., 2019) reported 40%, 78%, 51.1%, 51.61%, and 42.6% ESBL producing *K. pneumoniae* isolates, respectively. Further, (Bajpai et al., 2017; Gupta et al., 2020; Zubair et al., 2012) and (Datta et al., 2019) reported 45%, 96%, 67.8%, and 59% ESBL producers among Enerobacteriaceae isolates. (Sahoo et al., 2019; Shahi et al., 2013; Varaiya et al., 2008) reported 44%, 75%, and 48.38% proportion of ESBL producing *E. coli* isolates. These results suggest that antibiotic resistant bacteria with ESBL and MBL mechanism of resistance are widespread among different bacteria.

Antibiotic resistance in bacteria could be linked with the genetic background. For example, beta-lactamases encoded by the genes TEM_{ESBL} and SHV_{ESBL} are involved in beta-lactam antibiotic resistance (Poirel et al., 2008). Similarly, bacteria carrying MBL encoded by OXA-40 are resistant to Carbapenem antibiotics (Walsh et al., 2005). In the present study, we observed no relation between the presence of antibiotic resistance mechanism of the isolates and the presence of the particular gene involved in beta-lactamase production. Further, there was no correlation between the presence of ESBL or MBL mechanism of resistance in the isolates and the presence of the particular gene. Previously, (Sahoo et al., 2019) reported very low prevalence of ESBL producing genes (TEM_{ESBL} - 10%, SHV_{ESBL} - 5%, and CTX-M_{ESBL} - 5%) in *K. pneumoniae* isolates. However, overall prevalence of TEM_{ESBL}, SHV_{ESBL}, and CTX-M_{ESBL} genes in different isolates was 76.25%, 40%, and 71.87%, respectively (Sahoo et al., 2019). (Shukla et al., 2023) reported the presence of CTX-M_{ESBL} in 80.38% *K. pneumoniae*isolates and TEM_{ESBL} in 74.16% *K. pneumoniae* isolates. (Chaudhry et al., 2016) reported the high prevalence of TEM_{ESBL} (75%), SHV_{ESBL} (84.6%), and CTX-M_{ESBL} (76.9%) in different isolates while 100% ESBL producing *K. pneumoniae* isolates were carrying these genes. Moreover, (Bajpai et al., 2017) reported the presence of TEM_{ESBL} (48.7%), SHV_{ESBL}

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(5.1%), and CTX-M$_{ESBL}$(7.6%) in*E. coli* and *K. pneumoniae* isolates. A study by (Zubair et al., 2012) reported that CTX-M$_{ESBL}$ (81.8%) was the most prevalent gene in isolates followed by TEM$_{ESBL}$ (50%) and SHV$_{ESBL}$ (46.9%). In *K. pneumoniae* isolates, we observed the highest prevalence of TEM gene followed by CTX-M$_{ESBL}$ and SHV$_{ESBL}$ irrespective of the presence of ESBL antibiotic resistance mechanism. Among the genes involved in MBL production, NDM-1$_{bla}$ was the most prevalent gene in *K. pneumoniae* isolates followed by KPC$_{bla}$, OXA-48$_{bla}$, and VIM$_{bla}$. The prevalence of MBL producing genes (NDM-1$_{bla}$, KPC$_{bla}$, OXA-48$_{bla}$, and VIM$_{bla}$) varied in different studies. The highest prevalence of NDM-1$_{bla}$ gene in *K. pneumoniae* isolates was reported by (Nagaraj et al., 2012) (75%) followed by(Saseedharan et al., 2018) (57.14%),(Rahman et al., 2014) (45%), (Jaggi et al., 2019) (34.6%), (Yadav et al., 2023) (20%), and (Shukla et al., 2023) (5.74%). KPC$_{bla}$ was the least prevalent gene in *K. pneumoniae* isolates (Shukla et al., 2023) while it was not detected in *E. coli* and *K. pneumoniae* isolates by (Nagaraj et al., 2012). The highest proportion of OXA-48$_{bla}$ in *K. pneumoniae* isolates was reported by (Chaudhry et al., 2016) (57.7%), followed by (Shukla et al., 2023) (46.41%), and (Yadav et al., 2023) (50%). (Nagaraj et al., 2012) reported the prevalence of VIM$_{bla}$ gene (13.8%) in *E. coli* and *K. pneumoniae* isolates. Cumulatively, these results suggest that the prevalence of the genes involved in beta-lactam antibiotic resistance iscommon in pathogenic bacteria and their prevalence varied in different studies. Further, a significant proportion of Indian population is diabetic and composed of diverse ethnic groups. Proportion of diabetic patients and associated complications also vary in different geographic zones (Kale et al., 2023; Pradeepa and Mohan, 2021). Recently, (Shukla et al., 2023) studied the state-wise distribution of the genes involved in ESBL and Carbapenem resistance in the genomes of the*K. pneumoniae* isolates from India and revealed that CTX-M$_{ESBL}$ is the most prevalent gene followed by TEM$_{ESBL}$, OXA$_{bla}$, and NDM$_{bla}$. The present study revealed that the TEM$_{ESBL}$ was the most prevalent gene in *K. pneumoniae* isolates followed by CTX-M$_{ESBL}$ and NDMin the western Indian state Maharashtra. These results suggest that there are significant variations in the geographic distribution of the genes involved in antibiotic resistance. The relation between the prevalence of the genes involved in beta-lactam antibiotic resistance and their geographic distributions need to be further investigated considering the grade of urbanization, and ethnicity of the population. Further, the possibility of the involvement of other genes in antibiotic resistance cannot be denied as the prevalence of the genes (presently investigated) involved in ESBL and MBL antibiotic resistance was similar in all isolates irrespective of the presence of these antibiotic resistance mechanism. Contrastingly, (Chaudhry et al., 2016) reported that 100% ESBL positive *K. pneumoniae* isolates were carrying associated genes (TEM$_{ESBL}$, SHV$_{ESBL}$, and CTX-M$_{ESBL}$).

The present study revealed that most of the *K. pneumoniae* isolates were resistant all antibiotics tested except partial resistance toImipenem and Amikacin which are Carbapenem and Aminoglycoside antibiotics, resoectively. Similarly, (Saseedharan et al., 2018) and (Patil et al., 2022) reported that *K. pneumoniae* isolates were resistant to Carbapenem and Aminoglycoside antibiotics. A study by(Sood, 2014) reported that *K. pneumoniae* isolates were vulnerable to Polymyxin antibiotics. Additionally, *K. pneumoniae* isolates were also reported to be sensitive to Tigecycline antibiotics belonging to glycylcycline class (Patil et

al., 2022).These results highlight the necessity of the monitoring of antibiotic resistance in common pathogens over the time and evaluation of their genetic basis of antibiotic resistance.

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Table 3 Biochemical test results of 161 patients included in the present study. Values are expressed as average of all patients and range of the values in given in parenthesis.

Test	Values
HBA1C	7.34 (6.1-11) mmol/mol
Blood Sugar	220.59 (106-350) mg/dL
Urea	28.41 (17.8-55.1) mg/dL
Creatinine	0.98 (0.6-2) mg/dL
Sodium	140.80 (120-148) mEq/L
Potassium	4.11 (3.6-5.1) mEq/L

Table 4 Antibiotic resistance in*K. species* isolates with ESBL and MBL production ability. Values in the table represent proportion and the values in parenthesis are actual number of isolates.

	ESBL (+ve)	ESBL (-ve)	MBL (+ve)	MBL (-ve)
Ampicillin	93.90 (77)	96.20 (76)	95.94 (71)	94.25 (82)
Amoxicillin	87.80 (72)	83.54 (66)	86.48 (64)	85.05 (74)
Piperacillin	56.09 (46)	55.69 (44)	52.70 (39)	58.62 (51)
Cefuroxime	91.46 (75)	86.07 (68)	87.83 (65)	89.65 (78)
Ceftriaxone	87.80 (72)	84.81 (67)	86.48 (64)	86.20 (75)
Cefoperazone	74.39 (61)	56.96 (45)	58.10 (43)	72.41 (63)
Cefepime	70.73 (58)	65.82 (52)	67.56 (50)	68.96 (60)
Imipenem	45.12 (37)	22.78 (18)	24.32 (18)	42.52 (37)
Meropenem	71.95 (59)	69.62 (55)	71.62 (53)	70.11 (61)
Amikacin	42.68 (35)	49.36 (39)	48.64 (36)	43.67 (38)
Gentamicine	71.95 (59)	58.22 (46)	58.10 (43)	71.26 (62)
Ciprofloxacin	90.24 (74)	81.01 (64)	79.72 (59)	90.80 (79)

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Table 5 Presence of various genes in *K. species*isolates with respect to sex of the patients and resistance mechanism. Values are presented in percentile and the numbers in parenthesis are actual numbers of the *K. species*isolates (ESBL: Extendedspectrum beta-lactamase; MBL: Metallo-beta-lactamase).

	Total (161 patients)	**Male (110)**	**Female (51)**	**ESBL +ve (88)**	**ESBL -ve (73)**	**MBL +ve (79)**	**MBL -ve (82)**
TEM$_{ESBL}$	72.04 (116)	71.81 (79)	72.54 (37)	71.59 (63)	72.60 (53)	72.15 (57)	71.95 (59)
SHV$_{ESBL}$	42.23 (68)	44.54 (49)	37.25 (19)	43.18 (38)	41.09 (30)	41.77 (33)	42.68 (35)
CTX-M$_{ESBL}$	48.44 (78)	51.81 (57)	41.17 (21)	45.45 (40)	52.05 (38)	50.63 (40)	46.34 (38)
NDM-1$_{bla}$	48.44 (78)	47.27 (52)	50.98 (26)	45.45 (40)	52.05 (38)	46.83 (37)	50 (41)
KPC$_{bla}$	36.02 (58)	34.54 (38)	39.21 (20)	35.22 (31)	36.98 (27)	36.70 (29)	35.36 (29)
OXA-48$_{bla}$	16.77 (27)	17.27 (19)	15.68 (8)	14.77 (13)	19.17 (14)	17.72 (14)	15.85 (13)
VIM$_{bla}$	11.18 (18)	9.09 (10)	15.68 (8)	9.09 (8)	13.69 (10)	15.18 (12)	7.31 (6)

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Figure 3 Proportion of antibiotic resistant *K. species* isolates. (A) Overall proportion of isolates resistant to different antibiotics. (B) Proportion of antibiotic resistant isolates obtained from male and female diabetic patients.

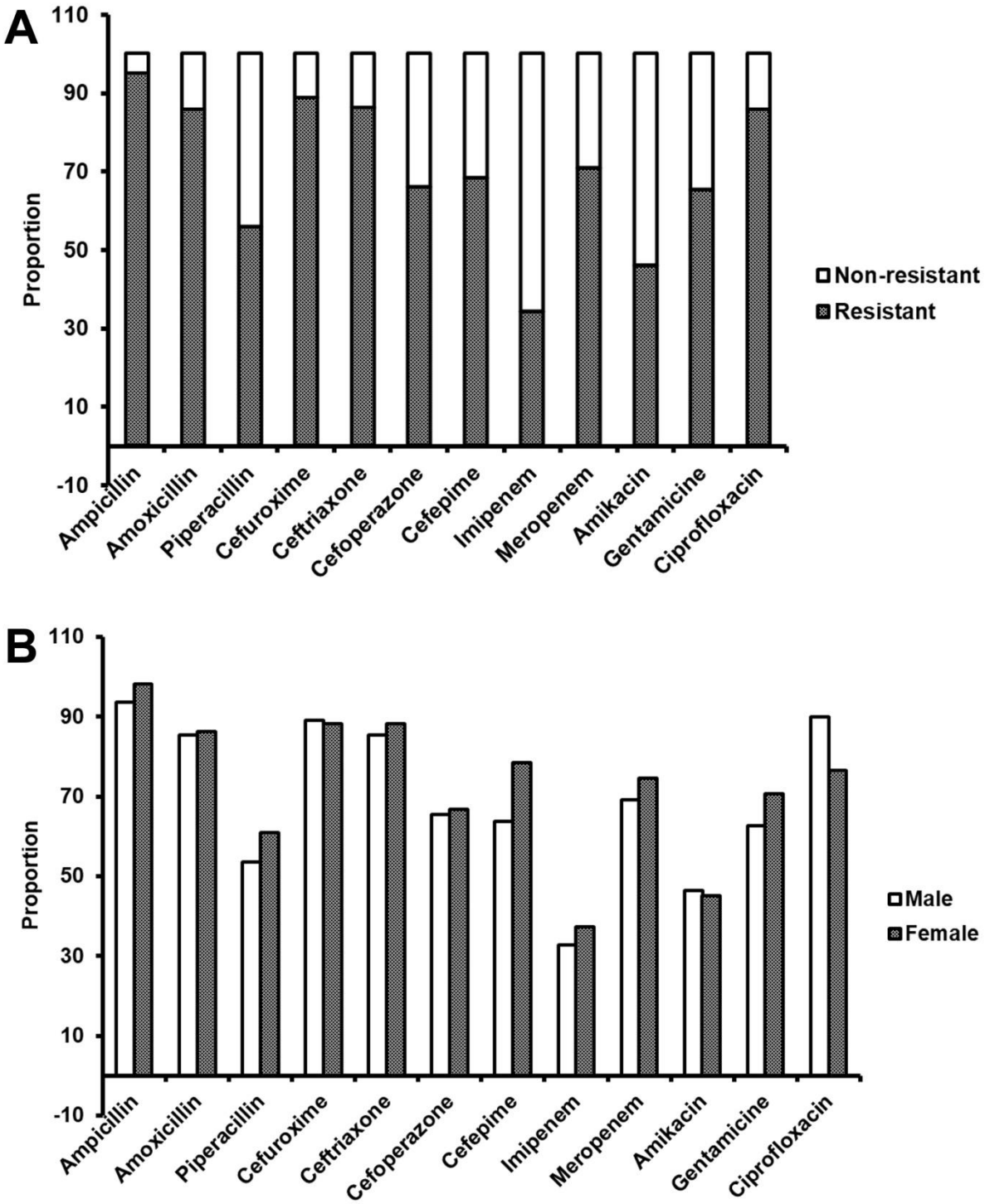

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Figure 4 Heat map depicting the proportion of antibiotic resistant *K. species* isolates with different genes involved in antibiotic resistance.