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The Role of EDTA in Biofilm Eradication of *Klebsiella pneumoniae* Isolated from Wound Infections

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Abstract: *Klebsiella pneumoniae* is one of the main pathogens which cause wound infections. Biofilm-producers of these bacteria have a high level of resistance to antibiotics and this leads to complications for the treatment of several infections. The study tested the effect of ethylenediaminetetraacetic acid (EDTA) on the biofilm formation by multidrug-resistant (MDR), strong biofilm producer *K. pneumoniae* isolates from Baghdad hospitals, Iraq. The Minimum Inhibitory Concentrations (MICs) by Microtiter Plate Assay with resazurin dye and the ability for *in vitro* biofilm formation by Microtiter plate assay using crystal violet were detected in MDR *K. pneumoniae* isolates in the presence of eight concentrations of EDTA (4 to 512 µg/ml). Out of 45 *K. pneumoniae* isolates, 35 (77.7 %) were Multi-Drug Resistant (MDR) and 25 (55.5%) were strong biofilm producers. It was found that all isolates of *K. pneumoniae* (100 %) were resistant to Ampicillin and Cephalexin, while these isolates exhibited a low-level resistance against Tigecycline, Meropenem and Imipenem. The results of Minimum Inhibitory Concentrations revealed that the effect of EDTA on the growth of *K. pneumoniae* isolates was recorded at concentrations (32-512 µg/ml). The highest antibiofilm activity by EDTA was demonstrated at the subinhibitory concentration (256 µg/ml) with biofilm eradication percent (94.28%), while at very low concentrations (8 µg/ml), it was found an obvious eradication effect on biofilm (82.11 %). The study suggests that EDTA plays an important role in the early stage of biofilm formation with a clear effect on the growth of MDR *K. pneumoniae.*

Keywords: *Klebsiella pneumoniae*, EDTA, Biofilm eradication.

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Introduction

Klebsiella pneumoniae is one of the major threats to the healthcare and regarded as a nosocomial pathogen exhibited multidrug resistance worldwide and has the ability to form biofilm with many various virulence factors (1). The multidrug resistance among pathogenic bacteria has emerged globally due to extensive use of antibiotics. The antibiotic resistance was mainly due to mobile genetic elements, horizontal gene transfer, transformation, and transduction (2). *K. pneumoniae* has developed several mechanisms for resistance to different antimicrobials by many mechanisms for developing the multidrug resistance is efflux pump systems and biofilm formation capacity (3). Ethylenediaminetetraacetic acid (EDTA) has been used as a powerful anticoagulant preventing clot formation *in vitro* (4). However, the EDTA most often used for reduced the antimicrobial properties (5). Also, it has shown the ability to disrupt *in vivo*- and *ex vivo*-generated biofilms (6).

The aim of the present study to evaluate the role of EDTA as antibiofilm agent to eradicate the formation of biofilm among multidrug

resistant *K. pneumoniae* isolated from patients with wound infections.

Materials and Methods

Isolation and identification of *K. pneumoniae*

This study was performed at Hospitals in Baghdad, Iraq, between June 2020 and March 2021, where, 150 wound swabs were collected from patients with wound infections and transported to the laboratories of Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad.

Antibiotic Susceptibility Test

Antimicrobial susceptibility test was conducted by using disc diffusion method. Briefly, *K. pneumoniae* overnight growth were prepared on McConkey agar and then resuspended in Mueller-Hinton broth (Oxoid). The turbidity of the suspension is adjusted to an equivalent 0.5 McFarland and this suspension was used to inoculate on Mueller-Hinton agar (Oxoid) plates. The antibiotics discs used in this study as the following:, Imipenem (IPM), Meropenem (MEM), Ceftazidime (CAZ), Cefotaxime (CTX), Ciprofloxacin (CIP), Tigecycline (TGC), Tetracycline (TE), Ampicillin (AM), Aztreonam (ATM), Cephalexin (CL), Piperacillin (PI), Cefipime (FEP), Trimethoprim / Sulfamethoxazole (TS), Gentamicin (CN) and Cefoxitin (FOX), (MAST, UK) were placed on the medium. The agar plates were incubated at 35 °C for 24 h. and then the inhibition zone was measured and interpreted by the percent of susceptible, intermediate, or resistant isolates as defined by CLSI breakpoint interpretative Criteria (7).

Minimum inhibitory concentrations (MICs)

The microdilution method (Microtiter Plate Assay with Resazurin Dye) was used for determination MICs as described by the Clinical and Laboratory Standards Institute (7). Briefly, 1: 2 serial dilutions of EDTA in Mueller Hinton Broth (MHB) were placed in a 96-well round-bottom plate at concentrations ranging from 4 to 512 µg/ml. The bacterial inoculum was prepared from a subculture of *K. pneumoniae* in LBB incubated for 18–24 hours at 35 ± 2°C before to the test. The bacteria suspension was diluted to $1x10^8$ colony forming units (CFU)/ml, and then a 1:200 dilution in MHB was performed to obtain a final concentration of $5x10^5$ CFU/ml. The diluted bacterial suspension was added to the 96-well plate containing the serially diluted EDTA. The final volume of 200 µL per well consisted of 100 µl of the compound and 100 µL of diluted bacteria suspension. Negative and positive growth controls were performed by adding only MHB or *K. pneumoniae* with MHB to the wells, respectively. After incubation for 24 h at 37 °C, resazurin (0.015 %) was added to all wells (20 µl per well), and further incubated for 2–4 h for the observation of colour change. On completion of the incubation, columns with no colour change (blue resazurin colour remained unchanged) were scored as above the MIC value. At the end of the incubation time, MIC was determined as the lowest compound concentration at which no bacterial growth was observed.

Biofilm formation

Klebsiella pneumoniae was cultivated overnight in MHB at 37 °C and then diluted to $1*10^8$ CFU/ml in fresh MHB. Each well of 96-well (flat bottom) polystyrene microtiter plates

was filled with 100 µl of bacterial solution. The media was gently aspirated after the incubation, and the wells of the plates were washed three times with 150 µl phosphate buffered saline (PBS) to eliminate unattached bacteria. Crystal violet (CV) staining was employed to determine the biofilm biomass. For fixation, 100 µl of 99% methanol was poured per well for 10 minutes, aspirated, and plates were allowed to dry. For 20 minutes, wells were dyed with 100 µl of 0.01% CV. Three washes with sterile PBS were used to remove excess colorant. Finally, bound CV was solubilized in 33% acetic acid, and optical absorbance at 630 nm was measured with a microtiter plate reader (BioTek Instruments, Winooski, USA) (8).

Effect of EDTA on *in vitro* biofilm formation

The ability of *in vitro* biofilm formation was determined using the microtiter plate assay in a 96-well microtiter plate, in the absence and presence of EDTA (4 to 512 µg/ml), in triplicates. The optical density was measured at 630 nm with ELISA reader (BioTek Instruments, Winooski, USA) and the degree of biofilm formation was estimated (9).

Results and Discussion

Out of 150 wound swabs, a total of 45 (30%) isolates of *K. pneumoniae* were collected from patients with wound Infections. CHROMagar Orientation, Blood agar and McConkey agar were used for isolation *K. pneumoniae*. These isolates were identified using traditional bacteriological methods and biochemical testing, with VITEK 2 system (bioMerieux, France), according to the manufacturer's recommendations. The antibiotics susceptibility test for all 45 *K. pneumoniae* isolates were achieved by Disc diffusion test on Mueller-Hinton agar according to CLSI (2019) (7), the results were summarized in Table (1).

Table (1): Percentages of antibiotics resistance rate of 45 *K. pneumoniae* isolates against 15 antimicrobial agents.

Antibiotic	Resistant No. (%)
Ampicillin	45 (100 %)
Cephalexin	45 (100 %)
Cefotaxime	41 (91.1 %)
Meropenem	4 (8.8 %)
Imipenem	6 (13.3 %)
Piperacillin	39 (86.6 %)
Aztreonam	34 (70.1 %)
Cefipime	34 (70.1 %)
Cefoxitin	36 (80.0 %)
Ceftazidime	31 (68.8%)
Trimethoprim / Sulfamethoxazole	27 (60.0 %)
Ciprofloxacin	24 (53.3 %)
Gentamicin	22 (58.4%)
Tetracycline	26 (57.8 %)
Tigecycline	4 (8.8%)

The antibiogram for *K. pneumoniae* isolates revealed high level resistance to most of the antibiotics under test. It was found that

all isolates of *K. pneumoniae* (100 %) were resistant to Ampicillin and Cephalexin. The present study showed a high resistance to Cefotaxime (91.1%), Piperacillin (86.6 %) and Cefoxitin (80.0 %), also obvious resistance was recorded for the antibiotics; Aztreonam and Cefipime. The moderate resistance was observed for Cetazidime, Trimethoprim/ Sulfamethoxazole, Gentamicin, Tetracycine and Ciprofloxacin. The current study demonstrated that *K. pneumoniae* exhibited a low-level resistance against Tigecycline (8.8%), Meropenem (8.8 %) and Imipenem (13.3 %). Among 45 isolates of *K. pneumoniae*, 35 (77.7 %) were resistant to more than 3 classes of selected antibiotics, in other words, Multi-Drug Resistant (MDR) *K. pneumoniae*.

The local study of Aljanaby *et al*.(10), which investigate the prevalence of multi-drug resistance among *K. pneumoniae* isolated from inpatients with urinary tract infection and burns infections in Al-Kufa hospital in Al-Najaf province, Iraq, the highest resistance rate was observed for amoxicillin and amoxicillin+ clavulanic acid (97.67%), while the lowest resistance rate was observed for imipenem (9.30%). The most common resistance associated-genes were *bla*SHV (86.04%). The spread of resistance among *K. pneumoniae* isolates present a significant danger to public health, where, the global emergence and spread of genes of antimicrobial resistance such as ESBL and carbapenemase genes represent one of the main causes for the increasing of resistance. The carbapenems have long been deemed as the last therapeutic option of antibiotics used to treat the life threat infections caused by multidrug-resistant gram-negative bacteria. The rapid global emergence of *K. pneumoniae* resistant strains may be due to the extensive use and misuse of antibiotics (11). Some important reasons for the increasing rates of antibiotics resistant isolates in Iraq include lack of standardized criteria to determine drug resistant isolates, limited laboratory facilities, and poor sanitation. Therefore, the appropriate antimicrobial therapy, and improvement of hygiene condition will lower the emergence of antibiotics-resistant bacterial strains.

The effect of EDTA on the growth of *K. pneumoniae* isolates (n=8) for determination the MICs of these isolates with different concentrations of EDTA (4-512 µg/ ml), and the results were illustrated in Table 2 and figure 1. The present study showed that the average of MICs of eight *K. pneumoniae* isolates (KM1-KM8) were 32-512 µg/ml, and there was no activity of the lower concentrations (4-16 µg/ml) on the studied isolates.

Table (2): The Minimum Inhibitory Concentrations (MICs) of *K. pneumoniae* isolates by using different concentrations of EDTA.

The isolate code	Minimum Inhibitory Concentration (MIC) (µg/ ml)							
	EDTA concentration (µg/ ml)							
	4	8	16	32	64	128	256	512
KM1	+	+	+	+	+	+	-	-
KM2	+	+	+	+	-	-	-	-
KM3	+	+	+	+	-	-	-	-
KM4	+	+	+	+	+	-	-	-
KM5	+	+	+	+	+	-	-	-
KM6	+	+	+	+	+	+	-	-
KM7	+	+	+	+	+	+	+	-
KM8	+	+	+	-	-	-	-	-

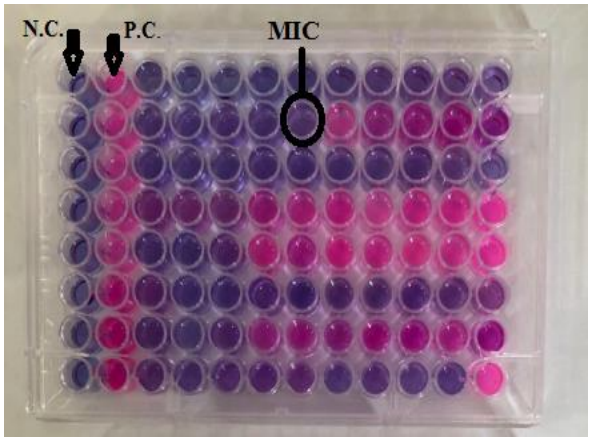

Figure (1): The Minimum Inhibitory Concentrations (MICs) of *K. pneumoniae* isolates by using different concentrations of EDTA (Microtiter Plate Assay with Resazurin Dye).

One of the studies revealed that MIC of EDTA disodium was 2048 µg/ml, where the EDTA is a nonantibiotic component and showed MBEC value of 8192 µg/ml (12). The EDTA showed bacteriostatic effect against planktonic cells of the *Staphylococcus epidermidis* isolates (MIC = 0.25-0.5 mmol/l; MBC = 10.0->25.0 mmol/l; MBC/MIC = 20, 30, 40, >50). The adhesion process and also formation of the biofilm was inhibited by EDTA at concentrations 1.0-2.0 mmol/l (2-8 x MIC) (13). EDTA was reported to have an antibacterial activity by destroying the outer membrane of bacterial cells, and prevention of biofilm formation by chelation of several divalent cations which are essential for stabilization of the biofilm (14).

The results of biofilm eradication among *K. pneumoniae* isolates (n=8) using different concentrations of EDTA (4-512 µg/ml) were summarized in table3, where the highest biofilm eradication percent was demonstrated at the concentration 256 µg/ ml (94.28%) for the isolate KM7, while the lowest concentration of EDTA (4 µg/ ml) exhibited biofilm eradication between (58.07- 71.69%).

Table (3): Effect of EDTA at different subinhibitory concentrations on biofilm formation of *K. pneumoniae* isolates.

The isolate code	Biofilm eradication (%)							
	EDTA concentration (µg/ ml)							
	4	8	16	32	64	128	256	512
KM1	64.07	76.35	84.72	86.81	90.43	93.60	-	-
KM2	62.34	74.60	79.41	82.55	-	-	-	-
KM3	61.66	72.64	77.80	79.08	-	-	-	-
KM4	58.59	63.22	61. 75	63.63	84.89	-	-	-
KM5	58.07	63.39	69.73	71.12	80.11	-	-	-
KM6	64.70	64.27	71.99	81.70	86.32	92.69	-	-
KM7	71.69	82.11	85.32	85.82	87.04	90.65	94.28	-
KM8	60.82	73.44	80. 88	-	-	-	-	-

The findings of Gawad *et al.* (15) investigated the effect of a non-antibiotic adjuvant, ethylenediaminetetraacetic acid (EDTA) on the biofilm formation by multidrug resistant (MDR) strong

biofilm producer Uropathogenic *Escherichia coli* (UPEC) from Egypt and revealed that EDTA with concentrations (10 and 20 mM) and Gelatin- EDTA coat inhibited biofilm formation by strong and moderatebiofilm producing UPEC by 45.8, 78.8, and 81.1%, respectively. One of the studies determined the effect of a composition comprising ovotransferrin (OT), protamine sulfate (PS) and ethylenediaminetetraacetic acid (EDTA) on biofilm formation by catheter-associated bacteria demonstrated that 79-95% reduction in *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilm formation was observed in a clear vinyl urinary catheter treated with the composition (16).

Biofilm formation is dependent on cell-to-cell adhesion and cations, particularly calcium, are thought to have an important role in bonding of polymer molecules in the biofilms, leading the cohesion of the polymer layer. Earlier studies have shown that EDTA at 50 mM is useful in disrupting the biofilm. Contrary to this, we noted that EDTA at 10 mM was found to be effective in disruption of bacterial biofilm when used alone (17; 18). The findings of Raheem and Ghaima (2021) (19) demonstrated the role of EDTA as antibiofilm and antifungal agent against *Candida albicans* isolated from patients with vulvovaginitis.

Conclusion

The present study showed that the antibiofilm disruption effects of EDTA suggest that this material could be useful for the development of promising antimicrobial agents for treatment of antimicrobial-resistant Gram-negative pathogens especially in wounds infections.

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