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MOLECULAR CHARACTERIZATION OF PATHOGENIC BACTERIA ISOLATED FROM URINARY TRACT INFECTION

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Abstract

Four isolates were obtained from urinary tract infections that belong to *Escherchia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa*. These microorganisms were identified phenotypically by oxidase, catalase, urease, indole, methyl red, Voges-Proskauer and citrate tests. As a molecular typing and characterization tool, enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) was employed in which different bands in number and size were obtained. In order to identify nitrogen fixation ability of isolates, only *K. pneumoniae* was shown to have this ability as PCR targeting *nifDK* were carried out.

Key words: Molecular typing, Nitrogen fixation, ERIC-PCR, UTI.

Introduction

Molecular diagnosis employs methods to analyze genotypes depending on estimating DNA, RNA and proteins in certain infectious diseases or health cases (Bakermans and Madsen, 2002). The traditional methods for identifying pathogenic microorganisms *i.e.* biochemical and cultural approaches, require effort and time and may be of low sensitivity in addition to cases where a microbe is slow in growth or sometimes difficult to grow under normal laboratory conditions (Allee, 1996). New molecular biology techniques are being successfully introduced such as polymerase chain reaction, northern and southern blot, single nucleotide polymorphs, Restriction fragment length polymorphism and pyrosequencing (Adane *et al*., 2016).

Urinary tract infections have become the most prevalent nosocomial infection and community acquired disease in all ages. In the study of Hammoudi, (2013), two hundred eighty urine samples were collected from out- patients with symptoms of this infection in AL-Karama teaching hospital in Baghdad city from 1/3/2009 to 1/7/2009. The isolated microbes were identified by biochemical tests to be *Klebsiella spp.* (30.8%) followed by *E. coli* (22.2%) and the Gram-positive pathogens were *Staphylococcus aureus* (27.2%). 125 urine samples obtained from 25 males and 100 females were shown to have bacterial infections with Gram negative bacteria in the study of Kareem and Rasheed, (2011). In 44% of cases, *E. coli* were isolated, in 32.8% *Klebsiella pneumoniae*, in 13.6% *Proteus mirabilis* and *P. vulgaris* and 9.6% *Pseudomonas aeruginosa*.

Nitrogen is essential element in supporting life on earth since it is present in amino acids, proteins and other important biocompounds. Microbes perform biological nitrogen fixation which converts atmospheric N_2 into usable forms (NH_4 or NO_2) by other living organisms such as plants (Timothy, 1999; Egamberdieva and Z. Kucharova, 2008). The aim of this study is to isolate, identify and molecular diagnose bacteria isolated from urinary tract infection and identification of nitrogen fixation genes.

Materials and methods

Isolation of microorganisms

Bacteria were isolated from urinary tract infection specimens and identified according to Forbes *et al*., 2007. Morphological and biochemical tests included Gram stain, Growth on Maconkey agar, Oxidase, catalase, urease, IMViC tests.

DNA isolation, PCR and electrophoresis

DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, USA). Rep-PCR method for finger printing was employed according to Mohapatra *et*

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Table 1: Identification tests of microorganisms.

Microorganism	Oxidase	Catalase	Urease	Indole	Methyl Red	Voges Proskauer	Citrate
E. coli	-	+	-	+	+	-	-
K. pneumoniae	-	+	+	-	-	+	+
P. mirabilis	-	+	+	-	+	-	+
P. aeruginosa	+	+	-	-	-	-	+

al., (2007). Primers used ERIC1F 5´-ATGTAAGCTCCT GGGGATTCAC-3´ and reverse 5´- AAGTAAGTGACT GGGGTGAGCG - 3´ using Intron's *Maxime* PCR PreMix Kit. Reactions were initial denaturation 95°C for 5 min., 35 cycles of 94°C of denaturation for 1 min, 51°C annealing for 2 min. and extension for 68°C for 2 min. final extension for 65°C for 10 min.

Electrophoresis was performed according to Sambrook and Russell, (2001), 3 µl of the processor loading buffer (Intron / Korea) has been mixed with 5 µl of the DNA to be electrophoresis (loading dye). An Electric current of 7 v\c2 has been used for 1-2 h until the dye reached to the other side of the gel. The gel has been analyzed by the UV with 336 nm after placing the gel in a container containing on 30µl Red safe Nucleic acid staining solution with 500 ml distilled water. 100 bp DNA marker was used (Intron/Korea).

Genetic map and cluster analysis

Photo capt program was used to calculate molecular weight of bands resulted from PCR reaction and comparing it with the DNA ladder (Cerasela *et al*., 2011). The data arranged in a table and the genetic distance was determined according to the following equation (Nei and Li, 1979):

$$\text{Genetic Distance} = 1-\left(\frac{2 \times N_{xy}}{N_y + N_x}\right)$$

Where N_{xy} is the similar bands between two isolates x and y, N_x is the total bands in isolate X and N_y is the total bands in isolate Y. Cluster analysis was drawn according to UPGMA (Sneath and Sokal, 1973) using Numerical taxonomy system, NTSYS-pc software.

Identification of nitrogen fixation ability using PCR

nifDK genes were detected according to Lagueree *et al*., 1996 method. Primers used FGPD807 5'-CACTGC TACCGGTCGATGAA-3' and FGPK492´ 5´-GATGACC TCGGCCAT- 3´. Intron's Maxime PCR PreMix Kit was used and PCR reaction involved: initial denaturation 95°C for 3 min., 35 cycles of 94°C of denaturation for 1 min, 63°C annealing for 1 min. and extension for 72°C for 2 min. final extension for 72°C for 3 min.

Table 2: Genetic distance of isolates.

	E. coli	***P. aeruginosa***	***P. mirabilis***	***K. pneumoniae***
1	0			
2	0.75	0		
3	0.625	0.42857	0	
4	1	0.55556	0.55556	0

Results and Discussion

Four isolates were identified by cultural and phenotypic characterization; these are *E. coli, K. pneumoniae, P. mirabilis* and *P. aeruginosa* (Table 1).

Hadi *et al*., (2014) study was conducted at the urology wards in several Basra hospitals for 90 patients. Patients were infected with *E. coli* (the highest percentage), *Pseudomonas* Spp., *Proteus* Spp., *Klebsiella* Spp., *S. aureus* and *Streptococcus* spp. In Tikrit A study was employed during the period from June 2015 to the end of January 2016 by Alsamarai, (2019), showed that women infected with *E. coli*, *S. aureus*, *K. pneumoniae* and *P. mirabilis*. Fifty urine samples were collected from patients with urinary infection in Al-Diwanyah teaching hospital and also fifty beef samples were collected from the different market in Al-Diwaniyah and *K. pneumoniae* was identified by 16S rRNA using PCR (Klaif *et al*., 2019).

ERIC sequences were used to type the isolated genetically. Table 2 show the genetic distance calculated using commercial software and the cluster analysis is in fig. 1. The method show different bands on each isolate.

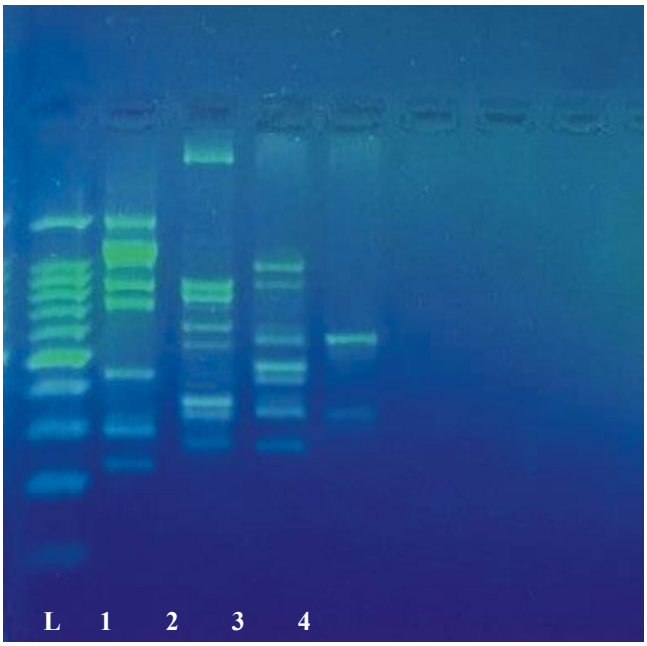

Fig. 1: PCR product. The PCR products was subjected to electrophoresis on 2% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. L: DNA ladder (100); 1=*E. coli*, 2=*P. aeruginosa*; 3=*P. mirabilis* and 4=*K. pneumoniae*.

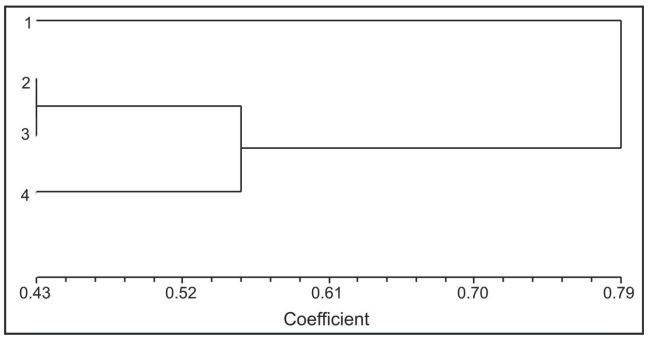

Fig. 2: Genetic map of ERIC-PCR repetitive sequences. 1=*E. coli*; 2=*P. aeruginosa*; 3=*P. mirabilis* and 4=*K. pneumoniae*.

Repetitive extragenic palindromic-PCR (rep-PCR) is a method that uses oligonucelotide primers that are complementary to repeated sequences spread in the genome of bacteria so by using the technique of polymerase chain reaction, this method can amplify varied regions of DNA flanked by the repeated sequences thus causing the formation of amplicons that are specific for the given strain. These repeated sequences may be divided into four classes: the enterobacterial repetitive intergenic consensus (ERIC) sequences, the repetitive extragenic palindromic (REP) sequences, the BOX sequences and the polytrinucleotide $(GTG)_5$ sequences (Versalovic *et al*., 1991; Versalovic *et al*., 1994; Rademaker and de Bruijn, 1997).

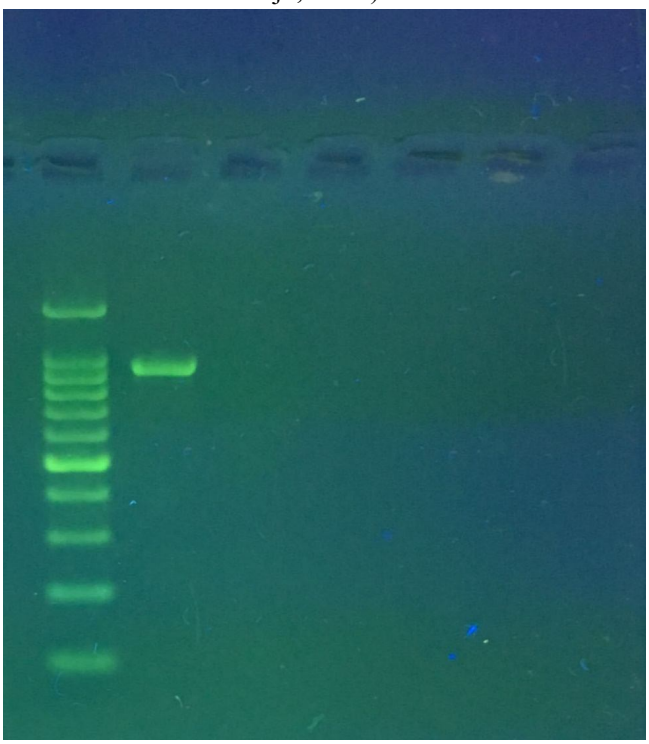

Fig. 3: PCR product of *K. pneumoniae*. The band size 980 bp. The product was electrophoresis on 2% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).

In the study of Bakhshi *et al*., (2018), fifty *Shigella* spp. were isolated from 3779 stool specimens of children having diarrhea conditions. Among the isolates, 92% were identified as *Shigella sonnei*, while 6% characterized as *S. flexneri* and 2% were characterized as *S. boydii.* The polymerase chain reaction of the enterobacterial repeated sequences (ERIC-PCR) gave rise to the 50 isolates into five ERIC types, which could be grouped into five clusters.

In the study of Ardakani and Ranjbar, (2016), 98 *E. coli* samples of urinary tract infections were obtained from patients admitted to Baqiyatallah Hospital. DNA molecular typing based on ERIC-PCR was used to type the *E. coli* isolates. The method obtained 4-17 bands, in the range of 100 to 3000 base pairs. The isolates were grouped into six clusters. It was found that ERIC-PCR had good differentiation power in genotyping *E. coli* strains isolated from the urinary tract infections.

Michelim *et al*., (2008) made an assessment of the discriminatory power related to different molecular techniques of typing (RAPD, ISSR, ERIC-PCR, BOX-PCR and rep-PCR) on *P. mirabilis*. Genomic typing patterns and cluster analysis showed that RAPD, BOX-PCR and ERIC-PCR can differentiate *P. mirabilis* from *E. coli*, *Hafnia alvei* and *Morganella morganii*. With the exception of rep-PCR, these methods have medium to high range as efficient in *P. mirabilis* in their discriminatory powers. The study indicated that a combination of ERIC-PCR and BOX-PCR is a fast and reliable alternative for discrimination in epidemiological studies regarding *P. mirabilis* pathogens isolated from infections.

Using PCR we were able to detect nitrogen fixation ability of *K. pneumoniae,* fig. 3. Nitrogen fixation was initially described from molecular biology point of view in *Klebsiella oxytoca* strain M5a1 first identified as *K. pneumoniae*.There are three structural genes two of them *nifD* and *nifK* encodes for Molybdenum nitrogenase subunit and nifH which encodes for iron protein subunit. Other genes also contribute for the nitrogenase synthsis. These are *nifH, nifD nifK, nifY, nifB, nifQ, nifE, nifN, nifX, nifU, nifS, nifV, nifW* and *nifZ* (Arnold *et al*., 1988; Franche *et al*., 2009). The nucleotide sequence was obtained for part of the *K. pneumoniae nifD* gene and all of *nifK* gene which encodes for α- and β-subunits of the nitrogenase MoFe protein respectively through the work of Holland *et al*., (1987) and also completed by Ioannidis and Buck, (1987) for the whole *nifHDK*.

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