MINAR International Journal of Applied Sciences and Technology
ISSN: 2717-8234
Article type: Research Article

Received: 15/10/2022 **Accepted:** 01/11/2022 **Published:** 01/12/2022

SCREENING OF ANTIBIOTIC RESISTANCE BETWEEN BIOFILM PRODUCER AND NON-BIOFILM PRODUCER OF *KLEBSIELLA PNEUMONIAE* ISOLATES

Zheen A. ABDULLAH[1]

Assistant lecturer, Pharmacognosy department, Pharmacy College, Hawler medical university,

Erbil, Iraq

Aisha K. Ahmed

BA. Pharmacy College, Hawler medical university, Erbil, Iraq

Abstract

Background: Klebsiella pneumoniae is a common cause of nosocomial infections. Antibiotic resistance and the ability to form biofilm, as two key virulence factors of *K. pneumoniae*, are involved in the persistence of infections.
Aim: determine the rate of (10) antibiotics activity against isolated *K. pneumoniae* then evaluate the ability of isolated bacteria to produce biofilm by using Congo red agar method and studying the antibiotic resistance pattern between biofilm producer and non-biofilm producer of *K. pneumoniae* isolates.
Material and methods: over a period of 4 months a total of 37 *k. pneumoniae* isolates were collected. Antibiotic susceptibility was determined by the disc diffusion method according to CLSI. Biofilm production was assessed by Congo red agar method.
Results: Antibiotic susceptibility profile showed a variable level of resistance. The highest resistance was reported with tetracycline (56.8%). And the highest susceptibility was reported with imipenem (85.6%) and meropenem (85.6%). Biofilm by Congo red agar method was detected in 19 isolates with a percentage of (51.35%) however, 18 (48.65%) of isolates were non biofilm producer. The association of antibiotic resistance between biofilm-producing and non-biofilm-producing *K. pneumoniae* isolates was evaluated by the Chi-square test and the p-value was found to be 0.97 (statistically non-significant).
Conclusion: our study revealed that the *K. pneumoniae* isolates differed in their responses against used antibiotics, moreover they are varied in their ability to produce biofilm and the resistance pattern between biofilm producer and non-producer was no significant. More studies are needed to characterize the pattern of antibiotic resistance between biofilm producer and non-biofilm producer of *K. pneumoniae* isolates and universal efforts should be increased to prevent the prevalence of multi- antibiotic resistant bacteria and eliminate the hospital born bacteria that are causing a great rise in mortality.

Keywords: *Klebsiella Pneumoniae*, Antibiotic Resistance, Biofilm.

http://dx.doi.org/10.47832/2717-8234.13.11

[1] Zheen.mardani@hmu.edu.krd, https://orcid.org/0000-0002-6680-3346

Background: *Klebsiella pneumoniae* belongs to the *Enterobacteriaceae* family and is Gram-negative, encapsulated, non-motile, and rod-shaped (Podschun and Ullmann, 1998; Murray *et al.*, 2007). It has emerged as a significant healthcare-associated pathogen in the last two decades, accounting for 14–20 percent of infections involving the respiratory tract, lower biliary duct, surgical wounds, and urinary tract (De Rosa *et al.*, 2015). Fimbriae, antiphagocytic capsule (CPS), LPS, membrane transporters, and siderophores are among the virulence factors, allowing *K. pneumoniae* to survive and evade the immune system during infection (Clegg and Murphy, 2016). Antibiotic resistance and ability to produce biofilm, are two key virulence factors of K. pneumoniae, which involved in the persistence of infections (Shadkam *et al.*, 2021). *K. pneumoniae*'s ability to build biofilms (Vuotto *et al.*, 2014; Chung, 2016), which are aggregation of different bacterial cells and covered by self-produced extracellular matrix (Singh *et al.*, 2021). This matrix made up of proteins, exopolysaccharides, DNA, and lipopeptides. (Nirwati *et al.*, 2019). shields the bacterium from both the immune system of the host and antimicrobial agents (Bandeira *et al.*, 2014). Infections caused by multidrug-resistant (MDR) carbapenem-resistant *K. pneumoniae* strains being increasingly isolated in hospital settings (Coque, *et al.*, 2008; Grundmann *et al.*, 2010). Due to plasmid-encoded extended-spectrum beta-lactamases (ESBLs) and carbapenemases, MDR and extensively drug-resistant (XDR) *K. pneumoniae* strains are resistant to a wide range of β-lactams (penicillins, third- and fourth-generation cephalosporins, carbapenems and monobactam), and also to other antibiotic classes such as aminoglycosides and fluoroquinolones (Rawat and Nair, 2010). In contrast to carbapenemase, *Enterobacteriaceae* efflux pumps and porins have been shown to be almost clearly involved in the production of biofilm as well as antibiotic resistance (Kvist, *et al.*, 2008; Gaddy, *et al.*, 2009). There has also been evidence of a link between antibiotic resistance and the ability of *K. pneumoniae* to build biofilms (Yang and Zhang, 2008). The antibiotic resistant have become a worldwide problem and there is still quite limited data related to biofilm producing capacity and antibiotic resistance of *K. pneumoniae* in Erbil- Iraq. Thus the aims of present study were to determine the rate of (10) antibiotics activity against isolated *K. pneumoniae* then evaluate the ability of isolated bacteria to produce biofilm by using Congo red agar method and studying the antibiotic resistance pattern between biofilm producer and non-biofilm producer of *K. pneumoniae* isolates.

Methods

Sampling and bacterial isolation:

A total of 37 samples of *K. pneumoniae* were collected within four months (November 2021 to March 2022) from patient's urine in Maternity Teaching Hospital in Erbil-Iraq. Bacterial isolates were initially identified by culturing them on MacConkey agar and Gram staining then confirmed by Vitek2 compact system. Each *K. pneumoniae* isolate was preserved in 20% glycerol at -70°C.

Antimicrobial susceptibility testing:

According to the clinical and laboratory standards Institute protocol (CLSI; M100-30th ed.), Antibiotic susceptibility test used to evaluate the susceptibility of isolated bacteria against 10 antibiotics by using disc diffusion (Kirby Bauer) method. The tested inoculums were suspended in sterile normal saline and adjusted to the 0.5 McFarland standard before being dispersed onto the surface of Mueller-Hinton agar (MHA) (LAB M Limited topely house, UK) for ceftazidime (CAZ: 30µg),Cefixime (CFM:5µg) imipenem (IPM:10µg), meropenem (MEM:10µg), ciprofoxacin (CIP: 10µg),amikacin (AN: 10µg), gentamicin (GM; 10µg), Tetracycline (TE: 10µg) Amoxicillin/CLA (AMC:20/10 µg), Levofloxacin (LEV: 5µg)

(Bioanalyse, Turkey). After overnight incubation the zones of inhibition were measured as described by (Wayne, 2005).

Biofilm formation test:

This test was performed using Congo red agar method which is described by Freeman et al. as a simple qualitative method to detect biofilm production. the tested bacteria were inoculated onto the surface of the Congo red agar medium and incubated at 37ºC for 24-48 hours. On Congo red agar medium, biofilm producers create black colonies (strong), red (moderate) while non-biofilm producers form pink/white colonies (Majeed, 2011).

Data analysis:

Descriptive statistical analysis (including means and percentages to characterize data) was done by using SPSS software version 16 for windows .To provide a comparison between groups the chi-square test was used. Results with P <0.05 were considered significant.

Results:

Bacterial isolation:

In total, 37 non-duplicative clinically-relevant *K. pneumoniae* were collected from urine of female's patients, their ages ranged between 22-36 years. Antibiotic susceptibility pattern of the isolates showed a variable level of resistance; 56.8% to tetracycline, 54.1% to cefixime, 51.4% to amoxicillin/clavulanic acid and 51.4% to ciprofloxacin. On the other hand the most effective antibiotics were imipenem, meropenem and gentamycin with percentage 86.5%, 86.5% and 64.9% respectively (Table.1). Overall high susceptibility was observed for ceftazidime (54.1%), amikacin (51.4%) and levofloxacin (51.4%).

Table (1) Number and Percentage of antibiotic susceptibility pattern of isolated *K. pneumoniae*.

Antibiotics	Resistance	Intermediate	Sensitive
Tetracycline	21 (56.8%)	1 (2.7%)	15 (40.5%)
Imipenem	5 (13.5%)	-	32 (86.5%)
Ceftazidime	16 (43.2%)	1 (2.7%)	20 (54.1%)
Cefixime	20 (54.1%)	-	17 (45.9%)
Gentamycin	12 (32.4%)	1 (2.7%)	24 (64.9%)
Amoxicillin/CLA	19 (51.4%)	10 (27%)	8 (21.6%)
Ciprofloxacin	19 (51.4%)	-	18 (48.6%)
Amikacin	13 (35.1%)	5 (13.5%)	19 (51.4%)
Levofloxacin	17 (45.9%)	1 (2.7%)	19 (51.4%)
Meropenem	5 (13.5%)	-	32 (86.5%)

Biofilm production:

A total of 37 positive culture of *K. pneumoniae* were taken for this study. Out of these 37 *K. pneumonia*e isolates, biofilm by congo red agar method was detected in 19 isolates with percentage (51.35%), in which 5 (13.51%) were strong biofilm producer in addition to 14 (37.84%) were moderate biofilm producer, however 18 (48.65%) of isolates were non biofilm producer as shown in figure 1.

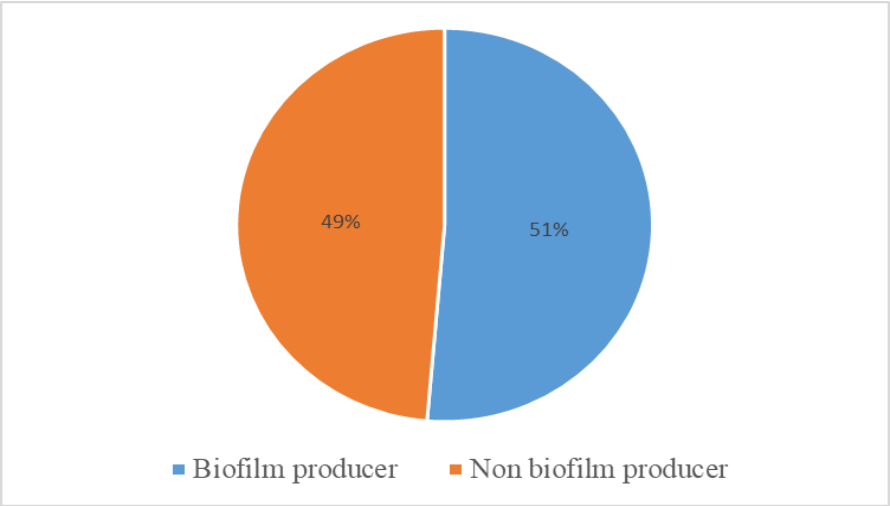

Figure (1) percentage of biofilm and non-biofilm production by *K. pneumoniae* isolates

The association of antibiotic resistance between biofilm producing and non-biofilm producing *K. pneumoniae* isolates was evaluated by Chi square test and the p value was found to be 0.97 (statistically non-significant) (Table 2).

Table (2) Antibiotic resistance pattern between biofilm producing and non-biofilm producing isolates of *K. pneumoniae* (statistically non-significant)

Antibiotics	Biofilm producer resistant isolates	Non biofilm producer resistant isolates
Tetracycline	10	11
Imipenem	2	3
Ceftazidime	8	8
Cefixime	12	8
Gentamycin	7	5
Amoxicillin/CLA	10	8
Ciprofloxacin	10	8
Amikacin	8	5
Levofloxacin	11	6
Meropenem	2	3

Discussion:

Klebsiella pneumoniae is the second most repeatedly isolated species from Urinary tract infection, after *Escherichia coli* (Stamm *et al.*, 1991). The ability of these bacteria to adhere to host structures is considered needful for the development of infection (Struve *et al.*, 2008; Lin *et al.*, 2010). As these bacteria are highly and increasingly resistant to many antibiotics, preventing the spread of these bacteria is a key goal in the healthcare setting. Indeed identification of isolated *K. pneumoniae* was depended on Vitek2 analysis with the Gram-negative (GN) card which was designed for use with this system. VITEK2 system is an automated machine designed to provide rapid and accurate phenotypic identification for most clinical microorganisms while the VITEK 2 card is an effective means of rapidly and accurately identifying many gram negative Enterobacteriaceae. This colorimetric Gram-negative (GN)

identification card, contains 47 tests (Ligozzi *et al.*, 2002). On other hand, antibiotic resistance is a main clinical problem in treating infections caused by these bacteria. The resistance to antibiotics has increased over the years however the resistance rates differ from one zone to another (Farrell *et al.,* 2003; Mathai *et al.*, 2001). In fact in this study the samples were isolated from patients at maternity teaching hospital, which is one of the hospitals in Erbil city of Iraq and the study of antibiotic resistance patterns of *K. pneumoniae isolates* done by using the Kirby Bauer method (figure 2). Furthermore, isolated bacteria gave variable resistance to tested antibiotics, and the highest resistance was reported to tetracycline (56.8%), cefixime (54.1%), amoxicillin/CLA (51.4%), and ciprofloxacin (51.4%). The results for amoxicillin/CLA and tetracycline were in agreement with Varghese *et al.*, 2016 results which recorded 62.9% and 45.7% resistance respectively. On other hand bacterial isolates showed less resistance to amikacin (35.1%), ceftazidime (43.2%), and levofloxacin (45.9%) when compared to the above-mentioned antibiotics, similar results for amikacin was reported by Varghese *et al.*, 2016 with resistance profile of (34.3%) and by Dumaru *et al.*, 2019 with a result of (40.81%). In the current study the most effective antibiotics were imipenem, meropenem, and gentamycin, with susceptibility percentages of 86.5% for both imipenem and meropenem and 64.9% for gentamycin. The results for imipenem and gentamycin were strongly supported by the findings of Varghese *et al.*, 2016 with susceptibility profiles of 85.7% and 60% respectively, but the study was not supportive enough for meropenem, with a percentage of 65.7%.

Figure (2) Detection of Antibiotic resistance

The reason for the high resistance to these antibiotics observed in this study may be due to unreasonable use of these antibiotics, transition of resistant isolates of bacteria among people, and consumption of food from animals that have taken antibiotics.

Though *K. pneumoniae* is a leading cause of Gram negative nosocomial infections and it is associated with a high mortality rate, not much is known about its pathogenic planner beyond the role of capsule. In the established pattern of *K. pneumoniae* pathogenesis, the anti-phagocytic polysaccharide capsule is the most studied virulence factor and most distinctive characteristic in contrast only a few another factors including pili and lipopolysaccharide have been suggested as virulence factors, however their contribution to host pathogenesis has not been examined in detail. The ability of *K. pneumoniae* to associate into communities in biofilms, is central to their pathogenicity as they give protection from bactericidal molecules present on host tissues (Hennequin and Forestier, 2007). The formation of biofilm protects *K. pneumoniae* against immune responses of the host, the action of antibiotics, and promote its persistence (Vuotto *et al.*, 2014). The result of biofilm production in present study indicated that each isolated *K. pneumoniae* had a different

potential to form biofilm under the same condition, this result was similar to that obtained by Preethi *et al.*, 2021. In addition to that biofilm-producing bacteria are usually related with antibiotic resistance, retrogression, and chronic infections which are of great concern while starting therapy (Deotale *et al.*, 2015). The cells existing within the biofilm can break up from one site and initiate the infection at a different site therefore suitable aggressive antibiotic therapy should be founded thereby eradicating and interfering with the formation of biofilm (Wasfi *et al.*, 2012). On other hand, the antibiotic resistance pattern between the biofilm and non-biofilm producing isolates was statistically non-significant (p value=0.97) this present result was disagree with that obtained by Shanmugam *et al.*, 2017 however it was similar to the result of Cepas *et al.*, 2019.

Therefore, self-medication, failure to act in accordance with therapy, and the selling of counterfeit antibiotics may all be part to the rise in antibiotic resistance in the world. Bacterial resistance can be overcome in a variety of ways so it is important to understand that antibiotics should only be provided based on sensitivity testing results, as incorrect antibiotic exposure leads to the emergence of resistant strains. Furthermore, these antibiotics should be taken for a sufficient amount of time, in the right combination, and at the right dose. Moreover to achieve proper and effective therapy, sanitation and patient awareness are also required.

Conclusion:

The antibiotic resistance patterns of the isolates of *K. pneumoniae* investigated, varied. The highest resistance was reported with tetracycline (56.8%), and the lowest resistance was reported with imipenem and meropenem (13.5%). Among 37 *K. pneumoniae* isolates 19 isolates were biofilm producers with a percentage (51.35%), in which 5 (13.51%) were strong biofilm producers and 14 (37.84%) were moderate biofilm producers, although 18 (48.65%) were non-biofilm producer. Furthermore the resistance pattern of the isolates between biofilm producer and non-producer was not significant. More studies are needed to characterize the pattern of antibiotic resistance between biofilm producer and non-biofilm producer of *K. pneumoniae* isolates and universal efforts should be increased to prevent the prevalence of multi- antibiotic resistant bacteria and eliminate the hospital born bacteria that are causing a great rise in mortality.

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