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Research Article

EVALUATION OF EFFLUX PUMP ACTIVITY AND BIOFILM FORMATION IN MULTIDRUG RESISTANT *KLEBSIELLA PNEUMONIAE* ISOLATES IN TANTA, EGYPT

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ABSTRACT

Nosocomial and community acquired infections that caused by multidrug-resistant (MDR) *Klebsiella pneumoniae* isolates are widespread recently resulting in high morbidity and mortality due to limited number of treatment options with effective antibiotics. The aim of this study is to evaluate the antibiotic resistance profile, biofilm formation and efflux pump activity of MDR *K. pneumoniae* isolates collected from different hospitals in Tanta, Egypt. A total of 70 *K. pneumoniae* isolates characterized by standard biochemical tests and confirmed by MALDI-TOF/MS were screened for antibiotic susceptibility, efflux pump activity and biofilm formation. Isolates displayed high resistance to penicillins, cephalosporins, trimethoprim-sulfamethoxazole and the majority of tested fluoro/-quinolones and decreased resistance to imipenem, amikacin, chloramphenicol, tigecycline and colistin. Out of 70 *K. pneumoniae* isolates, 2 isolates exhibited Pan Drug-Resistance (PDR) profile while 57 (81.4%) and 11 (15.7%) exhibited MDR and Extensively drug-resistance (XDR) profiles, respectively. Sixty-four (91.4%) isolates exhibited efflux pump activity while all tested isolates had the ability to form biofilm with varied degrees as 40 (57.1%), 26 (37.1%), and 4 (5.7%) isolates were strong, moderate and weak biofilm producers, respectively. Also, a strong relation between efflux pump activity and biofilm formation per isolate was detected. In conclusion, Multidrug resistance, biofilm formation and efflux pump capabilities in *K. pneumoniae* have serious public health implications in the management and control of infections caused by this bacterium. Therefore, a multifaceted approach and precise planning are recommended in controlling these infections.

KEY WORDS: *Klebsiella pneumoniae*; Efflux; Biofilm; Antibiotic resistance

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative, rod-shaped, nonmotile, encapsulated member of the family Enterobacteriaceae[1]. *K. pneumoniae* has become an important healthcare-associated pathogen, causing about 14–20% of the community-acquired and nosocomial infections in the last two decades[2]. With the emergence of hypervirulent and antibiotic resistant *K. pneumoniae* strains, their caused infections lead to high morbidity and mortality most frequently in high-risk individuals including immunosuppressed, neonates and the elderly[3,4]. The increase in multidrug-resistant (MDR) *K. pneumoniae* in recent years is primarily resulted from the irrational use of antibiotics in treatment and prevention of infections caused by this bacterium[5]. There are several mechanisms of antibiotic resistance and creating MDR *K. pneumoniae* isolates including efflux pumps and biofilm formation[6]. Efflux pumps are protein-based structure that extrude the antibiotics from within cells that possess them in an energy dependent manner[7]. Biofilm is an aggregate of microorganisms in which cells are embedded in a matrix of extracellular polymeric substances (EPS). It has been associated with antibiotic resistance and persistence observed in several pathogenic bacteria including *K. pneumoniae* and their prevalence has been shown to be a major contributor to a number of distinct nosocomial infections that are difficult to be treated[8,9]. Hence this study aimed at evaluating the efflux pump activity, biofilm formation and antibiotic resistance profile of *K. pneumonia*e isolates recovered from clinical samples in Tanta, Egypt.

MATERIALS AND METHODS

Ethics statement

All experiments were conducted in compliance with the ethical standards. The experimental protocols and consent forms were revised and accepted by the Research Ethics Committee, Faculty of Pharmacy, Tanta University, Egypt.

Bacterial isolation and identification

A total of 70 *Klebsiella pneumoniae* isolates were recovered from different clinical specimens, including urine, sputum, wound pus, burn, stool and blood, from patients admitted to the Tanta University Hospital and the National Cancer Institute, Tanta University. These isolates were examined microscopically and identified using a panel of standard biochemical tests according to the methods suggested by MacFaddin[10]. Also, these isolates were further confirmed by culturing on chromogenic agar medium. Confirmation of identity was performed using MALDI-TOF/MS with score values > 1.9, following the manufacturer recommendations (Bruker Daltonics, Germany).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the recovered *K. pneumoniae* isolates to various antimicrobials was determined by Kirby-Bauer disc diffusion method except for tigecycline and colistin that was determined by broth microdilution method, and results were interpreted in accordance with the recommendations of Clinical and Laboratory Standards Institute (CLSI) and the European

Committee on Antimicrobial Susceptibility Testing (EUCAST) for tigecycline[11,12]. The antimicrobials tested by disc diffusion method were ampicillin (10 µg), amoxicillin/ clavulanate (20/10 µg), piperacillin/tazobactam (100/10 µg), cefuroxime (30 µg), cephalexin (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefoxitin (30 µg), aztreonam (30 µg), imipenem (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), pefloxacin (5 µg), norfloxacin (10 µg), ofloxacin (5 µg), levofloxacin (5 µg), gatifloxacin (5 µg), amikacin (30 µg), gentamycin (10 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg), tetracycline (30 µg). All antibiotic discs were purchased from Oxoid, England. *K. pneumoniae* ATCC 13883 was used as a reference strain.

Multiple antibiotic resistance (MAR) index studies in *K. pneumoniae* isolates

The MAR index values for each isolate were calculated using the following formula:
MAR index for each isolate = Number of antibiotics to which the isolate was resistant/ Total number of antibiotics to which the isolate was exposed[13]**.**

Screening of efflux pump activity

Efflux pump activity of *K. pneumoniae* isolates was determined using the Ethidium Bromide (EtBr)-agar Cartwheel method[14]. Trypticase Soy agar (TSA) plates containing 0 mg/L, 0.25 mg/L, 0.5 mg/L, 1 mg/L, 1.5 mg/L, 2 mg/L and 2.5 mg/L concentrations of EtBr (Sigma, USA) were prepared fresh and kept protected from light. The tested bacterial cultures are prepared in liquid media, adjusted to 0.5 of a McFarland standards, swabbed on the EtBr-TSA plates forming a cartwheel pattern and incubated at 37ºC for 16 h. After which bacterial culture were viewed under an UV trans-illuminator (Kowell, Spain) and the minimum concentration of EtBr (MC_{EtBr}) that produces fluorescence of the bacterial mass was recorded.

Screening of biofilm formation

The ability of isolates to form biofilm was determined by the microtitre plate technique[15]. For each isolate, 3 wells of a 96-well plastic tissue culture plate were filled with 180 µl of LB broth supplemented with 1% glucose and 20 µl of the overnight culture that diluted to a final optical density 600 (OD600) equals 0.1. Sterile LB broth supplemented with 1% glucose was used as a negative control. After incubation at 37°C for 18 h, the content of each well was discarded, and wells were washed with phosphate buffered saline (PBS) for three times. Wells were dried for 1 h at 60°C, stained for 15 min with 180 µl of 2% crystal violet and washed again three times with PBS. Then, 180 µl of 33% (v/v) glacial acetic acid were added to each well to solubilize the dye bound to adherent cells and the optical density (OD) was measured at 570 nm using an ELISA auto-reader (Sunrise Tecan, Austria). The OD cut-off (ODc) was described as three standard deviations above the mean OD of the negative control. The isolates were classified based on their adhesion capabilities into the following groups: non-biofilm producers ($OD \leq ODc$), weak biofilm producers ($ODc < OD \leq 2 \times ODc$), moderate biofilm producers ($2 \times ODc < OD \leq 4 \times ODc$) and strong biofilm producers ($4 \times ODc < OD$).

Statistical analysis

The relationship between efflux activity and biofilm formation was conducted using chi-square test by SPSS software for Windows (version 20; SPSS Armonk, NY: IBM, USA). Statistical significance was defined as P value less than 0.05.

RESULTS AND DISCUSSION

The extensive use of antimicrobial agents has led to a high prevalence of MDR *K. pneumoniae* strains that caused several worldwide life-threating hospital and community acquired infections[16]. In our study, multiple antibiotic (9-24) resistance was observed among the tested isolates. This phenomenon of multi-resistance was also observed in several other studies[17-19]. The frequency of resistant *K. pneumoniae* isolates was markedly high for 11 of the tested antibiotics as all (100%) isolates were resistant to ampicillin, cefuroxime, nalidixic acid and ciprofloxacin while 95.7%, 91.4%, 88.6%, 84.3%, 81.4%, 80% and 78.6% of the tested isolates were resistant to pefloxacin, norfloxacin, piperacillin/ tazobactam, trimethoprim-sulfamethoxazole, cephalexin, ofloxacin and cefotaxime, respectively (Fig. 1). In partial agreement with our results, Mohamed et al. (2018) in Egypt also reported a high incidence of resistance to nalidixic acid (100%), ciprofloxacin (100%), ampicillin (100%), cefuroxime (100%), piperacillin/ tazobactam (90%) and trimethoprim-sulfamethoxazole (80%) among biofilm producing *K. pneumoniae* isolates[20]. Moreover, the frequency of resistant *K. pneumoniae* isolates was between 65% and 75% for six of used antibiotics (amoxicillin/clavulanate 74.2%, ceftriaxone 72.9%, cefoxitin 71.4%, aztreonam 67.1%, levofloxacin and gentamicin 65.7%). Comparable incidences of resistance to ceftriaxone (79%), cefoxitin (79%) or aztreonam (65.5%) were reported in another study in Egypt[21]. This observed high frequency of resistant isolates to penicillins and cephalosporins in our research and that of others could be due to the massive prescription and often misuse of these broad-spectrum antibiotics in Egypt for treatment of nosocomial and community acquired infections which often associated with extended- spectrum β- lactamase production. Also, relatively similar incidence (69.2%) of resistance to gentamicin were reported by Wassef et al. (2015)[22].

The frequency of resistant *K. pneumoniae* isolates was less than 65% for tetracycline (60%), gatifloxacin (57.1%), amikacin and imipinem (44.3%), chloramphenicol (37.1%), tigecycline (20%) and colistin (2.9%) as shown in Fig. 1. Comparable results were reported by Ranjbar et al. (2019)[23] who recorded that 54% and 43.4% of their *K. pneumoniae* strains isolated from hospital-acquired infections were resistant to tetracycline and chloramphenicol, respectively. Increased chloramphenicol susceptibility than the past indicated that routine exposure of bacteria to newly developed antibiotics triggers a reversal of susceptibility to outdated antibiotics[24]. A relatively similar (48.1%) incidence of resistance to imipenem has been also previously reported in China[25]**,** this high incidence of resistance is considered an alarming notice as these antibiotics are the last line antibiotics for treatment of such bacterial pathogens. In Egypt, Helmy and Kashef, (2017)[26] reported amikacin incidence of resistance in 41% of *K. pneumoniae* isolates a finding that was nearly similar to that of our study (44.3%). We observed that our isolates were more resistant to gentamicin than toward the aminoglycoside amikacin, a finding that could be explained by the widespread use of gentamicin as an empirical therapy for the management of most of the nosocomial infections[27]. Comparable incidence of resistance for tigecycline was reported by Heidary et al. (2017) who reported 15% incidence of resistance to tigecycline[28]. This relatively lower incidence of tigecycline resistance recorded in our study and reported by others might indicate that tigecycline is potentially one of the most effective antimicrobial agents used in the treatment of *K. pneumoniae* infections.

Table 1: Relation between efflux pump and biofilm formation

Biofilm formation	Efflux pump							
	Positive		Negative		Total		Chi-square	
	N	%	N	%	N	%	X^2	P-value
Weak	1	1.6	3	50.0	4	5.7	26.566	<0.001*
Moderate	23	35.9	3	50.0	26	37.1		
Strong	40	62.5	0	0.0	40	57.1		
Total	64	100.0	6	100.0	70	100.0		

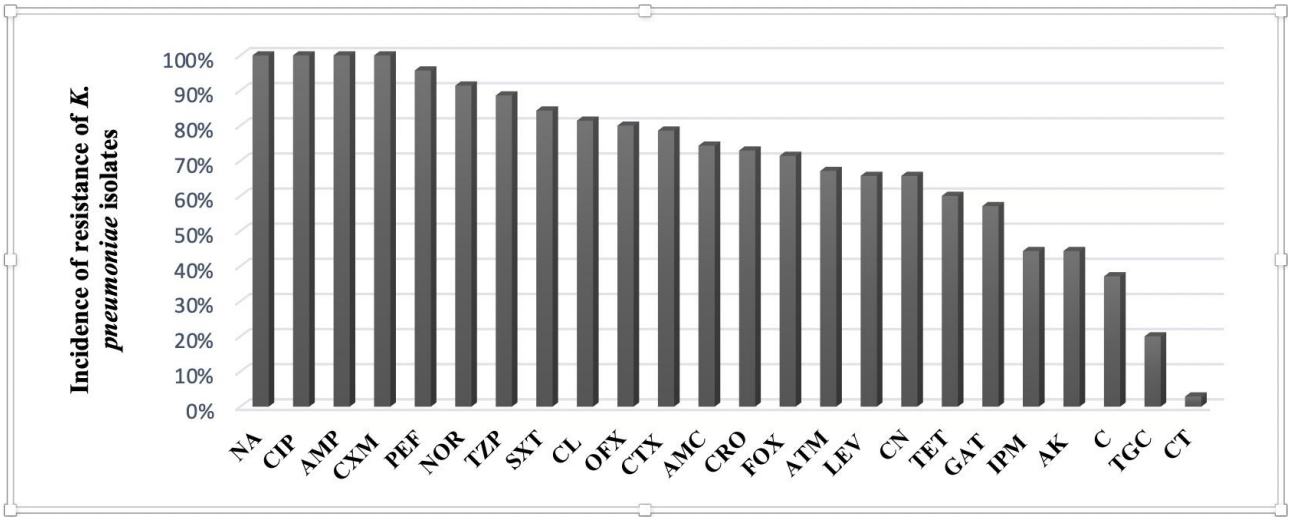

Figure 1: The percentage of resistant isolates to each antimicrobial agent

NA: Nalidixic acid, CIP: Ciprofloxacin, AMP: Ampicillin, CXM: Cefuroxime, PEF: Pefloxacin, NOR: Norfloxacin, TZP: Piperacillin/tazobactam, SXT: Trimethoprim/sulfamethoxazole, CL: Cefalexin, OFX: Ofloxacin, CTX: Cefotaxime, AMC: Amoxicillin/clavulanate, CRO: Ceftriaxone, FOX: Cefoxitin, ATM: Aztreonam, LEV: Levofloxacin, CN: Gentamicin, TET: Tetracycline, GAT: Gatifloxacin, IPM: Imipenem, AK: Amikacin, C: Chloramphenicol, TGC: Tigecycline and CT: Colistin sulfate

Although the incidence of colistin resistance was the lowest among our isolates, it is considered to be a threat alarm because colistin-resistant Klebsiella infections are potentially fatal. There are other studies in Egypt that recorded the high incidence of resistance to colistin as Tohamy et al. (2018)[29] and Zafer et al. (2019)[30] who reported 12.5% and 9.4% incidence of colistin resistance among their MDR *K. pneumoniae* isolates.

According to Mthembu (2008), if the MAR index value > 0.2 this means that such isolates were originated from environments where antibiotics were excessively used[31]. So, all of our tested *K. pneumoniae* isolates seemed to be originated from environments where antibiotics were overused as indicated by their high MAR index values (≥ 0.38).

As reported by Magiorakos et al. (2012), the isolate that have shown resistance to at least one agent in three or more antimicrobial classes is considered multidrug-resistant (MDR). The isolate that showed resistance to at least one agent in all but two or fewer antimicrobial classes is considered of extensively drug-resistance (XDR) profile. Pan drug-resistant (PDR) isolates are those showed resistance to all agents in all antimicrobial classes[32]. Accordingly, the majority (81.4%) of our isolates showed an MDR profile. The XDR and PDR profiles were detected in 15.7% and 2.9% of the tested isolates, respectively. Rates as high as 71% and 95.8% of MDR *K. pneumoniae* isolates were also observed in Egyptian studies performed by Wasfi et al. (2016)[33] and Kamel et al. (2018)[18], respectively. Also, Taha & Omar, (2019)[34] detected PDR profile in 18.5 % of collected *Klebsiella* spp. isolates. This high rate of antimicrobial resistance can be due to the lack of strict policies that regulate the antibiotic use in Egypt[35].

Several mechanisms have been introduced overtime by bacterial pathogens in preventing the action of antibiotics. Possessing active efflux pumps is considered one of such mechanisms that was detected in 64 (91.6%) of *K. pneumoniae* isolates that showed fluorescence at EtBr concentration ≥ 2 mg/L which considered to phenotypically exhibit efflux pump activity. Also, Akinpelu et al. (2020)[36] reported that all MDR *K. pneumoniae* have an efflux pump activity. Thus, it could be suggested that active efflux pump played an important role in the observed antibiotic resistance.

K. pneumoniae is known to form biofilm that increases its virulence and resistance to antibacterial treatments[37]. In our study, all the tested isolates were biofilm formers but with varied degrees as 40 (57.1%), 26 (37.1%) and 4 (5.7%) were classified into strong, moderate and weak biofilm formers (Table 1). Also, the study reported by Vuotto et al. (2017) found that 100% of their *K. pneumoniae* isolates were biofilm producers where 71%, 12.5% and 16.7% were strong, moderate and weak biofilm formers[38]. In addition, our study reported a strong relation between efflux pump activity and biofilm formation per isolate which was highly significant ($P < 0.001$) as shown in Table 1. In agreement with our results, Reza et al. 2019 reported that the efflux pump activity and biofilm formation are connected to each other with a whole resulting effect of amplified antibiotic resistance[9].

CONCLUSION

In this study, susceptibility testing of our isolates revealed a worrying trend of high prevalence of MDR *K. pneumoniae* strains. Moreover, the biofilm formation and efflux pump activities by such isolates may be a major barrier in treatment of *K. pneumoniae*-associated infections. Therefore, a multifaceted

approach and precise planning are recommended in controlling these infections. Our findings also support the idea that demonstrated the influential role of efflux pumps in bacterial biofilm formation and the increased antimicrobial resistance as we noticed that all strong biofilm formers *K. pneumoniae* isolates also exhibited active efflux pump activity. In addition, 3 of the four weak biofilm formers *K. pneumoniae* isolates did not phenotypically exhibit active efflux pump activity. Furthermore, two isolates (UR111 and SP70), recovered from urine and sputum, respectively, that were strong biofilm formers and exhibited efflux pump activity displayed pan resistance to all tested antimicrobials.

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