Molecular Identification of Pathogenic *Klebsiella Pneumoniae* Strains Producing Biofilm

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Abstract

Klebsiella pneumoniae is an opportunistic pathogen causing nosocomial infections with production of biofilm. In the current study, 73 samples were collected from both patient genders of different ages suffering of burn and wound injuries that referred to four hospitals in Baghdad city from Aug. to Nov. 2019. The samples were cultured on MacConkey agar and 60 bacterial isolates were obtained. Primary identification by cultural and microscopic examinations declared that all isolates belong to genus *Klebsiella.* Then the 60 isolates were identified by Vitek 2 system which insured they were belonged to *Klebsiella pneumoniae.* The identified isolates were screened for ability to produce biofilm by Microtiter Plate technique and Congo red agar method. Results showed that only 4 isolates (3 of wounds and 1of burns) were able to form biofilms. Upon such important characteristic, the four isolates were submitted to the *16S rRNA* gene amplification test by using PCR technique and results declared that they are certainly belong to Klebsiella *pneumoniae*. The "National Center for Biotechnology Information (NCBI)" verified and documented that they are new strains of *Klebsiella pneumoniae* to be discovered in this study for the first time and registered them as; KPWIQ25, KPWIQ49, KPWIQ51 and KPBIQ19 strains of *Klebsiella pneumoniae*.

***Keywords**: 16S rRNA, sequences, PCR, Strains, Biofilm, health; pathogen.*

Introduction

In hospitalized patients, *K. pneumoniae* is a frequent reason of antibacterial-resistant opportunistic infections. Naturally, it is resistant to multiple antimicrobials; serious clinical concerns have stimulated the interest in *K. pneumoniae* research and especially the application of genomics [(1)]. *K. pneumoniae* bacterium is a Gram-negative pathogen that has the capability to form biofilms, Microorganism living in a biofilm sometimes have completely different properties from free-floating (planktonic) microorganism of identical species, as has been shown by completely different approaches [(2)], together with microarray analysis studies representing one of the main causes in hospital infections [(3)]. *K. 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 bacteria causing wide series of infections and rendering increasingly recurrent acquisition of antibiotics resistance [(4)]. This microorganism accounts about one-third of whole Gram-negative infections like surgical wound infections, pneumonia, infections to the urinary tract, cystitis, endocarditis in addition to septicemia [(5)].

Among hospitalized individuals, *K. pneumoniae* leads to significant morbidity and mortality universally. The pathogenesis principle mechanism in hospital environments relates biofilms formation, foremost on implanted medical equipment's [(6)]. Biofilm arrangement bears a few stages to make a develop biofilm, which concede essential connection

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to the surface, microfilm development, mushroom shape development of biofilm, and discharging motile microscopic organisms inside separation organize [7].

Due to the importance and wide spread of burn and wound infections caused by *K. pneumoniae* and the limitation studies on new strains of this bacterium, the current study was planned for the aim of searching and identification of a new pathogenic strains of *K. pneumoniae* which are able to form biofilms.

Materials and Methods

Samples collection

A gross of 73 samples were obtained from patients of several ages and both genders suffering from burn and wound injuries who referred to Baghdad Teaching Hospital, Ghazi Al-Hariri Hospital for Surgery Specialist, Specialty Burn Hospital and Al-Kindy Teaching Hospital in Baghdad city from Aug.to Nov. 2019. The samples were taken by sterile disposable cotton swabs and kept in Brain-Heart Infusion broth (Himedia, India) before culturing on MacConkey agar (Mast group, UK) and incubated at 37°C for 24h.

Isolation and primary identification of bacteria

Colonies from bacterial cultures were described for their morphological properties including shape, size, color, texture…etc. [8]. Then, a touch from a colony was smeared and stained by Gram method to observe cells under the microscope for their Gram reaction, shape, arrangement [9].

Identification of *K. pneumoniae* isolates by VITEK 2 system

The VITEK 2 (bioMérieux, France) system is a computerized microbiology program using growth-based technology. The suspension of **b**acteria was arranged depending on the recommendations of manufacturers. From overnight pure culture, a sufficient number of colonies were transferred and suspending in sterile saline (3.0 ml) into polystyrene test tube. By using the DensiChek meter, turbidity was regulated to 0.5 McFarland. Later, the same suspension was applied in GN-ID (bioMérieux, France) with VITEK 2 system. Finally, cassette of GN-ID was loaded to the VITEK 2 chamber jointly with the tubes of specimen suspension [10].

Detection of *Klebsiella pneumoniae* biofilm

By Microtiter Plate (MtP) method

Microtiter Plate is a quantitative assay used to deduct biofilms through the microplate reader. In the method, each of the bacterial isolates was grown on MacConkey agar (Mast group, UK) at 37°C for 24h. After incubation, part of a grown colony was suspended in physiological solution, then concentration of all isolate suspensions were equilibrated with 0.5 McFarland. A portion (180 µl) of Mueller-Hinton (MH) broth enriched with 1% glucose was put in each of the 96 wells, and then, 20 µl of bacterial suspension was added to it. The microtiter plate was incubated at 37°C for three periods (24, 48 and 72h).

The isolates of which their biofilms formed on the walls of microplate wells were stained with crystal violet for 15 min (Afco, Jordan). Cells not forming biofilms in the wells were discharged by washing twice with phosphate-buffered saline (Euroclone, Italy) and wells were dried at 60°C for 1h. After drying, dye of biofilms that lined the walls of the microplate was resolubilized by of 96% ethanol (ROMIL pure chemistry, UK) and then microplate was spectrophotometrically measured at 570nm by using GloMax Explorer Microplate Reader (Promega Corporation, USA).

The non-inoculated wells containing sterile MH broth augmented with 1% glucose were used as blanks (negative controls). Absorbance values of the blanks were used to detect if isolates form biofilms or not [11].

By Congo red agar (CRA) method

Congo red agar assay is a qualitative method for screening of biofilm producing microorganisms which depends on colonies color change grown on CRA medium. This medium was prepared by dissolving 0.8g Congo red (Alfa Aesar, USA), 36g sucrose (Oxoid, UK) and 37g Brain-heart infusion (Himedia, India) agar in D.W. then the volume was completed to 1L by D.W. The medium was inoculated the isolate culture and incubated for 24h at 37°C. After incubation, black colonies are considered as biofilm formers while pink ones are non-biofilm producers [11].

Identification of *K. pneumoniae* by 16S rRNA analysis: [12]

(All materials of this analysis were from Promega, USA).

Extraction of bacterial DNA

The pure colonies were re-suspended completely in 200µl of Buffer CL, and 20µl of Proteinase K solution (20 mg/ml) was added before adding to the cell pellets. The tube was mixed by vortex and incubated first at 56˚C for 30min and second at 70˚C for same time. After incubation, 200µl of Buffer BL was added to the sample and mixed vigorously before incubation at 70˚C for 30 min. After that, 200µl of absolute ethanol was added, and mix thoroughly. All mixtures were transferred to the mini-column, centrifuged for 1 min at 6,000 x g and the collection tube was replaced by a new one. Then, 600µl of Buffer BW was inserted to the mini-column, centrifuged for 1 min at 6,000 x g and the collection tube was replaced with a new one again. A portion of 700µl Buffer TW was centrifuged for 1 min at 6,000 x g. The pass-through was discarded and the mini-column was reinserted back into the collection tube. The mini-column was centrifuged at 13,000 x g, the mini-column was placed into a fresh tube and 100µl Buffer AE was added before incubation for 1 min at room temp. Finally, it was centrifuge at 5,000 rpm for 5min.

Quantitation of DNA

Quantus Florometer was used to detect the fineness of isolates for downstream applications. For DNA (1 µl), diluted Quantiflor (199 µl) Dye was prepared. DNA concentration values were inspected after five minutes of incubation within an ordinary room environment.

Agarose Gel Electrophoresis:

The presence of amplification was confirmed, agarose gel electrophoresis was adopted after Poly Chain Reaction (PCR) amplification.

a) Solutions: DNA ladder marker, 1XTAE buffer, and 10mg/ml Ethidium bromide.

b) Agarose preparation: In a beaker, 100 ml of 1X TAE was taken, 1 gm agarose was mixed with the buffer, then all particles of the gel were dissolved by boiling the solution in the Microwave. Then, 1µl of Ethidium Bromide was mixed with the agarose and mixed by the blender. The solution was cooled down to 50 °C

c) The horizontal agarose gel casting: After both edges were fixed, agarose solution was flipped into the gel plate with tapes of cellophane and the agarose was solidified at room temperature for 30min. The barrier was gently expelled and the gel arranged in the plate. When the buffer reached 4 mm atop the gel surface, the platter was loaded with 1X TAE-electrophoresis buffer.

d) Mixture of reaction: The 25µl mixture of PCR amplification consists of; (12.5 µl Master mix, 2 µl DNA template, 1 µl forward primer, 1 µl reverse primer and 8.5 µl nuclease free water). The protocol of PCR started with initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec., annealing at 60 °C for 40 sec., extension 72 °C for 1 min. and final extension at 72 °C for 7min (fig. 1).

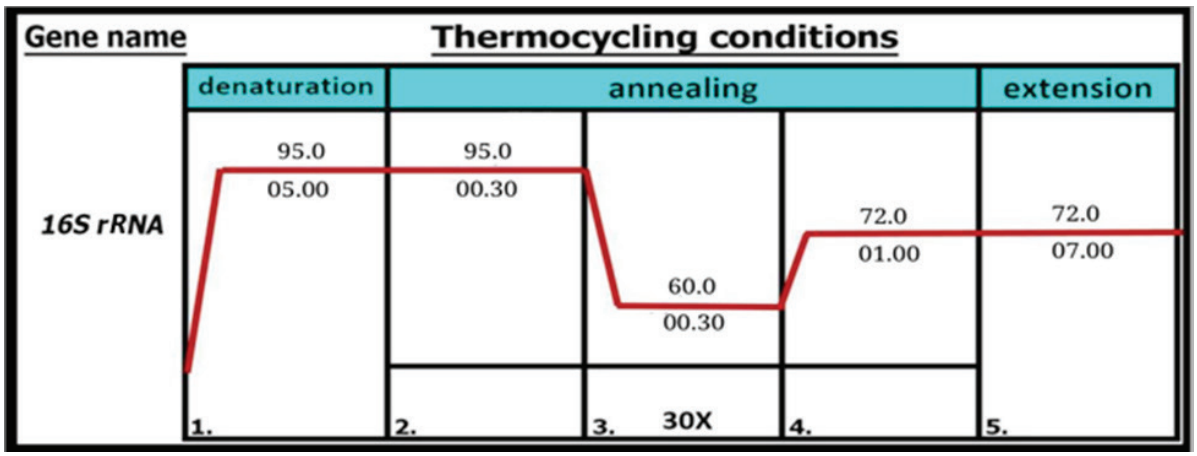

Figure: (1): Thermocycling conditions diagram of PCR monitor for primer.

e) Loading of DNA: Products of PCR were stacked. For Poly Chain Reaction item, 10µl was padded to well. For 75min electrical power was switched on at 100v/ mAmp. Towards plus Anode poles, DNA moved from Cathode. Investing Gel imaging system stained bands of the Ethidium bromide in gel were visualized.

Standard Sequencing

Automated DNA sequences and the product of PCR were sent for Sanger sequencing by Macrogen Corporation/ South Korea using ABI3730XL. The results obtained are shown in table 1. Afterward, it was analyzed depending on genius software and registered by the National Center for Biotechnology Information (NCBI).

Table (1): Names and sequences of primers.

Name of primer	Sequences	Annealing Temp. °C)	Product size bp)	Ref.
27F	5`-AGAGTTTGATCCTGGCTCAG-3`	60	1500	(Hashim and AlKhafaji, 2018)
1492R	5`-TACGGTTACCTTGTTACGACTT-3`			

Statistical Analysis

Figures were drawn by Graph pad prism version 8 (Graph pad software Inc., La Jolla, CA, USA). Calculations and determination of forming biofilm, means and negative control standard deviation has been done according to [11] by using Microsoft Excel program.

Results and Discussion

Isolation and primary identification of bacteria

From a total of 73 clinical swab samples, 42 were obtained from patients suffering of wound and 31 of burn injuries. After grown on MacConkey agar, 60 bacterial isolates were obtained; 36 (60%) from wounds and 24(40%) from burns. Colonies of the isolates appeared as large, round, mucoid and pink in color; such characteristics are similar to those described by [13] for *Klebsiella* spp. Microscopic examination of the suspected *Klebsiella* isolates revealed that they were Gram-negative short rods according to [9].

Identification of *K. pneumoniae* by VITEK 2 system:

By using VITEK 2 system, all the 60 bacterial isolates were identified as *Klebsiella pneumoniae* with a probability of 99%.

Biofilms produced by *Klebsiella pneumoniae*:

The results of Microtiter plate (MtP) for biofilm production by *Klebsiella pneumoniae* are shown in fig. (2). 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 (2): 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 24 h, results illustrated in fig. (3A) 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 (fig. 3B). All isolates were let to grow for extended periods of incubation (48 and 72 h) and also found that they were unable to produce biofilms.

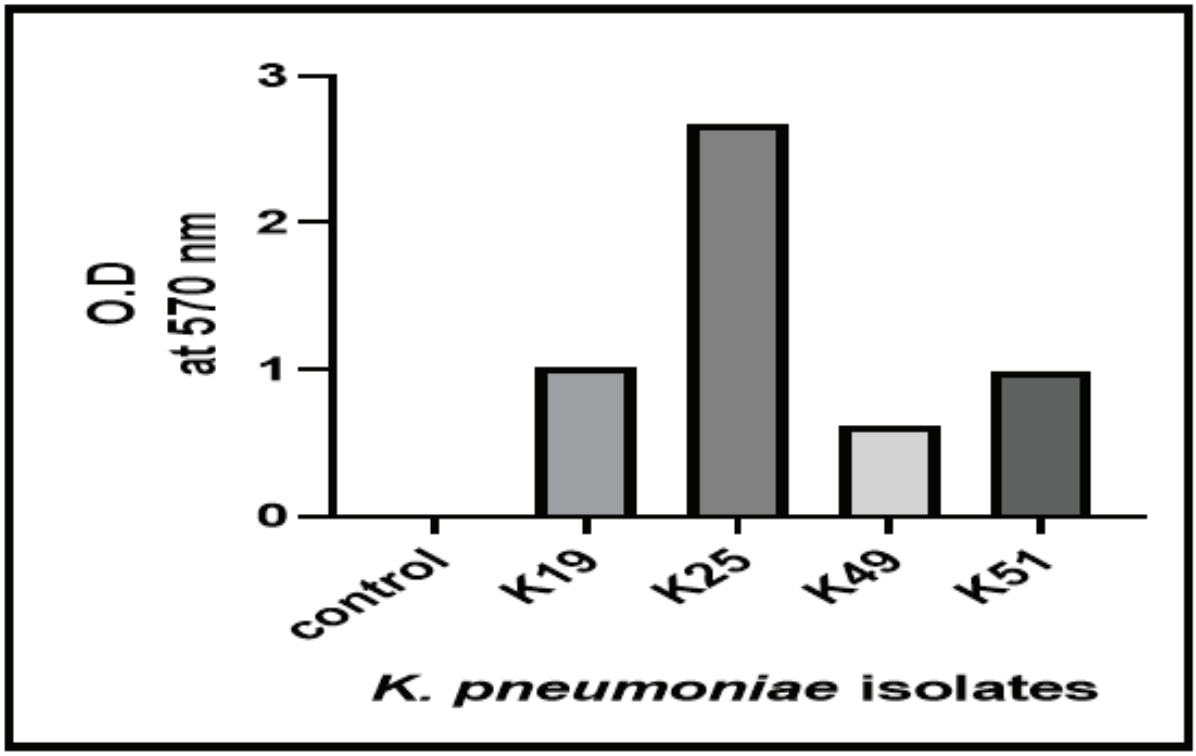

Figure (3): *Klebsiella pneumoniae* isolates after incubation on Congo red agar at 37 °C for 24 hrs. A: growth of isolate given positive result for biofilm production, and B: growth of non-biofilm producing isolate.

Results of [14] showed that most of the *Klebsiella* spp isolates collected from numerous clinical centers in Baghdad Province had potency to attach on the smooth surface (glass as well as plastic surfaces) but in various grades and different levels of producing biofilm by using microtiter plate method. However, a trial by [15] found that there is a relationship between color of the colonies and the strength of biofilm foundation; in which dark black reveals heavy biofilm formation, while red colonies declared the absence of biofilm production; they described the Congo red agar as specified method to characterize biofilm-forming bacteria morphologically.

Identification and registration of new strains of *Klebsiella pneumoniae*

Depending on the 16S rRNA-based molecular identification of clinical isolates and to confirm the identification of *Klebsiella* spp to subspecies level, the amplification of *16S rRNA* gene was conducted by 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) as written within (table 2).

Table (2): Gene bank locus registered by the "National Center for Biotechnology Information (NCBI)" based on 16S rRNA molecular identification for the new strains of *Klebsiella pneumoniae* isolated from Iraqi patients suffering burns and wounds.

Strain symbol	Gene bank locus	Source of strain
KPBIQ19	MT102627	burn
KPWIQ25	MT102629	wound
KPWIQ49	MT102630	wound
KPWIQ51	MT102634	wound

In this study the molecular technique were used to confirm the identification of *Klebsiella pneumoniae* strains. where, also [16] performed sequence analysis by using the National Center for Biotechnology Information (NCBI) blast tool and searched in sequences of the Gene Bank database for comparison to identify the *K. pneumoniae* strain (symbolled SRP2), isolated from paper mill waste, Canada.

Conclusions

After screening all isolates for biofilm production, only four of them were able to form biofilm. The four biofilm producers were discovered for the first time in this study to be new strains of *K. pneumoniae* and nominated by NCBI as KPBIQ19, KPWIQ25, KPWIQ49 and KPWIQ51 *K. pneumoniae* strains.

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

Conflict of Interest: Non

Funding: Self-funding

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