Original article

Identification of carbapenems resistant genes on biofilm forming *K. pneumoniae* from urinary tract infection

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ABSTRACT

The multi-drug resistant effect of the Gram negative bacteria K. pneumoniae was identified by disc diffusion method using specific UTI panel discs of Kleb 1 HX077 and Kleb 2 HX090 HEXA. Among the multidrug resistant bacteria, the carbapenem resistant (CR) effect of the K. pneumoniae was screened by specific carbapenem detection antibiotics of HEXA HX066 and HX0103 HEXA by disc diffusion method. In addition, the effective antibiotics were further performed against K. pneumoniae by minimum inhibition concentration method. Further, the carbapenemase genes of VIM 1 and IMP 1 were detected from the isolated strains by multiplex PCR method. Furthermore, the biofilm forming ability of selected carbapenem resistant K. pneumoniae was initially identified by tissue culture plate method and confirmed by exopolysaccharide arrest ability of congo red agar assay. Finally, our result was proved that the identified K. pneumoniae is carbapenemase producing strain, and its virulence was extended with strong biofilm formation.

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1. Introduction

Urinary tract infections (UTIs) are one of the leading human infections accounting 20% of death worldwide (Almalki and Varghese, 2020). According to the UTIs survey agency report, 15% of adults and more than 50% of women's were affected at least one-time in their life history (Kumar and Das, 2016). The extensive usage, improper prescription and overuse of misguided antibiotics were the main sources for cause UTIs in human beings (Sophie and Jean-Christophe, 2020). Based on the UTIs guidelines, the cut-off values for antibiotic resistant Gram negative bacteria to a specific antibiotics is referred as 10–15%, if this values are increased, it is considered that the bacteria developed resistance against antibiotics and this antibiotics are unusual. The health condition that associated with multidrug resistant bacteria in the urine, bladder and kidney are defined as drug resistant bacteria (Ahmed et al., 2019).

According to the WHO announcement, the antibiotic resistant bacteria were categorized based on the priority list. In first ever critical list, all the carbapenemase and extended spectrum beta lactamases (ESBLs) producing Gram negative bacteria, third generation cephalosporins resistant bacteria and ciprofloxacin resistant Gram negative bacteria (Vladimir et al., 2020). All the carbapenemase and ESBLs producing bacteria have similar mechanisms including damage of cell wall synthesis, alter the efflux pump, inhibition of protein and nucleus materials, and interfere the signaling molecules and disassembling of exopolysaccharide (Di et al., 2019; Plantinga et al., 2020). All these factors are leading to cause recurrent infections in urinary tract. Antibiotic resistant infections is a long term infections that cause economic burden to Government and peoples due to the longer hospital stays, increased morbidity and mortality (Chong, 2019). Some of the antibiotics resistant bacteria may affect in target sites including efflux pump, ESBLS, QS, cell wall synthesis and biofilm formation. Other bacteria may interfere the cytotoxic effect of the available drug nature by activating

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the metabolic pathways such as aminoglycoside, phosphorylated transferase. Some bacterial genes were evolved automatically as different types of mutation may stimulated by unfavorable environmental conditions such as pH, temperature, NaCl, nutrients, carbon and nitrogen sources (Houcem et al., 2016; Mariam et al., 2011).

In the antibiotic resistant, carbapenem (imipenem, meropenem) resistant Gram negative bacteria are an emerging thread worldwide, as they are resistant to almost all classes of currently available antibiotics including fluoroquinolones, cephalosporin and carbapenem except colistin and few aminoglycosides (Miguel et al., 2014). Among the Gram negative bacteria, carbapenem resistant *K. pneumoniae* is an important bacterium, which produces the class A carbapenemase. It has the ability to inhibit the different varieties of clavulanate and hydrolysis the penicillin and cephalosporins highly compared with carbapenems. The classes A carbapenemases are a one kind of ESBLs producer, which produce a recurrent infections with rapid dissemination due to the hydrolysis of β-lactam antibiotics by stimulates the β-lactamases (Shriparna and Ranadhir, 2006).

The basic mechanism of carbapenems are acquisition of resistance genes on mobile genetic elements, and another way is through mutations in genes that change the expression and/or function of chromosomally encoded proteins (Ramachandran et al., 2020). Sometimes, the *K. pneumonia* was highly resistant to carpabenems antibiotics due to the over production of carbapenemase, ESBLs, extramural function of IMP and MRP genes and increased production of efflux pump (Brandi et al., 2011). The extended mechanism of carbapenem resistant *K. pneumonia* is biofilm formation, which is a self-produced exopolysaccharide production, stimulated the intercommunicated signaling molecules. It protected the cells form antibiotics and acted as a physical barrier of the cells. In the stage of biofilm, the bacteria exhibited 1000 times more resistance than common resistance bacteria (Tada, xxxx; Rajivgandhi et al., 2081). Accordingly, this study was initiated to detect the imipenem and meropenem resistant genes of *K. pneumonia* strains in UTI patients by phenotypic and genotypic method and to evaluate the biofilm forming efficiency by microtiter plate and congo red agar method.

2. Materials and methods

2.1. Collection of samples

The 20 numbers of clinical pathogens *K. pneumoniae* strains were obtained from Department of Microbiology laboratory, K.A. P.V. Government Medical College & Hospital, Tiruchirappalli, Tamil Nadu, India. All the chemicals, media, plates and antibiotic discs of this study were purchased from Sigma Aldrich, Mumbai, India.

2.2. Detection of MDR bacteria

The MDR effects of all bacteria were performed against specific Gram negative bacteria antibiotic panel disc of Kleb 1 HX077 and Kleb 2 HX090 including amoxyclav (AMC-30 μg), ampicillin (AMP-10 μg), ciprofloxacin (CIP-5 μg), co-Trimoxazole (COT-25 μg), gentamicin (GEN-10 μg), norfloxacin (NX-10 μg) and ciprofloxacin (CIP-5 μg), gentamicin (GEN-10 μg), erythromycin (E-15 μg), vancomycin (VA-30 μg), lincomycin (L-15 μg) and penicillin-G (P-10 μg) for HX090 were used in the HEXA discs method by Kirby-Bauer disc diffusion method followed by Clinical & Laboratory Standards Institute guidelines (CLSI Guidelines), followed by the method of Maruthupandy et al. (Maruthupandy et al., 2018).

2.3. Detection of carbapenem resistant activity

The CR ability of selected MDR *K. pnemoniae* was performed against specific carbapenem detection discs of HX066 and HX103 (CLSI Guidelines) including cefepime (CPM-30 μg), cefoperazone (CPZ-75 μg), ceftriaxone (CTR-30 μg), cefoxitin (CX-30 μg), imipenem (IPM-10 μg), ticarcillin/clavulanic acid (TCC-75/10 μg) and ciproflaxacin (CIP-5 μg), imipenem (IPM-10 μg), meropenem (MRP-10 μg), ertapenem (ETP-10 μg), cefoperazone/sulbactum (CFS-75/30 μg) piperacillin/tazobactam (PIT-100/10 μg) by Kirby-Bauer disc diffusion method (Rajivgandhi et al., 2018).

2.4. Minimum inhibition concentration test

All specific carbapenem detection powder antibiotics was used to detect their inhibition ability against selected carbapenem resistant antibiotics by minimum inhibition concentration test using 96-well microtitre plate (Kumar and Das, 2016; Sophie and Jean-Christophe, 2020). The phenotypic positive antibiotics of ciproflaxacin, amoxyclav and co-Trimoxazole, imipenem, meropenem were used in this study to detect the minimum inhibition concentration. Briefly, all the selected antibiotics were diluted in distilled water and prepared a working concentration of 10–100 mg/mL. The 10 μL of overnight matured carbapenem resistant *K. pneumonia* culture was taken in all the 96-well plates before poured with nutrient broth 100 μL concentration. Next, the various concentrations 10–100 μg/mL of selected antibiotics were added into the wells and mixed well. After 30 min mixing, the samples were maintained at overnight incubation for room temperature. After overnight incubation, the wells were monitored for turbidity formation, and which lowest concentration was inhibited the maximum growth by turbidity in the wells were noted and indicated as a best result.

2.5. Detection of carbapenem resistant genes by multiplex PCR

The multiplex PCR analysis was used to detect the available resistant genes in the selected *K. pneumonia* strain with the following modification of Rajivgandhi et al. (Ramachandran et al., 2020). Briefly, the 24 h old selected *K. pneumoniae* strain of rapid DNA was prepared and heated in 100 mL D.DH$_2$O at 95℃ for 15 min. Next, the cell suspension of the liquid culture was centrifuged at 5,000 rpm for 10 min. The template DNA materials containing supernatant was separated and used for PCR amplification by thermal cycler (Scavenling, India). For the detection of Verona integron-encoded metallo-β-lactamase (VIM), and imipenemase (IMP) genes identification, we have designed a specific universal primers 5′- ATAATTTGCCGGGATACATTTGCAG-3, 5′- CATGGCTCA TATATAATAGACAGAC-3′ and 5′- ATG TGC AGY ACC AGT AAR GTKATG GC − 3′, 5′ - TGG GTR AAR TAR GTS ACCAGA AYC AGC GG- 3′ for IMP and MRP were used respectively for forward and reverse of this study. For the amplifications process, the entire solutions were made with 25 μL including 2 X Promega PCR master mixes (Germany), each primer was taken in the concentration of 0.2 μM and μL template DNA, followed by denaturation at 95 °C for 5 min. The program was set to 30 cycles with 95 °C for 1 min time interval and followed by annealing temperature 55 °C for 1 min and extended to 72 °C for 10 min. Finally the sample mixture was maintained at 4 °C. The amplified PCR product was run X TE buffer of 1.5% agarose gel electrophoresis. After run the gel, the received material was treated by EtBr and viewed the observation of dye in UV-transilluminator. Finally, the molecular weight of the amplified product was identified and compared with 100 bp molecular weight marker (Merck, India).

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2.6. Detection of biofilm formation

The microtiter plate assay was performed to quantify the biofilm production in the selected against carbapenem K. pneumoniae (Houcem et al., 2016). Aliquot 24 old staled culture into the fresh luria bertani broth (LB) containing 96-well polystyrene plate (Himedia, India) with 0.1% glucose as supplement. Previously reported E. coli BDUMS 3 (KY617770) was acted as a positive control for biofilm formation and without addition of bacterial culture containing well-acted as a blank. The plate was incubated at 37 °C for 24 h. After incubation, 0.2 mL phosphate buffered saline (PBS) was used to remove free floating bacteria with two times, and followed by 200 μg/mL of 0.5% crystal violet solution and permitted to attach the stain in adhering cells. The plate was subsequently washed with PBS and fixed with 95% ethanol for 15 min. Finally, the plate was read at 540 nm using using ELIZA reader (Shimadzu, Japan). Based on the adherent ability of O.D values, both the bacteria were quantified as biofilm positive or negative strains.

2.7. Congo red agar assay (CRA)

Biofilm forming ability of K. pneumoniae was further cross checked by CRA plate method with the following modification of Perez et al. (Perez et al., 2019). After making CRA, the different time interval (6, 12, 24 h) of bacteria was quadrantly streaked on CRA plate and incubated at 37 °C for 24 h. After incubation, all the plates were monitored for biofilm positive or negative colonies based on the color variations. CLSI guidelines reported that the biofilm positive bacteria exhibited dark black color colonies due to the production of exopolysaccharide, and non-biofilm producer exhibited red colonies (absence of exopolysaccharide). Whereas, the low pink color colonies expose the crystalline morphology and while black color colonies exhibited a darkening which indicate, the variation of dry crystalline.

3. Result

3.1. Detection of MDR bacteria

The disc diffusion assay result of Kleb 1 HX077 and Kleb 2 HX090 exhibited that the selected K. pneumoniae was more resistant against almost all the performed antibiotics when compared with other 19 K. pneumoniae strains (Fig. 1a). After 24 h incubation, the ciprofloxacin of Kleb 1 HX077 and Kleb 2 HX090 showed 14 and 12 mm zone of inhibition against selected K. pneumoniae. The amoxyclav and co-Trimoxazole exhibit 10 mm zone of inhibition and other antibiotics did not show any inhibition against tested K. pneumoniae (Fig. 1b). Based on the CLSI guidelines, the exhibited zone of ciproflaxacin, amoxyclav and co-Trimoxazole were not produced the sufficient role against K. pneumoniae and it also sensitive to K. pneumonia (Fig. 1c, d). The low zone of inhibition revealed that the antibiotics loss their inhibitory effect against tested bacteria (Wang et al., 2017). The tested uropathogen was not considered sensitive towards the antibiotics that created low inhibitions zones, which likely arises from target site modifications of these antibiotics (Piazza, A., Comandatore, F., Romeri, F., Pagani, C., Mattioni Marchetti, V., Brilli, M., Panelli, S., Migliavacca, R., Ridolfo, A., Olivieri, P., Gismondo, M.R., Bandi, C., Rimoldi, S.G., 2018). The result proved that the bacteria may cause various infection due to the over productions of exopolysachharide (EPS), ESBL, urease, biofilm formation, quorum sensing (QS) and efflux pump (Sharma et al., 2018).

Further, specific carbapenem detection discs of HX066 and HX0103 exhibited less than CLSI reported zone was also observed. In result, 8 and 10 mm zone of inhibition observed around imipenem discs of both HX066 and HX0103 were observed. Whereas,

meropenem of HX0103 was exhibited only 8 mm zone of inhibition (Fig. 2c, d). In addition, ESBL detection discs (ceftazidime, cefepime, cefoperazone, ceftriaxone, cefoxitin, ticarcillin/clavulanic acid, cefoperazone/sulbactum and piperacillin/tazobactam), carbapenems (imipenem, meropenem and ertapenem) and fluoroquinolones (ciprofloxacin) class antibiotics were not performed better against selected K. pneumonia (Fig. 2a, b). All the classes of antibiotics were ineffective and lost their transferring molecules, which was clearly confirmed by selected K. pneumoniae MDR character, especially carbapenems. Both the Hexa disc methods were proved that the selected K. pneumoniae was MDR, especially CR (Fig. 1e). The exhibited result statement was agreed by previous report of Rajivgandhi et al. (Rajivgandhi et al., 2019). Carbapenems resistant bacteria has been shown to be more critical to treat and pose an emerging threat pose a during infections due to a severe lack of viable treatment options (Francesca et al., 2015). A important mechanism in resistance of carbapenems are mutation of chromosomal genes including regulator and promoter genes. In addition, the antigen presenting sites of bacterial cell wall was surrounded by more teichoic acid and EPS, which produce some essential components to susceptibilities of external drugs (Rajivgandhi et al., 2081). Also, increase the replication, transcription, repair, supercoiling and recombination (Wang et al., 2017).

3.2. Minimum inhibition concentration

After overnight incubation, the antibiotic disc of ciprofloxacin was inhibited 56% at 100 mg/mL concentration against tested carbapenem resistant K. pneumoniae. Whereas, the other tested antibiotics of amoxyclav and co-Trimoxazole, were exhibited 43% and 45% of inhibition against tested carbapenem resistant K. pneumoniae respectively at 100 mg/mL. Surprisingly, the antibiotics of imipenem and meropenem were exhibited 60% and 52% of inhibition respectively at 100 mg/mL concentration (Fig. 3). Compared with all the antibiotics, the imipenem and meropenem were inhibited the bacterial growth very highly. The result was indicated that the antibiotics imipenem and meropenem were suitable for carbapenem inhibition effect. In addition, based on the observation, the more turbidity was observed in the imipenem and meropenem treated wells. The other wells were also exhibited the turbidity in their respective treated wells. The observed result was interpreted with CLSI guidelines, and received all the antibiotics were exhibited sensitive effect against tested bacteria. In the result of 56, 43 and 45 inhibitions of ciprofloxacin, amoxyclav and co-Trimoxazole were not reached the inhibition level of K. pneumoniae by CLSI guidelines (Emel et al., 2019). In addition, 60 and 52% inhibition of imipenem and meropenem were also showed with sensitive due to the production of carbapenemase enzymes (Barbara and Tiffeny, 2018). The carbapenemase enzymes were deactivating the antibiotics when it contact with bacterial cell surface. Finally, the bacteria could develop the multi-drug resistant effect within the cytoplasm and other parts. It effectively damaged the cytoplasmic formed materials and stimulated the more leakages in infected places (Norelle et al., 2018). This effect was indicated that the bacteria developed the resistant effect against foreign antibiotics due to the production of carbapenemase, ESBLs, QS, biofilm and other protein stimulation (Griangsak and Somnuk, 2013). Hence, the present result was indicated that the selected K. pneumonia was fluoroquinolones, third generation cephalosporins and carbapenems resistant and could be possible that the carbapenemase resistant genes present in the strain.

3.3. Detection of carbapenam resistant genes by multiplux PCR method

Multiplex PCR method is an important method to detection of multi-drug resistant genes form pathogenic bacteria (Rajivgandhi

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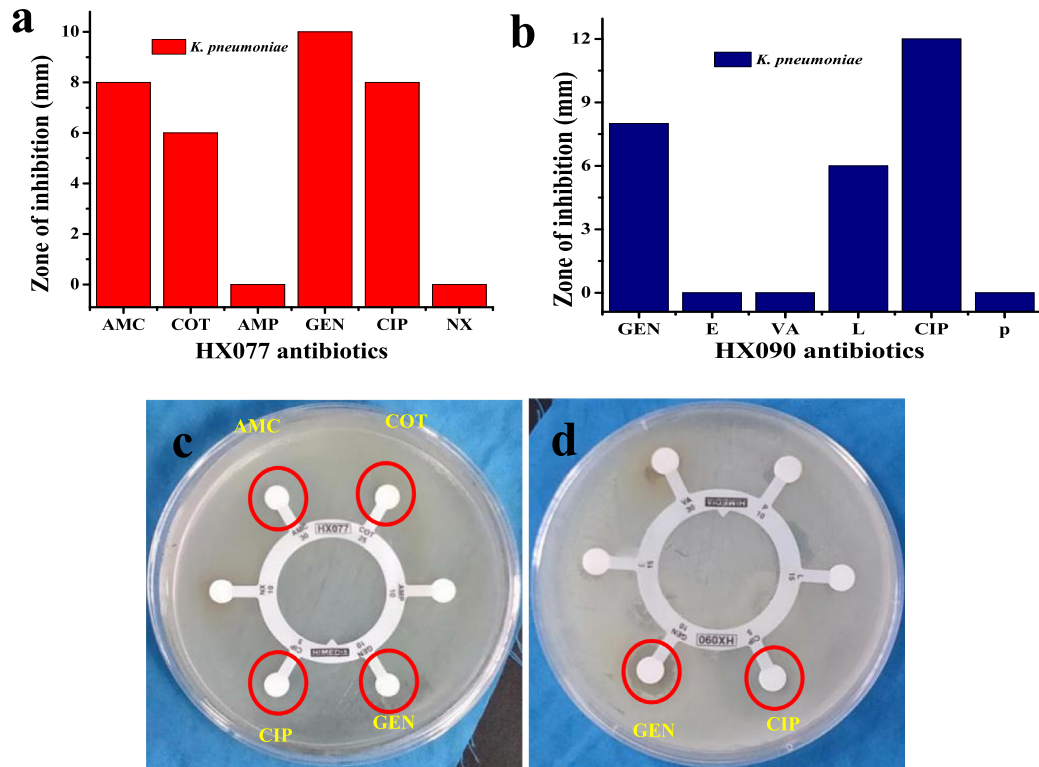Saudi Journal of Biological Sciences 28 (2021) 1750–1756

Fig. 1. Detection of multi drug resistant *K. pneumoniae* from tested samples using Kleb 1 HX077 and Kleb 2 HX090 HEXA discs, the differentiation of zones (a, b) and zone of inhibition plate by disc diffusion method (c, d).
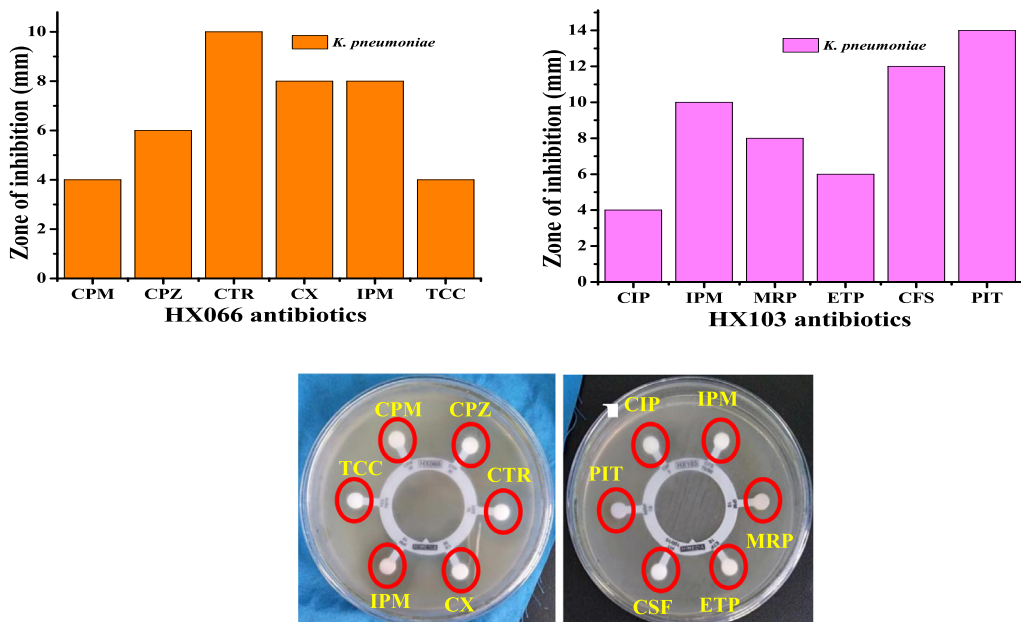

Fig. 2. Screening of carbapenem resistant *K. pneumonia* from tested samples using specific carbapenem identification HX066 and HX0103 HEXA discs, differentiation of diffused zones (a, b) and zone of inhibition plate by disc diffusion method (c, d).

et al., 2019). After run by 1% agarose gel electrophoresis, the purity of the DNA and their molecular weights were identified after the comparison with known genes molecular weight (Jiri et al., 2020). In this method, we have used the primers of IMP-F, IMP-R and MRP-F and MRP-R for detection of IMP and MRP resistant genes in *K. pneumonia*. After proper evaluation, the result was agreed the phenotypic result, and proved that the genes of IMP and MRP was present in the tested *K. pneumonia* strain. The IMP and MRP genes were able to produce the multi-drug resistant proteins in bacteria and it helped to develop the multi-drug resistant
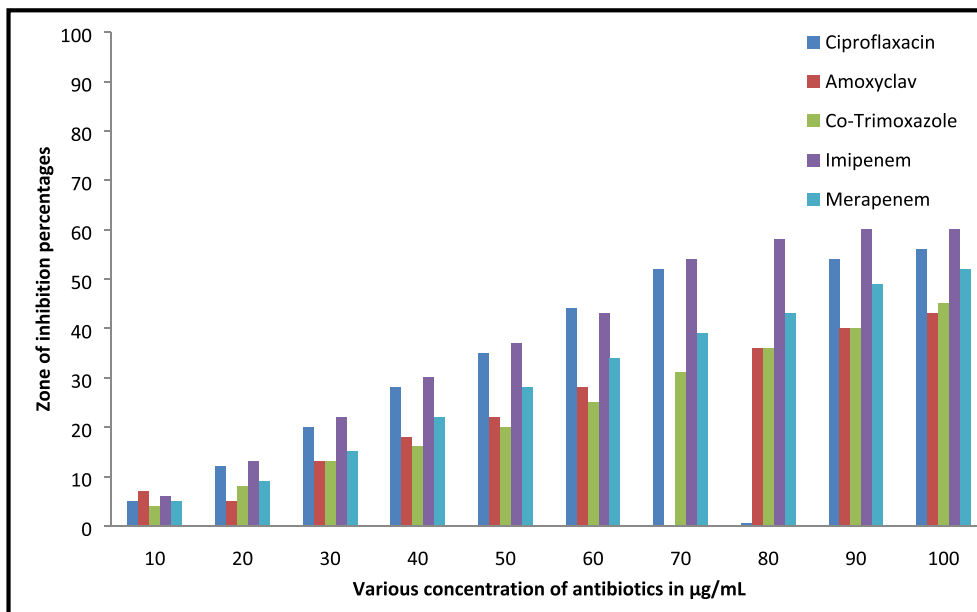
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Fig. 3. Minimum inhibition concentration of effective antibiotics against carbapenem resistant *K. pneumonia.*

effect against almost all the current antibiotics (Satoru and Masami, 2019). In our result, the carbapenem resistant genes were showed with 593 and 455 bp regions. Compared with control molecular marker, they were confirmed that the exhibited regions were IMP and MRP regions (Fig. 4). The result was also proved that the IMP and MRP genes were present in the phenotypically confirmed carbapenem resistant *K. pneumonia.* Recently, the researcher was reported that the IMP and MRP resistant bacteria has the ability to develop multi drug resistant effect against all the bacteria due to the production of carbapenamases. These enzymes able to neutralize the beta lactam ring in antibiotics due to the damage of oxy-amino chide chines (Samia et al., 2017). When the antibiotics enter into the bacterial cell wall, the antigen presenting genes were reacted, and stimulated the antibiotics denature genes continuously. Due to this effect, the antibi-

otics were lost their inhibition ability against bacteria and leads to sensitive nature [34]. Recent years, the researchers were worked against beta lactamase and carbapenamase enzymes for decrease the antibiotic resistant characteristic bacteria (Rajivgandhi et al., 2019). In 2019, the WHO announced three major classes of resistant bacteria, among the list carbapenamase producing *K. pneumonia* frequently comes under the first ever critical list (Yuxuan et al., 2020). The carbapenamase is a different kind of enzymes, it could able to deactivate the fluroquinolones, chaphalosporins and carbapenam antibiotics (Rajivgandhi et al., 2018). The result was agreed by US researchers and reported that the carbapenamse producing enterobacteriaceae is the emerging thread and we should more concern in prevention and eradication strategy (Wang et al., 2017). The similar result was reported by Rajivgandhi et al. (Rajivgandhi et al., 2019), carbapenemases pattern of multi-drug resistant *K. pneumonia* is worldwide problem, in particular IMP and MRP resistant *K. pneumonia* infection is creates increased mortality (Norelle et al., 2018). Internationally, the detection of carbapenemase resistant bacteria and their research is done only in phenotypic approach; particularly few authenticated reports were only published by Indian researchers. Due this lack of gene research, we have more concentrated in genetic research in carbapenem resistant bacteria. Thus, based on the phenotypic and genotypic results, the *K. pneumonia* has carbapenem resistant bacteria and it was indicated as carbapenem resistant *K. pneumonia.*
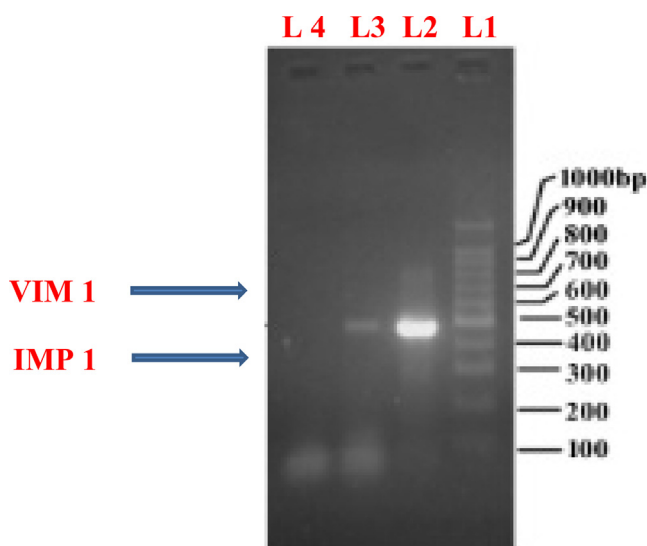
3.4. Time dependent biofilm formation

Biofilm positive result of CR *K. pneumoniae* was clearly identified in TCP assay method and result is given in Fig. 5**a, b.** The selected CR *K. pneumoniae* was identified with strong adherent ability, which exhibited 0. 33, 0.560 and 0.594 values at 6, 12, 24 and 48 h respectively. It was supported with positive control bacteria *P. mirabilis* BDUMS 1(KY617769), it exhibited O.D value of 0.260, 0.357 and 0.463 at 6, 12, 24 and 48 h respectively. All the time intervals of both the biofilm positive strains clearly indicate, selected bacterial strain was strong biofilm producer. Whereas, non-biofilm producing *K. pneumoniae* MTCC 3384 was identified with weak biofilm result, which exhibited 0.100, 0.104, 0.165 and 0.191 at 6, 12, 24 and 48 h respectively. Based on the O.D values,
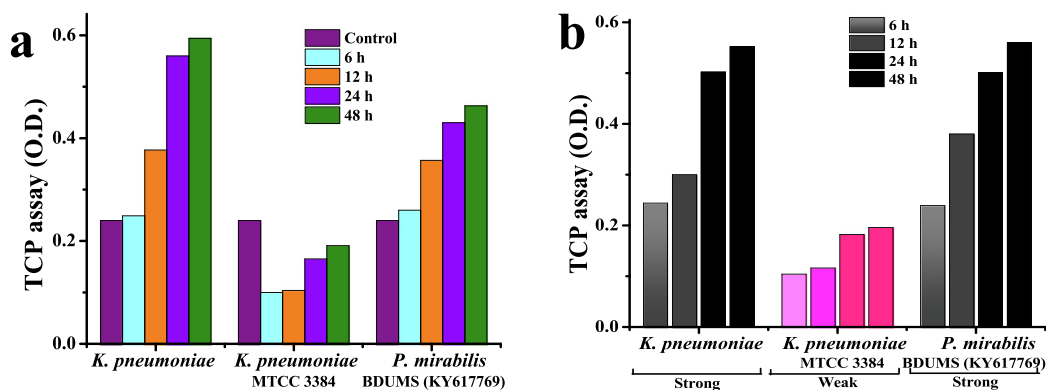

Fig. 4. Detection of carbapenem resistant genes from tested *K. pneumoniae* through multiplex PCR method. The 16srRNA genes, VIM 1, IMP 1 and MRP 1 amplicons were added into the Lanes.

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Fig. 5. Time dependent biofilm formation of carbapenem resistant *K. pneumoniae* by TCP method (a, b).

selected CR *K. pneumoniae* could produce strong biofilms with more adherence ability when compared with control either produced no biofilms or biofilms with weak adherence (Perez et al., 2019). In TCP assay, the strong adherent cells were utilized crystal violet satin due to the presence of biofilm forming cells of the negative charged cell wall. It is a primary acceptable method used to count viable bacterial count by staining method (Panda et al., 2016).

In addition, TCP assay result was further confirmed by CRA plate due to the color variation. After respective time interval of 3, 6, 12 and 24 h, black color production was increased in the CRS plates (Fig. 6a). After 24 and 48 h, the complete biofilm formation was observed in bright black color produced CRA plate. This result was confirmed and supported to TCP assay, which CR *K. pneumoniae* has biofilm producing ability. Whereas, non-biofilm producing *K. pneumoniae* MTCC 3384 culture inoculated plates exhibited pink color colonies at the respective time interval (Fig. 6b). Differences between the control and test results were identified by color variation, which depends on the exopolysaccharide production. If the bacterial colonies have biofilm promoter gene, it easily observe sugar molecules from CRA medium and produced black color colonies. If the bacteria don't have the ability of biofilm production, it cannot observe the sugar molecules and exhibited pink color colonies instead of black color. Observed result was supported by previous report of (Noumi et al., 2018). EPS play a role in cell protection, attachment, maturation, cell to cell interaction for biofilm production (Nasser et al., 2017). Similarly, our previous report of biofilm positive *P. mirabilis* BDUMS 1(KY617769) study (Fig. 6c) also clearly resembled with current study in TCP and CRA methods (Rajivgandhi et al., 2081). Hence, results of both TCP and CRA methods suggest, selected carbapenem resistant *K. pneumoniae* as a strong biofilm producer.
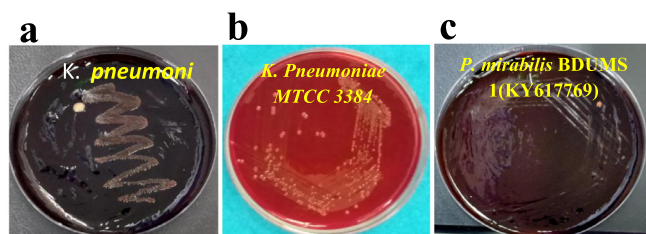
4. Conclusion

Based on the phenotypic disc diffusion method, the result was concluded that the selected *K. pneumoniae* strain was carbapenem resistant bacteria. The MIC method was confirmed the resistant level of the antibiotics. In addition, the carbapenemase genes IMP and MRP were detected present in the bacterial sample and confirmed by multiplex PCR method. Further, the extended resistance characteristic feature of carbapenem resistant *K. pneumoniae* was confirmed by biofilm production using TCP and CRA methods. Finally our result was confirmed that the selected *K. pneumoniae* strain was carbapenemase and biofilm producer.

Ethical clearance

All the samples of this study was approved by the Ethics Review Committee (S.No of IEC Management office: DM/2016/101/55), Department of Microbiology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. The permission was sought from the Government Hospital and Microbiology laboratory authorities. The ethical principles of scientific research as well as related national laws and regulations were adhered to.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Fig. 6. Confirmation of biofilm formation of carbapenem resistant *K. pneumonia* by congo red agar plate assay. The black color production of tested *K. pneumoniae* (a), non-biofilm forming *K. pneumoniae* (b) and Reference positive biofilm producing *P. mirabilis* BDUMS 1(KY617769) (c).

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