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Original Research Paper

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ESTABLISHMENT OF A PCR BASED DIAGNOSTIC METHOD FOR THE IDENTIFICATION OF *KLEBSIELLA PNEUMONIAE* IN CLINICAL SPECIMENS TARGETING *RCSA* GENE IN EGYPT

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ABSTRACT Rapid diagnosis is crucial for successful treatment of nosocomial infections. Our research aims at developing a molecular-based method for the diagnosis of Klebsiella pneumoniae using PCR assay. Our technique is based on the detection of a specific marker gene for the organism. Using NCBI gene database, rcsA was used as a potential genetic marker for Klebsiella pneumoniae. Upon application of several bioinformatics tools, K1 and K2 primer sets were designed. The K2 primer set showed higher sensitivity (Ta = 57°C) with minimum detectable limit of 10-20 pg/µl of genomic DNA and 1 CFU/ml of bacterial culture without preceding DNA extraction. On the other hand, K1 primer pair was specific for K. pneumoniae and showed no detectable bands for any other common Gram-negative bacteria (Ta =60°C). In conclusion, our research provides a rapid, simple, specific and sensitive PCR assay which can be potentially used for routine diagnosis and infection control in hospitals.

KEYWORDS : Klebsiella pneumoniae, PCR, rcsA

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative bacterium of family *Enterobacteriaceae*. According to reported statistics, *Klebsiella pneumoniae*, which is known for being one of the main causes of nosocomial infections among other pathogens, causes UTIs, neonatal septicemia, wound infections and pneumonia (Podschun & Ullmann, 1998). Recently, there has been an increase in multi-drug resistance in *Klebsiella pneumoniae* due to extended spectrum beta lactamases, which provide resistance against beta lactams (Rosenberg *et al.*, 2014). A research conducted by Lee *et al.*, 2016, showed the endemic dissemination of *K. pneumoniae* carbapenemases of the oxacillinase-48 (OXA-48) type, figure 1. Therefore, a simple, rapid, specific, and cost effective diagnostic method is strongly required. Phenotypic methods including culturing on MacConkey agar, biochemical identification and API20E system are used for the detection of *Klebsiella pneumoniae*. However, they can be time consuming, not specific and costly (Paterson *et al.*, 2014). According to Ranjibar *et al.*, 2014, the molecular typing methods won over the traditional phenotypic methods for their specificity and rapidity. When it comes to molecular techniques, several methods can be used including phage typing, ribotyping which depends on the detection of the 16s and 23s ribosomal DNA using specific probes, multiplex PCR which allows the PCR detection of different genes using different primers simultaneously, Rep-PCR which involves the PCR detection of repetitive genes in the same genome and real-time PCR systems based on SYBR Green (Dong *et al.*, 2015). However all of these methods are complicated and expensive, hence a simple, rapid and cost-effective molecular diagnostic tool is required. In this research, traditional PCR assay was employed. Polymerase Chain reaction assay is composed of repetitive cycles of a three-step amplification of DNA using *Taq* DNA polymerase under temperature cycling conditions. PCR technology is known for its high sensitivity as it is able to generate a sufficient amount of copies from minute amounts of DNA for detection by conventional laboratory techniques (Garibyan & Avashia, 2013).

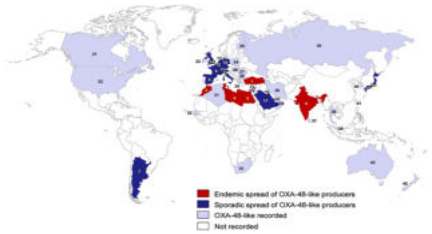


Figure 1. Epidemiological features of OXA-48-like-producing K. pneumoniae. (5): Egypt (Lee et al., 2016)

One of the most important virulence factors of *K. pneumoniae* is its ability to synthesize large amounts of CPS (Capsular Polysaccharide). Consequently, *rcsA* gene, which is a housekeeping gene that regulates the synthesis of CPS, was used as the genetic marker of *K. pneumoniae* (Dong *et al.*, 2015). Two sets of primers were designed and their sensitivity and specificity were evaluated using traditional PCR assay. Clinical isolates were also determined using traditional PCR assay.

MATERIALS

Microorganisms

Standard strains of *Klebsiella pneumoniae* ATCC 10031, *E. coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis*. *Klebsiella pneumoniae* were isolated and identified via biochemical tests in Microbiology lab of El Kasr El Ainy hospitals, Cairo, Egypt.

Media

MacConkey agar, Tryptic Soy Broth, L.B. broth and nutrient agar were prepared according to the manufacturer's instructions (Oxoid, UK).

DNA extraction kit

GeneJET Genomic DNA purification kit (Thermo Fisher Scientific, USA).

PCR kits and reagents

MyTaq™ Red Mix (Bioline, UK)

Gel electrophoresis reagents

Agarose powder (Oxoid, UK). 100 base-pair DNA ladder (Thermo Scientific Fisher, USA), Tris Acetate EDTA TAE buffer 50X, Ethidium bromide, Dimethyl sulfoxide and magnesium (Sigma Aldrich, Germany).

METHODS

Isolation of bacterial strains and genomic DNA extraction

10 clinical strains of *Klebsiella pneumoniae* and 1 standard strain of *Klebsiella pneumoniae* ATCC 10031were isolated using streaking method. The isolates were incubated overnight in L.B broth with agar at 37°C and genomic DNA was extracted from the isolate of *K. pneumoniae* standard strain using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Baltics UAB, USA).

Primer design

The *rcsA* gene sequence was downloaded from the NCBI gene database (https://www.ncbi.nlm.nih.gov/gene/11848468.). Further

analysis of *rcsA* gene was carried out using Primer-Blast (https://www. ncbi.nlm.nih.gov/tools/primer-blast/). Two primer sets, K1 and K2, were designed, Table 1, and further analyzed for risk of hairpin formation using OligoCalc (http://biotools.nubic.northwestern. edu/OligoCalc.html), efficiency using *in-silico* PCR (http://insilico.ehu.es/PCR/) and specificity of PCR amplicons using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast. cgi?PAGE_TYPE=BlastSearch). The two primer pairs were synthesized commercially (MRC PPU DNA sequencing and services, Dundee, Scotland, UK).

PCR detection of *rcsA* gene

Table 1. Properties of the designed two sets of primers for the rcsA gene.

Primers	K1-F	K1-R	K2-F	K2-R
Type	Forward	Reverse	Forward	Reverse
Sequence (5'-3')	ATACCCGGTTGGGATTGACG	CAGATTCTTCCGTACCCGCA	CGGCGACGCTGTTTGTTATC	CGACGATACCGTCTTCGCTT
Melting temperature	60.5°C			
GC content	55%			
Primer length	20 bp			
Start – End position	3416487 – 3416758		3416682 - 3416968	
PCR amplicon size	272		287	

The PCR reaction mixture (25 µl) consisted of 12.5 µl MyTaq™ Red Mix, 1 µl of each primer, 1 µl template DNA and completed with nuclease free water. The DNA was denatured at 94°C for 3 minutes and 35 cycles were performed. The cycles consisted of denaturation at 94°C for 30 seconds, annealing for 30 seconds at different temperatures according to the primers used and primer extension at 72°C for 1.5 minutes and final extension time was set at 72°C for 10 minutes. The thermal cycler used was Biometra thermal cycler.

Analysis of PCR products by agarose gel electrophoresis

The PCR amplicons were separated using 1% agarose (1 gm agarose powder and 100ml TAE buffer 1X) gel electrophoresis. 5µl of each PCR product was transferred to a separate well and 3µl of 100bp ladder was added. The gel was left to run in Biometra Compact Multi-wide DNA electrophoresis chamber for 45 minutes using a voltage of 100V and electric current of 134mA provided from Beijing Liuyi power supply. The gel was then visualized using UV trans-illumination (UltraBright Large Format UV Transilluminator, San Francisco, USA).

RESULTS

Optimization of PCR reaction

Annealing temperatures ranging from 51°C to 57°C at 2°C intervals were compared for optimal amplification of extracted DNA of *K. pneumoniae* standard strain. According to figure 2, K2 primer set showed stronger bands than K1 primer set at all annealing temperatures. We chose 57°C as the optimal annealing temperature. The bands formed were of size of 300bp approximately thus supporting the result of *in silico* PCR using K1 primer set, 272bp, and K2 primer set, 287bp.

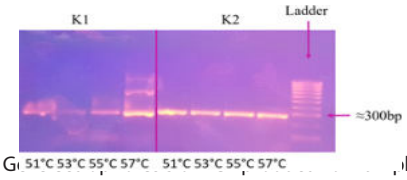
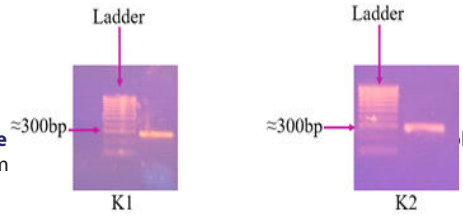


Figure 2. G lification of K. pneumoniae standard strain amplified by K1 and K2 primer sets at annealing temperatures 51°C, 53°C, 55°C and 57°C

Determination of sensitivity of K1 and K2 primer sets

As shown in figure 3, both primer sets were able to amplify 3µl DNA (maximum limit) of *K. pneumoniae* [redacted] pg/µl and 10^{-20} pg/µl for K1 and K2 respectively, figures 4 & 5.



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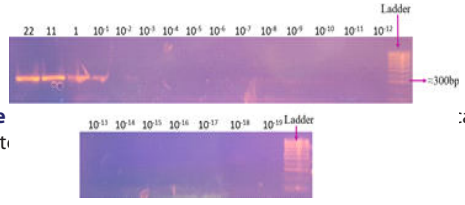

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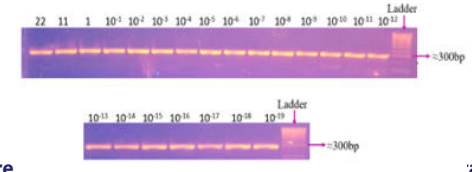

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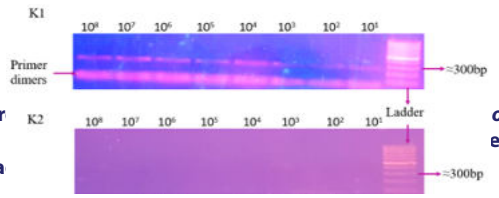
Upon 1 *umoniae* standard strain (adjusted with 0.5 McFarland standard to be equivalent to 10^8 CFU/ml), K1 primer pair was able to detect DNA, using colony PCR with an initial denaturation step which was extended to 10 minutes to extract DNA from the bacterial cells, in bacterial culture without preceding laboratory extraction until a concentration 10^1 CFU/ml was reached at annealing temperature 57°C. However, K2 primer pair failed to amplify DNA without preceding extraction, figure 6.

Table 2. Calculations of serial dilutions.

Primer set	K1	K2
Maximum detectable limit	Concentration of 1µl DNA (measured using Qubit 3.0 Fluorometer, Invitrogen, Thermo Scientific Fisher, USA) = 33.4 ng/ µl Concentration of 3 µl DNA = 100.2 ng/ µl	
Minimum detectable limit	DNA concentration at 6.6:4.4 serial dilution (6.6 µl DNA:4.4 µl WFI) = 33.4*(6.6/10) = 22.044 ng/ µl DNA concentration at 5:5 serial dilution = 22.044*(5/10) = 11.022 ng/ µl DNA concentration at two 10-fold serial dilutions = 11.0228(1/102) = 0.11022 ng/ µl ≈ 10-1 ng/ µl	DNA concentration at 6.6:4.4 serial dilution (6.6 µl DNA:4.4 µl WFI) = 33.4*(6.6/10) = 22.044 ng/ µl DNA concentration at 5:5 serial dilution = 22.044*(5/10) = 11.022 ng/ µl DNA concentration at 24 10-fold serial dilutions = 11.0228(1/1024) = 1.1022 x 10-23 ng/ µl ≈ 10-20 pg/ µl



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Verification of the specificity of K1 and K2 primer sets

Both primer pairs were able to amplify extracted DNA of 10 clinical isolates at annealing temperature 57°C with approximate band size of 300 bp, figure 7.

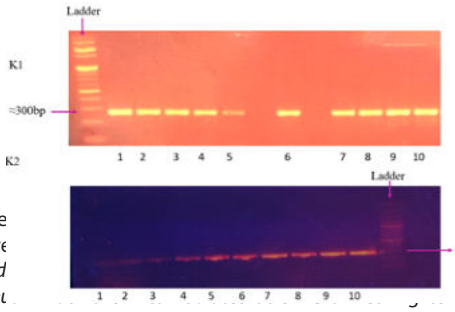

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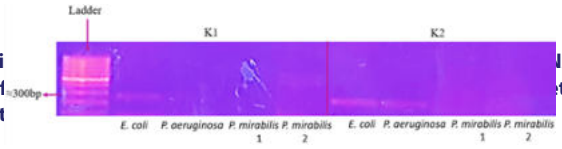
figure 8. However upon increasing the annealing temperature to 60°C, K1 primer pair was able to amplify *K. pneumoniae*, in contrast to K2 primer set which failed to amplify all Gram negative pathogens including *K. pneumoniae* even after addition of DMSO and Magnesium, figures 9, 10 & 11.



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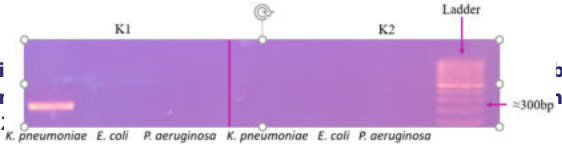

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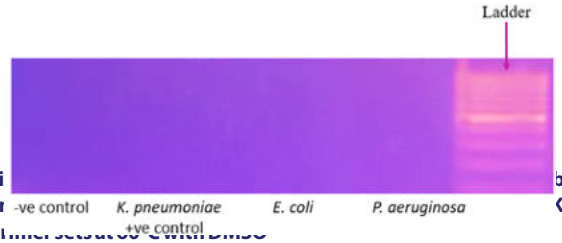

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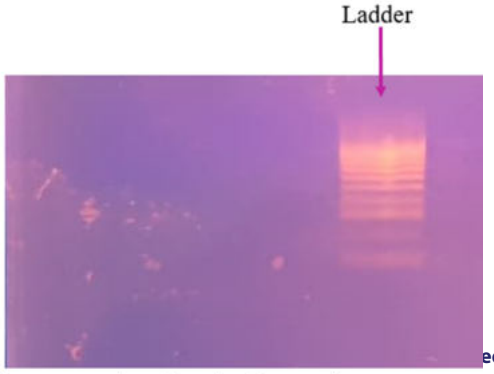

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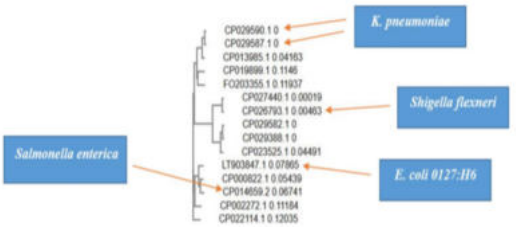
DISCUSSION

Klebsiella pneumoniae is one of the most common nosocomial pathogens which cause pneumonia, urinary tract and wound infections (Lin *et al.*, 2013). Additionally, *K. pneumoniae* also became the predominant causative pathogen of liver abscess in many Asian countries, particularly Korea and Taiwan, and has spread to western countries in the recent years (Dong *et al.*, 2015). The significance of *K. pneumoniae* as a nosocomial pathogen is due to some virulent factors, most importantly the synthesis of large amounts of capsular polysaccharide. Recently, OXA-48-like-producing *K. pneumoniae* has been reported with high prevalence in North African countries especially Egypt (Lee *et al.*, 2016). *K. pneumoniae* can be detected by phenotypic and genotypic methods. The phenotypic techniques are not sensitive and consume a lot of time whilst most genotypic methods are expensive and require skilled personnel. Consequently, traditional PCR was preferred in this research due to its advantages over other methods for being cost effective, simple, specific and sensitive. Although there are other genes which are specific to *K. pneumoniae* such as *phoE* and *tyrB*, they have mutants in their sequences. To determine the specificity of *rcsA* gene to *Klebsiella pneumoniae*, science of phylogeny, which represents the evolutionary history among all living organisms, was investigated (Woese, 2000). By taking a close look at the phylogenetic tree of the *Enterobacteriaceae* family obtained by aligning all the sequences of its members, the large phylogenetic distance between *rcsA* of *Klebsiella pneumoniae* and common Gram negative pathogens as *E. coli* and *Salmonella* sp., figure 12, proves the specificity of *rcsA* gene to *Klebsiella pneumoniae*. Taking primer design's rules such as: its length, Tm, Ta, GC% content in to consideration, two sets of primers K1 and K2 were designed. In a research carried out by Dong, *et al.*, 2015, they suggested that loop-mediated isothermal amplification (LAMP) method targeting the *rcsA* gene in *K. pneumoniae* clinical samples is considered 100-fold more sensitive than traditional PCR since their sensitivity and specificity results showed that the detection limit of LAMP assay and traditional PCR was 0.115 pg/µl and 115.0 pg/µl, respectively. However, our designed primers succeeded at being over 200-fold more sensitive to *K. pneumoniae* with a minimum detectable limit of 10^{-20} pg/µl than primers used for LAMP assay and PCR detection in the research conducted by Dong, *et al.*, 2015. Also, K1 and K2 primer pairs are specific and sensitive to *rcsA* housekeeping gene, responsible for regulating capsular polysaccharide synthesis, in *Klebsiella pneumoniae* strains only without preceding DNA extraction. Consequently, the designed primer sets helped to develop a rapid, accurate and simple PCR assay for the diagnosis of *Klebsiella pneumoniae* nosocomial infections within hours rather than days applicable for routine diagnosis and infection control in developing and low-incoming countries, especially Egypt.



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