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Original Article

Molecular Diagnosis of Clinically Isolated *Klebsiella pneumoniae* Strains by PCR-ELISA

Fatemeh Tayebeh[1, 3], Jafar Amani[1*], Shahram Nazarian[2], Mehdi Moradyar[3], Seyed Ali Mirhosseini[1]

Abstract

Klebsiella pneumoniae is the most important infectious bacteria in Enterobacteriaceae family and the most common bacteria causing Urinary Tract Infection (UTI) after *Escherichia coli*. Therefore, accurate and rapid identification of this bacterium in hospital infection is very important. In this study, PCR-ELISA method was used for detecting *Klebsiella pneumonia* clinical strains. For this purpose, 16S rDNA gene based specific primers were designed and the DIG-labeled PCR products were bound to streptoavidin-coated wells of a microtiter plate and detected by anti-DIG–peroxidase conjugate. Biotin-labeled DNA probe specific for 16S rDNA gene was used in PCR-ELISA. Sensitivity and specificity of PCR-ELISA method were determined by using Enterobacteria strains. 16S rDNA of *Klebsiella pneumoniae* was amplified using gene specific primers resulted in a fragment of the 260 bp. The results of PCR-ELISA showed that this technique does not cross-react with the bacteria in their families as well as the sensitivity of 6.0 ng were evaluated. PCR-ELISA is known as an accurate and rapid method for detection of the infectious agents and therefore can be used as a suitable substitute for all the above aspects because it is quite a sensitive, specific, and rapid method for detection of the *Klebsiella pneumoniae* strains.

Keywords: *Klebsiella pneumoniae*, 16S rDNA, Diagnostic Method, PCR-ELISA

1. Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
2. Imam Hossain University, Faculty of Science, Department of Biology, Tehran, Iran
3. Department of Biology, Damghan Azad University, Damghan, Iran

*** Corresponding Author**
Jafar Amani
Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
E-mail: jafar.amani@gmail.com

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Introduction

Klebsiella pnemouniae is one of the most important members of *klebsiella* genus in Enterobacteriaceae family [1, 2]. *Klebsiella pnemouniae* is well-known as an opportunistic pathogens causing a community-acquired bacterial pneumonia, urinary tract infection, septicemia, tissue and wound infections. This bacterium is one of the major causes of severe morbidity and mortality especially among the newborn. The *Klebsiella* and *Escherichia coli* are the most frequent microorganisms reported from neonatal sepsis and meningitis cases in developing countries [3, 4]. Gastrointestinal tract and the hands of hospital personnel are the main pathogenic reservoirs for transmission of *Klebsiella*. *K. pnemouniae* can survive for several hours on the hands of hospital personnel, which likely facilitates nosocomial spread (5).

Capsular Antigens, Fimbriae, Serum Resistance and Lipopolysaccharide are major pathogenicity factors of *K. pnemouniae* [2, 6, 7]. It is emerging worldwide as a major cause of bacteremia and drug resistant infections. Increasing levels of multiple-antibiotic resistance associated with this species pose a major emerging clinical problem [8]. β-lactamases are cephalosporinases that are resistant to β-lactam drugs [9]. At first Extended-spectrum β-lactamases (ESBLs) were detected in *klebsiella* species in the 1980s [10]. β-Lactams have been the mainstay of treatment for serious infections, and the most active of these are the carbapenems, which are advocated for use for the treatment of infections caused by extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, particularly *E. coli* and *K. pneumonia* [11]. The conventional methods of *K. pnemouniae* detection is mainly aimed at clinical sample, such as biological specimens and body fluid but these methods take a long time and are not very accurate [12].

Although Real-time PCR is a rapid, sensitive, and specific technique but it is very expensive [13]. Despite recent advances in molecular biology and in the development of commercially available phenotype-based identification kits, identifying bacterial strains remains a difficult task for many routine microbiological laboratories. PCR-ELISA is an accurate technique that is used for the detection of several pathogens. Researchers used PCR-ELISA for recognizing representative coliforms in water samples, direct identification of *Pseudomonas aeruginosa* from positive BACTEC blood culture bottles. Accordingly, Mousavi *et al*., evaluated a PCR-enzyme-linked immunosorbent assay for sensitive and rapid detection of *Vibrio cholerae* O1 from Iran [14]. In our previous study PCR-ELISA was used to detect genes encoding shiga toxins 1 and 2 from *E. coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC) [15].

There is no report regarding application of PCR-ELISA technique for detection of *K. pneumonia*. Among the several thousand genes within a bacterial genome, the 16S rRNA gene has served as the primary key for phylogeny-based identification when compared against well-curated 16S rRNA gene sequence databases [16].

In this study 16S rDNA gene-targeted specific primers and probe were designed. Specific target DNA was amplified using digoxigenin labeled nucleotides. The amplified PCR

product was hybridized to a biotin labeled probe and detection with specific antibody against conjugated digoxigenin was done. We identified specific sequences of 16S rDNA gene from *K. pneumoniae* with considerable sensitivity and short turnaround time.

Materials and Methods

Bacterial strains

Klebsiella pnuemuniae, *E. coli* O157, *Pseudomonas aeruginosa*, *Shigella dysenteriae* and Enterotoxingenic *E. coli* were provided from the reference laboratory of Iran and verified by biochemical and immunologic methods. Clinical samples in this study were selected from several hospitals in Tehran at 2014.

Primer and probe design

After studies on bacterial genome 16S rDNA was selected as target gene and primers were designed for amplification of a specific partial sequence of 16S rDNA gene (Table 1). The features designed primers such as GC content; Tm, ΔG etc were checked by DNASIS and Oligo software. The primer and probe sequences are presented in Table 1. The oligo nucleotides were supplied by CinnaClone (IRAN).

Table1. PCR primers and probe for detection of *K. pnuemuniae*.

Oligonucleotide	Sequence	Nucleotide position	Expect Product size (bp)
KP16F	GCAAGTCGAGCGGTAGCACAG	50-70	260 bp
KP16R	CAGTGTGGCTGGTCATCCTCTC	279-309	
KP16P	TAATACCGCATAACGTCGCA	161-180	

DNA extraction

Bacterial strains were cultured on Luria Bertani (LB) broth and incubated at 37°C for 16 hours. Bacterial culture was centrifuged in 4000 rpm for 6min and the pellet was suspended in 567 µl TE buffer, 30 µl SDS %10 and 3-5 µl Proteinase K then incubated at 37°C for 1 h.

After cell lysis, 100 µl NaCl and 80 µl CTAB was added and tubes incubated for 15 min in 65°C. DNA was purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Supernatant was collected after centrifugation at 13,000 rpm and 500 µl isopropanol was added, mixed gently and kept for 12 hours in -20°C. DNA pellet was obtained by centrifuging at 2000 rpm for 20 min, washed with 70% ethanol and dried. Finally DNA samples were solved in 100 µl TE buffer and to eliminate RNA, 3 µl RNase A was added and the tubes were incubated in 37ºC for 30 min. For analysis of extracted DNA gel electrophoresis was carried out.

Photograph of a stained gel was taken directly on a UV transilluminator by gel documentation system. The concentration of the DNA samples was determined spectrophotometrically at A260 by NanoDrop 2000, Thermo Scientific (USA). Purity ratio (A260:A280) was also determined by determining the absorbance value at 280 nm.

PCR reaction

PCR reaction was performed for amplification of 16S rDNA gene in a 25 µl total reaction mixture. Each reaction containing 0.4 µm of each primer, 200 µM of each dATP, dCTP, and dGTP, 190 µM dTTP, 10 µM DIG-11-dUTP (Roche Diagnostics), 0.5U of Taq DNA polymerase, 2.5 µl 10X buffer, 2 mM $MgCl_2$ and different concentrations of genomic DNA. PCR cyclic conditions were initiated with 95°C for 3min followed by 28 cycles of 95°C for 45 sec, 58°C for 45 sec, 72°C for 1 min, and a final extension at 72°C for 5 min. Finally, the PCR product was electrophoresed by 1% agarose gel. For gel electrophoresis, a mixture of 5 µl PCR products and 1µl of loading buffer was loaded into a well of a 1% agarose gel. 100 bp plus DNA ladder was used to determine the size of the PCR products. Electrophoresis was run at 80V for 30 min in 0.5X TBE buffer. Finally, the gel was stained with ethidium bromide and PCR products were visualized under UV light and its image was stored by the use of a gel documentation system.

For labeling of PCR product, the reaction of PCR was performed by dNTP mixture containing digoxigenin labeling mix (Digoxigenin dNTPs, Roche, Germany) with the same condition.

PCR-ELISA

PCR-ELISA, DIG-Detection kit was used to detect DIG-labeled PCR products. Microtiter wells were coated with one microgram streptavidin and placed overnight at 4ºC. Plates were washed three times with PBS plus 0.5% Tween20 (PBST). Wells were blocked with 3% BSA in PBS buffer and incubated for 2 h at 37°C.

After washing 10 µl of labeled product was added to 90 µl 1X SSC buffer and incubated for 10, 5 min in boiling water and on ice respectively. In the next step 10 µl of probe was added to the tube. After incubation for 2 hrs in 60ºC, 100 µl of the hybridization buffer were added to each well and allowed to remain at 37ºC for 1 h. Wells were washed with PBS three times before adding antibody. Diluted 1000-fold of anti-digoxigenin antibody conjugated with peroxides in PBST buffer, was added to each well and plate was incubated at 37°C for 1 h. Plates were then washed three times with PBST for 1 min each time. OPD substrate solution was added to each well and the plate was incubated at room temperature for 10 min, kept them in a dark environment. The reaction was stopped with 2.5 M H_2SO_4 and the optical density was measured at 492 nm using an ELISA reader (Dynex Technologies, Guornesey, Channel Islands and Great Britain). Finally, 100 µl from freshly prepared substrate solution contain 5 mg OPD was added in each well till color appearance. Enzyme–substrate reaction was stopped with sulfuric acid and the absorbance was measured at 492 nm [15].

Sensitivity and Specificity of PCR-ELISA

To determine the minimum genomic DNA concentration that can be detected by the method, serially diluted genomic DNA in TE buffer (pH 8) was use as PCR template and the product was analyzed on 1% agarose gel. To evaluate Specificity of PCR-ELISA, Genomic DNA of *E. coli* O157:H7, *Shigella dysentriae*, *Pseudomonas aeruginosa*, and Enterotoxigenic *E. coli* strains were extracted and 10-2 fold dilution was prepared as templates.

The PCR was carried out according to mentioned protocols and the products were analyzed by agarose gel electrophoresis and ELISA.

Detection of clinical samples by PCR-ELISA

In this study 30 clinical samples were collected from the laboratory of the different hospitals. The samples were cultured in the LB medium and chromosomal DNA was prepared. PCR was performed according to the previous plan in 30 cycles. PCR–ELISA was carried out.

Statistical analysis

Logistic regression analysis was used in percentage of PCR technique and antibody ELISA results with SPSS statistical software and Microsoft Excel 2007 for Windows.

Results

Reaction of PCR

The genome DNA of bacteria was extracted and analyzed on 1% agarose gel electrophoresis. The extracted DNA has an appropriate quality to perform PCR. The purity of the DNA samples was confirmed by A260/A280 absorbance ratio, which was 1.7-1.9. For PCR detection 16S rDNA specific primers was used. Several conditions and annealing temperature were examined for optimization of PCR reaction. For detection of PCR product, 5 µl from that was electrophoresed by 1% agarose gel and measured by 100 bp plus DNA ladder. The best annealing temperature which leads to amplification of 260 bp PCR products was 59°C (Fig. 1). For the labeling of digoxigenin, a PCR reaction was performed with digoxigenin labeling mix and the results were analysis on the 1% agarose gel (Fig. 1).

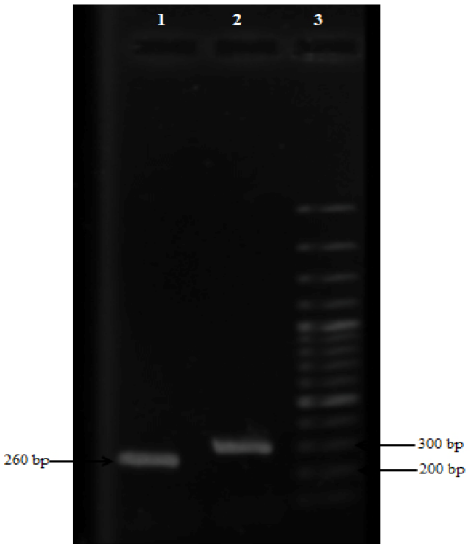

Figure 1. Agarose gel electrophoresis of PCR-amplified 16S rDNA gene. Lane 1: PCR product of 16S rDNA gene; Lane 2: DIG Labeled PCR product of the gene; Lane 3: 100 bp DNA Ladder plus as a standard molecular size marker.

The optimized PCR with specific primers was done for ETEC, *E. coli* O157, *Pseudomonas aeruginosa*, *Shigella dysenteriae*. PCR products with 260 bp size were amplified for *Klebsiella pneumonia* strain. As shown in Fig. 2, no PCR products were amplified from non-target pathogens.

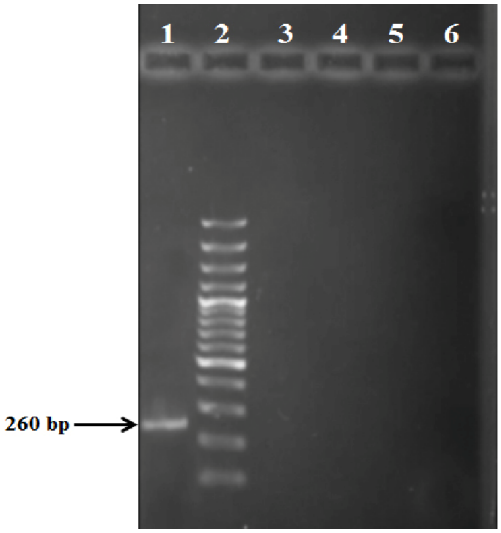

Figure 2. PCR Specificity for detection of *Klebsiella pneumonia*. Lane 1: PCR product of *Klebsiella pneumonia* 16S rDNA gene; Lane 2: 100 bp DNA Ladder as a standard molecular size marker; Lane 3-6: PCR product of ETEC, *E. coli* O157, *Shigella dysenteriae* and *Pseudomonas aeruginosa*, respectively.

PCR-ELISA specificity and sensitivity assay

To determine the sensitivity of PCR-ELISA assay, serial dilutions of *K. pneumonia* genomic DNA were subjected to the PCR-ELISA reactions. As shown in Figure 3, minimum detectable concentration of genomic DNA was 2 pg/µl. The specificity of the PCR-ELISA was analyzed using genomes of ETEC, *E. coli* O157, *Shigella dysenteriae* and *Pseudomonas aeruginosa*. No PCR products were amplified from non-target pathogens (Fig. 4).

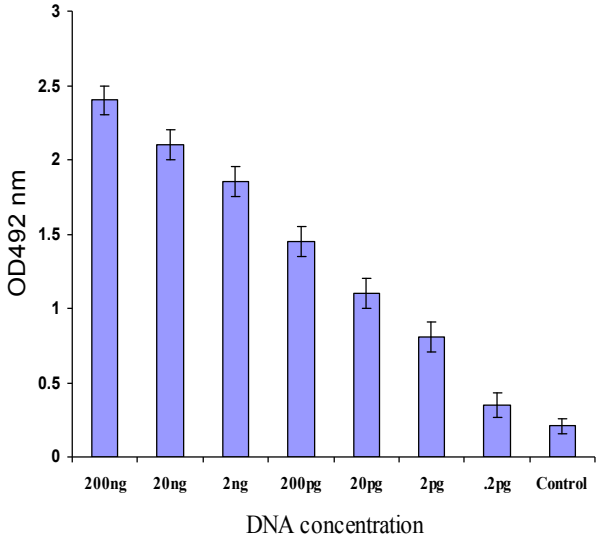

Figure 3. The sensitivity PCR-ELISA assay. Serial dilution of DNA extraction from 200 ng to 0.2 pg was used in each PCR-ELISA reaction.

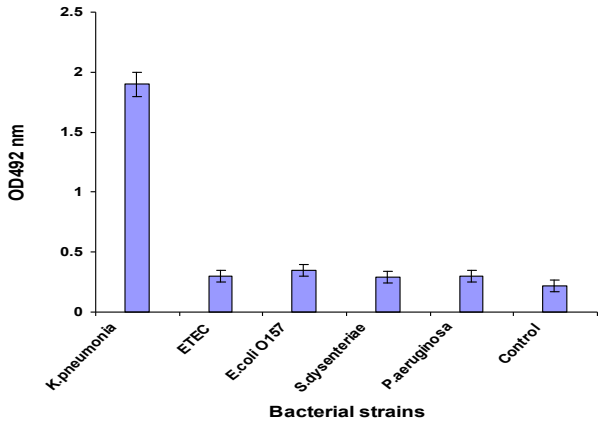

Figure 4. Specificity PCR-ELISA assay for detection of *K. pneumonia.*

Detection of Clinical Samples by PCR-ELISA

A total of 30 clinical samples were collected and screened for the detection of klebsiella pnemouniae strain. Five (16.6%) of samples was identified as *K. pnemouniae* (Fig. 5).

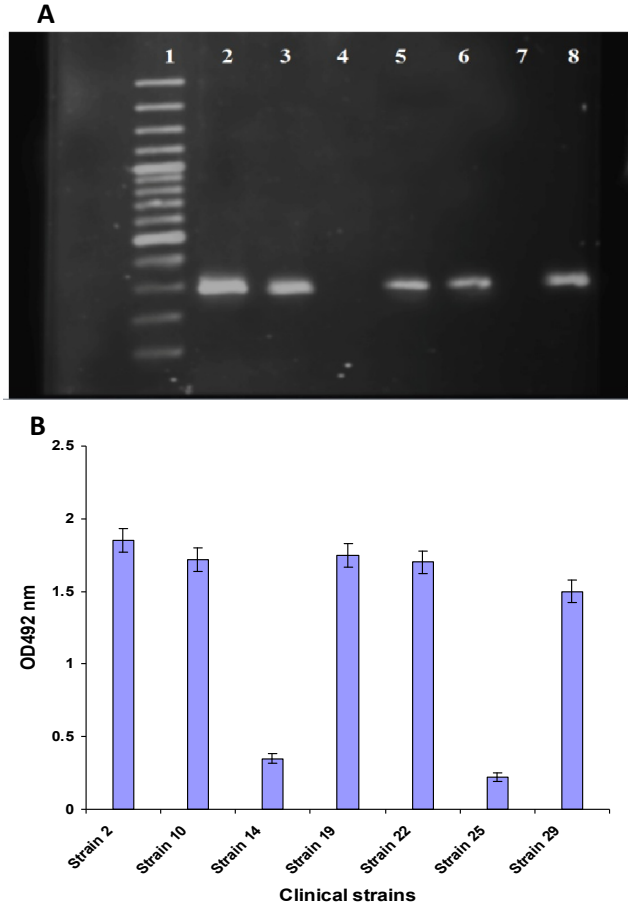

Figure 5. Detection of *Klebsiella pnemouniae* isolated from clinical samples by PCR and PCR-ELISA. (A) Agarose gel electrophoresis of PCR assay: Lane 1) 100bp DNA Ladder plus as; Lane 2,3,5,6,8) clinically *Klebsiella pnemouniae* samples. (B) Spectrophotometric analysis of PCR-ELISA. Negative and positive controls are indicated.

Discussion

Klebsiella pnemouniae is well-known as a hospital infectious pathogen, causing community and nosocomial infection, including pneumonia, urinary tract infection, septicemia, tissue infection, and wound infections. It can cause severe morbidity and mortality especially among the newborns [3, 4]. More specific to *K. pnemouniae* is its capacity to silently colonize patients or hospital personnel [5]. It is emerging worldwide as a major cause of bacteremia and drug resistant infections. Increasing levels of multiple-antibiotic resistance associated with this species pose a major emerging clinical problem [8]. The conventional methods for detection of *K. pnemouniae* is mainly aimed at clinical samples, such as biological specimens and body fluid, but these methods take a long time and are not very accurate [12]. Although Real-time PCR is a rapid, sensitive, and specific technique but it is very expensive [13]. Among the several thousand genes within a bacterial genome, the 16S rRNA gene has served as the primary key for phylogeny-based identification when compared against well-curated 16S rRNA gene sequence databases [16]. PCR-ELISA is a sensitive and specific technique that can identify different pathogens. With this method, multiple samples can be screened especially when the numbers of samples were high and the time was little [17].

Other researchers used PCR-ELISA for recognizing representative coliforms in water samples, direct identification of *Pseudomonas aeruginosa* from positive BACTEC blood culture bottles [12, 18].

As noted above, Mousavi et al., assessed a PCR-ELISA for detection of V. cholera O1 with using 10 bacterial strains and 50 samples from South Iran. Limit of detection in this assay was 0.5 pg of the genomic DNA, it is near to our results [14]. In our previous study PCR-ELISA was used to detect genes encoding shiga toxins 1 and 2 from *E. coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC) [15]. The result of previous studies showed that PCR-ELISA is more sensitive in comparison to PCR alone [14, 15, 17].

According to rapid speared of *K. pnemouniae* world wide and its antibiotic resistance, existence of rapid and sensitive techniques that can detect this bacterium with high specificity is necessary. In our study we used PCR-ELISA for rapid detection of *K. pnemouniae*. The PCR-ELISA method detects nucleic acid instead of protein and is a much more sensitive compared to conventional PCR assays, with lower detection limit and shorter analytical time. In our procedure we used DNA genomic DNA; we also decreased the number of the PCR cycles to five for reducing the reaction time.

Using specifically designed primers and a capture probe, the PCR-ELISA method described above was sensitive enough to detect 2 pg/µl of *K. pnemouniae* genome, and this level of detection was achieved within 3 hrs. Specificity of this method with other strains of bacteria was examined and the results showed that all the primers and probes which were used in this research were only assigned to *Klebsiella pnemouniae*.

Our PCR-ELISA approach for identification of *K. pneumoniae* resulted in significant time saving in comparison to traditional biochemical identification of *K. pneumoniae*.

Conclusion

PCR-ELISA also known as PCR-ELOSA is a useful technology alternative to real time and other methods. Detected sequence of nucleotides by antibody is a main objective of PCR-ELISA. Our data indicated that PCR–ELISA is highly specific, and using this method instead of conventional gel electrophoresis increased the sensitivity of the assay. By offering faster diagnosis and high sensitivity, there is high potential for PCR-ELISA to serve as a suitable technology for implementation in routine diagnostic laboratories. The results of the study show that PCR–ELISA method is quite sensitive, specific, and rapid for detection of *K. pnemouniae*.

Acknowledgements

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