Using Probiotics, Antibiotics and their Combinations to Eradicate Biofilms Formed by Four Pathogenic *Klebsiella Pneumoniae* Strains

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Abstract

Klebsiella pneumoniae is an opportunistic pathogen leading to nosocomial infections with production of biofilm. In the present study, 73 samples were collected from patients of different ages and both genders suffering from burn and wound injury that referred to four Hospitals in Baghdad from Aug. to Nov. 2019. All *Klebsiella* isolates were subjected to the primary identification; first by describing colonies grown on MacConkey agar and second microscopically after Gram staining of their cells. Then the isolates were identified by Vitek 2 system which insured that 60 of the isolates were found to belong to *Klebsiella pneumoniae.* All identified isolates were screened for ability to produce biofilm by using the Microtiter plate assay and the Congo red agar method, and the results showed that only 4 isolates (3 of wounds and 1of burns) were able to form biofilms. For the importance of these four isolates as the only ones able to produce biofilm, they were subjected to the 16S rRNA gene amplification test by using PCR technique. Results insured that they are new strains *Klebsiella pneumoniae* to be discovered for the first time in this study. They were verified and documented by the "National Center for Biotechnology Information (NCBI)" and registered as; KPWIQ25, KPWIQ49, KPWIQ51 and KPBIQ19 strains of *Klebsiella pneumoniae*. When the probiotic filtrates and antibiotics combination were investigated for eradication of biofilms formed by the four pathogenic strains, results showed that such combinations had great efficiency in the eradication.

***Keywords**: Klebsiella pneumoniae, Biofilm, Probiotics, Antibiotics, Combination.*

Introduction

Klebsiella pneumoniae is a member of such clinically considerable organisms that have obtained much public health apprehension. It is a significant *Enterobacteriaceae* behold as one of the opportunistic pathogens causing broad series of diseases and rendering increasingly recurrent acquisition of resistance to antibiotics [(1)]. Biofilm arrangement bears a few stages to make its develop, which concede essential connection to the surface, microfilm development, mushroom shape development of biofilm, and discharging motile microscopic organisms inside separation organize[(2)].

Several mechanisms possessed within probiotics by which they can lead to prevent infection, conferring health benefits, producing antimicrobial substances, inhibiting pathogens adherence, competition for lacked nutrients and limitation of invasion by bacteria[(3)]. All probiotics action mode is a strain-dependent, making it vital to be chosen and scientifically compare probiotics for their meant function [(4)]. Combination of some antimicrobial agents may lead to a synergistic relationship which is effective against various infections types as the progress of subsequent failure beside monotherapy resistance is prevalent correlated with the prohibition of the emergence of microbial resistance [(5)].

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Due to the importance and wide spread of burn and wound infections caused by *K. pneumoniae* and the limitation studies on using antibiotics and probiotics either together or individually against this pathogen, the present study was designed for the aim of investigating a new strains of this pathogen and comparing the antimicrobial effect of various types of antibiotics, probiotics and their combinations in order to select and apply the most efficient synergetic treatment.

Samples collection

A total of 73 samples were collected from patients of different ages and genders suffering from burn and wound injury who referred to Baghdad Teaching Hospital, Ghazi Al-Hariri Hospital for Surgery Specialist, Specialty Burn Hospital and Al-Kindy Teaching Hospital in Baghdad from August to November 2019. The specimen was taken by sterile disposable cotton swabs and kept in Brain Heart Infusion broth (Himedia, India) before culturing on MacConkey agar (Mast group, UK) then incubated at 37°C for 24h.

Isolation and primary identification of bacteria

A colony was showed from each positive culture of bacteria and its identification was depended on the morphology properties [6]. Then, colonies were stained by gram to observe a specific Gram reaction, shape and arrangement [7].

Identification of *K. pneumoniae* isolates

a) by VITEK 2 system

Isolates further identified by subjecting to VITEK 2 system [8].

b) by 16s RNA

The results has been analyzed depending on genius software and registered by the National Center for Biotechnology Information (NCBI) [9].

Detection of *Klebsiella pneumoniae* biofilm

By the quantitative method, Microtiter plate (MtP) has been applied for this purpose, each of the bacterial isolates was incubated on MacConkey agar at 37°C along 24h. Then 180 µl of Mueller-Hinton broth (Mast group, UK) was put in each of the 96 wells, and then, 20 µl of bacterial suspension was added to it, and incubated at 37°C for 24h. The isolates stained with crystal violet and washed twice with phosphate-buffered saline (PBS). The microplate was resolubilized by of 96% ethanol and then spectrophotometrically measured at 570 nm using Microplate Reader (Promega, USA). For the qualitative method, Congo red agar assay have been examined biofilm producing by these strains depending on colonies color change, black colonies are considered as biofilm formers while pink ones are non-biofilm producers [10] .

Preparation of probiotic filtrates

The unconcentrated filtrates of *L. casei* and *L. plantarum* isolates were obtained by growing 1ml of each isolate culture in 9ml MRS broth (Oxoid, UK) for 24h at 37°C [11]. After that, the concentrated filtrates were prepared by evaporating100ml of the unconcentrated in the oven at 45°C to reduce the quantity to gain the three-fold concentrated filtrate (12.5ml) .

Dispersal of *K. pneumoniae* biofilm by probiotics-antibiotics combination

The biofilms of *K. pneumoniae* strains were treated with ratios (50, 75 and 25) of combinations of three-fold concentrated filtrates of each of *L. casei* and *L. plantarum* and antibiotics. Plates were incubated at 37°C for 24h. The control wells were contained medium with bacteria only. Following propagation, each well was rinsed with 200 µl PBS, stained with crystal violet solution, then washed twice with PBS. After that, the microplate was re-solubilized by eluting from attached cells with 200 µl of 96% ethanol per well. Thereafter, the stained biofilms were measured at 570 nm by using Microplate Reader (Promega, USA). Each strain was examined for triplicate and mean was taken [10].

Statistical Analysis

Graph pad prism version 8 (Graph pad software Inc., La Jolla, CA, USA) has been used for figures. Calculations and determination of forming biofilm, means and negative control standard deviation has been done according to [10] by using Microsoft Excel program.

Results and Discussion

Isolation and primary identification of bacteria

A total of 73 clinical swab samples, 42 (57.53%) were

obtained from patients wound injuries and 31 (42.47%) from burns. After grown on MacConkey agar, 60; 36 (60%) were obtained from wounds and 24(40%) from burns. Colonies of the isolates appeared as large, round, mucoid and pink in color. Microscopic examination of the suspected *Klebsiella* isolates revealed that they were Gram-negative short rods.

Identification of *K. pneumoniae* by VITEK 2 system

By VITEK 2 system, all the sixty bacterial isolates were identified as *Klebsiella pneumoniae* and gave positive results with 99% of probability.

Biofilms produced by *Klebsiella pneumoniae*:

The results of Microtiter plate (MtP) for biofilm production by *Klebsiella pneumoniae* are shown in fig. (1). After incubation, only 4 isolates, 3 from wounds (symbolled K25, K49 and K51) and 1 from burns (symbolled K19) were able to form biofilms. Among these, K25 isolate was the strongest biofilm producer when its optical density reached 2.67. Adversely, isolate K49 was the weakest biofilm producer with an optical density of only 0.61.

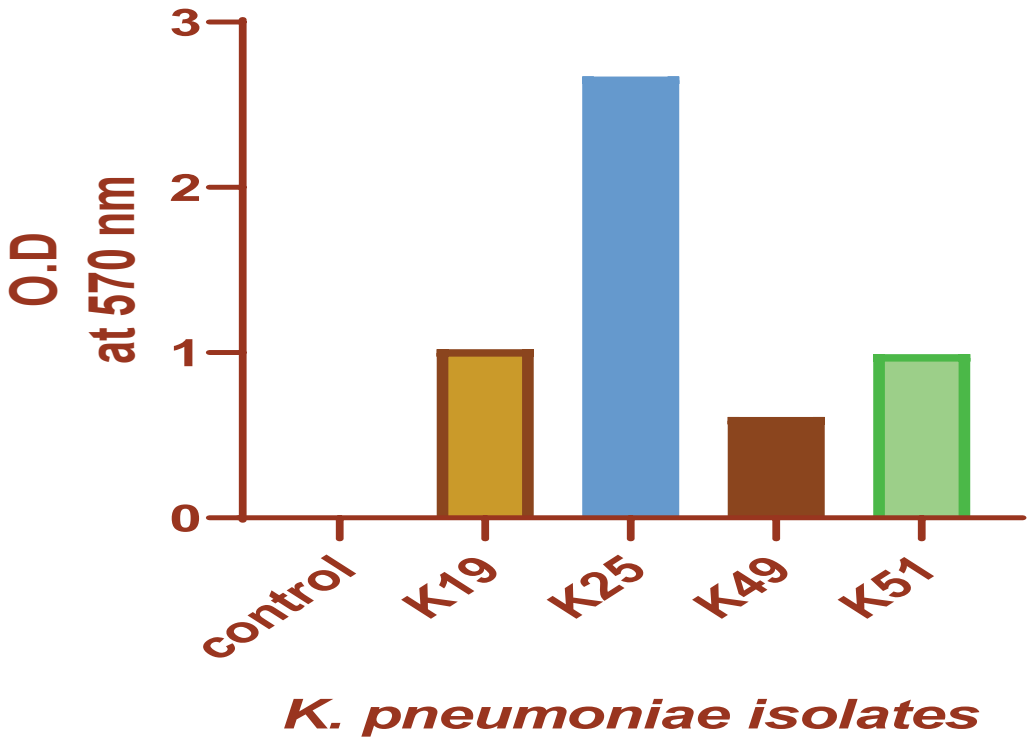

Figure (1): Optical densities of biofilms produced by *Klebsiella pneumoniae* isolates obtained from wound and burn infections by using Microtiter plate method.

After that, the Congo red agar assay was applied for qualitative evaluation of pathogenic biofilm. After incubation of *K. pneumoniae* isolates on the medium at 37 °C for 24h, results showed that only the four biofilm-producing *K. pneumoniae* strains was able to turn color of the medium to black, while others (the non-biofilm producing isolates) were unable to change the red color of medium.

Identification and registration of new strains of *Klebsiella pneumoniae*

The 16S rRNA gene amplification was performed using PCR technique to detect the positive result, 4 new

strains of *Klebsiella pneumoniae* namely (KPBIQ19, KPWIQ25, KPWIQ49 and KPWIQ51) were obtained from Iraqi burn and wound patients have been detected and registered in The National Center for Biotechnology Information (NCBI).

Effect of *Lactobacillus casei* filtrates against *K. pneumoniae* biofilms

Application of the three-fold concentrated filtrate of *L. casei* has eradicated the biofilms produced by three (KPWIQ25, KPWIQ49 and KPWIQ51) of the four *K. pneumoniae* strains, while it has no effect on one (KPBIQ19) strain (table 1). Moreover, highest eradication of biofilm was recorded by *L. casei* filtrate against KPWIQ25 *K. pneumoniae* strain when it led to a distinctive decrease the O.D (0.0) after it was 0.27 before treatment. Regarding KPBIQ49 strain, the *L. casei* filtrate reduced its O.D from 0.15 to 0.01, as well as KPWIQ51 inhibited its O.D from 0.13 to 0.01.

Table (1): Impact of *Lactobacillus casei* three-fold concentrated filtrate on *Klebsiella pneumoniae* strains (KPWIQ25, KPWIQ49, KPWIQ51 and KPBIQ19) isolated from burn and wound infections.

Treatment	Strains	OD average	ODC	2*ODC	4*ODC	strain OD	Results
With L. casei	KPBIQ19	0.21	0.09	0.18	0.36	0.12	+
	KPWIQ25	0.09	0.09	0.18	0.36	0.00	0
	KPWIQ49	0.08	0.09	0.18	0.36	0.01	0
	KPWIQ51	0.10	0.09	0.18	0.36	0.01	0
Control untreated	KPBIQ19	0.21	0.09	0.18	0.36	0.12	+
	KPWIQ25	0.36	0.09	0.18	0.36	0.27	++
	KPWIQ49	0.24	0.09	0.18	0.36	0.15	+
	KPWIQ51	0.22	0.09	0.18	0.36	0.13	+
Negative control		0.08	0.09	0.18	0.36		

0= No biofilm, + = Weak biofilm, ++= Moderate biofilm, +++= Strong biofilm; SD= 0.001

***Lactobacillus plantarum* filtrate against *K. pneumoniae* biofilms**

Lactobacillus plantarum three-fold concentrated filtrate was found to has no inhibitory on biofilms formed by the four *K. pneumoniae* strains. Adversely, it led to the enhancing the biofilms.

Effect of antibiotics against *K. pneumoniae* biofilms

Application of four antibiotics (AK, CAZ, GM and IMI) has eradicated the biofilms produced by four (KPBIQ19, KPWIQ25, KPWIQ49 and KPWIQ51) of the *K. pneumoniae* strains significantly, while no effect

registered when AK used against (KPWIQ49) strain and CAZ against (KPBIQ19) strain.

Effect of antibiotics-probiotics combination on *K. pneumoniae* biofilms

Biofilm produced by KPBIQ25 strain of *K. pneumoniae* has been eradicated by 10 of the 12 combinations (in ratios 50:50, 75:25, 25:75) of *L. casei* and each of AK, GM and IMI antibiotics when the recorded averages of biofilm O.D values were highly reduced from that average of the control (0.27), highest reduction in biofilm O.D (0.02) was obtained for the combination of *L. casei* filtrate and Gentamycin in the ratio of 50:50 (table 2).

Table (2): Effect of combinations of *Lactobacillus casei* filtrate with each of four antibiotics: Amikacin (AK), Ceftazidime (CAZ), Gentamycin (GM) and Imipenem (IMI) in three ratios (50:50, 75:25 and 25:75) on strain KPBIQ25 of *Klebsiella pneumoniae*.

Probiotic	Antibiotic	Probiotic: antibiotic ratio	Strain	OD Mean	ODC	2*ODC	4*ODC	Strain OD	Results
L. casei	AK	50:50	KPBIQ25	0.13	0.09	0.18	0.36	0.04	0
		75:25		0.17	0.09	0.18	0.36	0.08	0
		25:75		0.21	0.09	0.18	0.36	0.12	+
	CAZ	50:50		0.49	0.09	0.18	0.36	0.40	+++
		75:25		0.22	0.09	0.18	0.36	0.13	+
		25:75		0.52	0.09	0.18	0.36	0.43	+++
	GM	50:50		0.11	0.09	0.18	0.36	0.02	0
		75:25		0.13	0.09	0.18	0.36	0.04	0
		25:75		0.20	0.09	0.18	0.36	0.11	+
	IMI	50:50		0.19	0.09	0.18	0.36	0.10	+
		75:25		0.21	0.09	0.18	0.36	0.12	+
		25:75		0.16	0.09	0.18	0.36	0.07	0
Broth	None	Control	KPBIQ25	0.36	0.09	0.18	0.36	0.27	++
		Negative	NC	0.08	0.09	0.18	0.36		

0= No biofilm, + = Weak biofilm, ++= Moderate biofilm, +++= Strong biofilm; SD= 0.001

Additionally, biofilm produced by KPBIQ51 strain of *K. pneumoniae* has been eradicated by 10 of the 12 combinations (in ratios 50:50, 75:25, 25:75) of *L. casei* and each of AK, GM and IMI antibiotics when the recorded averages of biofilm O.D values were highly reduced from that average of the control (0.13). However, highest reduction in biofilm O.D (0.03) was obtained for the combination of *L. casei* filtrate and Amikacin in the ratio of 50:50. Furthermore, biofilm produced by KPBIQ25 strain of *K. pneumoniae* has been eradicated by 10 of the 12 combinations (in ratios 50:50, 75:25, 25:75) of *L. plantarum* and each of AK, GM and IMI antibiotics when the recorded averages of biofilm O.D values were highly reduced from that average of the control (0.27). However, highest reduction in biofilm O.D (0.02) was obtained for the combination of *L. plantarum* filtrate and Amikacin and Imipenem in the ratio of 50:50 as well as Imipenem in the ratio of 25:75 (table 3).

Table (3): Effect of combinations of *Lactobacillus plantarum* filtrate with each of four antibiotics: Amikacin (AK), Ceftazidime (CAZ), Gentamycin (GM) and Imipenem (IMI) in three ratios (50:50, 75:25 and 25:75) on strain KPBIQ25 of *Klebsiella pneumoniae*.

Probiotic	Antibiotic	Probiotic: antibiotic ratio	Strain	OD Mean	ODC	2*ODC	4*ODC	Strain OD	Results
L. plantarum	AK	50:50	KPBIQ25	0.11	0.09	0.18	0.36	0.02	0
		75:25		0.18	0.09	0.18	0.36	0.09	0
		25:75		0.61	0.09	0.18	0.36	0.52	+++
	CAZ	50:50		0.17	0.09	0.18	0.36	0.08	0
		75:25		0.16	0.09	0.18	0.36	0.07	0
		25:75		0.68	0.09	0.18	0.36	0.59	+++
	GM	50:50		0.13	0.09	0.18	0.36	0.04	0
		75:25		0.13	0.09	0.18	0.36	0.04	0
		25:75		0.14	0.09	0.18	0.36	0.05	0
	IMI	50:50		0.11	0.09	0.18	0.36	0.02	0
		75:25		0.14	0.09	0.18	0.36	0.05	0
		25:75		0.11	0.09	0.18	0.36	0.02	0
Broth	None	Control	KPBIQ25	0.36	0.09	0.18	0.36	0.27	++
		Negative	NC	0.08	0.09	0.18	0.36		

0= No biofilm, + = Weak biofilm, ++= Moderate biofilm, +++= Strong biofilm; SD= 0.001

A part of the results illustrated that the biofilm produced by KPBIQ51 strain of *K. pneumoniae* has been eradicated by 8 of the 12 combinations (in ratios 50:50, 75:25, 25:75) of *L. plantarum* and each of AK, GM and IMI antibiotics when the recorded averages of biofilm O.D values were highly reduced from that average of the control (0.13). The highest reduction in biofilm O.D (0.03) was obtained for the combination of *L. plantarum* filtrate and Gentamycin in the ratio (75:25) and Imipenem in the ratio of 50:50 (table 4).

Table (4): Effect of combinations of *Lactobacillus plantarum* filtrate with each of four antibiotics: Amikacin (AK), Ceftazidime (CAZ), Gentamycin (GM) and Imipenem (IMI) in three ratios (50:50, 75:25 and 25:75) on strain KPBIQ51 of *Klebsiella pneumoniae*.

Probiotic	Antibiotic	Probiotic/ antibiotic ratio	Strain	OD Mean	ODC	2*ODC	4*ODC	Strain OD	Results
L. plantarum	AK	50:50	KPBIQ51	0.26	0.09	0.18	0.36	0.17	+
		75:25		0.19	0.09	0.18	0.36	0.10	+
		25:75		0.40	0.09	0.18	0.36	0.31	++
	CAZ	50:50		0.39	0.09	0.18	0.36	0.30	++
		75:25		0.16	0.09	0.18	0.36	0.07	0
		25:75		1.53	0.09	0.18	0.36	1.44	+++
	GM	50:50		0.16	0.09	0.18	0.36	0.07	0
		75:25		0.12	0.09	0.18	0.36	0.03	0
		25:75		0.14	0.09	0.18	0.36	0.05	0
	IMI	50:50		0.12	0.09	0.18	0.36	0.03	0
		75:25		0.18	0.09	0.18	0.36	0.09	0
		25:75		0.14	0.09	0.18	0.36	0.05	0
Broth	None	Control	KPBIQ51	0.22	0.09	0.18	0.36	0.13	+
		Negative	NC	0.08	0.09	0.18	0.36		

0= No biofilm, + = Weak biofilm, ++= Moderate biofilm, +++= Strong biofilm; SD= 0.001

Conclusions

The four biofilm producers were discovered for the first time in this study to be new strains of *Klebsiella pneumoniae* and nominated by NCBI as KPBIQ19, KPWIQ25, KPWIQ49 and KPWIQ51 *Klebsiella pneumoniae* strains. Combinations of *Lactobacillus casei* filtrate with each of the Amikacin, Ceftazidime, Gentamycin and Imipenem antibiotics resulted in significant eradication of biofilm of *K. pneumoniae* four strains, while combinations of *L. plantarum* filtrate with same antibiotics did not do so.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: Non

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