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RANDOM AMPLIFICATION POLYMORPHIC DNA, A GOOD EPIDEMIOLOGIC SCREENING METHOD FOR NOSOCOMIAL ISOLATES OF *PSEUDOMOS AERUGINOSA.*

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ABSTRACT: *Pseudomonas aeruginosa is an aerobic Gram-negative bacterium which has emerged as one of the most problematic nosocomial pathogens. To characterizes P. aeruginosa strains that are widespread in patients in Hilla city,300 clinical and environment samples were collected from wounds, burn, ear, stool ,nose ,sputum and urinary tract infection taken from general hospitals of Hilla city. Methods for isolation and identifying P. aeruginosa based upon culture methods coupled with biochemical tests, were used in this study. The results show that, the selective medium (cetrimide agar) at 42˚C aerobically had highest recovery in the isolation of P. aeruginosa, they were produced greenish-yellow or blue pigment colonies, catalase and oxidase was positive whereas negative for methyl red, VogesProskauer and indole. A total of 34 amplified DNA fragmentsfrom 250 to 1500 bp)were observed using the 6 random primers,Amplification bands were exclusively revealed with four out of the six random primers (OPB-10,OPX-01,272,275) While(RAPD TYPING, 325) primers were failure to give amplification bands, and each of primer that successful giving amplification bands revealed different genetic patterns .*

Conclusion:RAPD-PCR analysis proved to be of great value in designing a variety of molecular based epidemiological studies that focuses on the identification and characterization of P.aeuroginosa

KEYWORDS: Pesudomonas Aeruginosa , Nosocomial ,RAPD , PCR

INTRODUCTION

Pseudomonas aeuroginosa is gram negative ,obligate an aerobic and non- sporulation, is ubiquities organisms widely distributed in soil,water and living hosts and motile through polar flagellum (Akanji*et al.,2011*) ..*P.*aeuroginosa can be lived or presented in many diverse environmental and it can be isolated from various living sources include plantes,animals and humam. The ability of *P.*aeuroginosa to survive on minimal nutritional requirements and tolerate a variety physical condition has allow to this bacterium living in both hospital community and environment (Pollack,1995) .*P.*aeuroginosa leading cause many infection include urinary tract infection, ear infection, eye infection, skin infection, central nervous system and bones &joints infections (*W*illenbrok et al .,2004) .the first step in *P.aeuroginosa* infection is that adherence of *P.*aeuroginosa to epithilum surface is mediated by pili, flagella and Alaginate (*C*oter*et al .,2010*) . the biofilm formation that helped *P.*aeuroginosa to escape from host defense mechanisms and resist to antibacterial action of antibiotics (Prasad *et al* .,2009) the second stepinclude colonization of *P.*aeuroginosa and produce several extracellular virulence factors which involved pyocyanin , hemolysine, alkaline protease ,elastease, neuraminidase, and exotoxins A,S,U,Y,T responsible for extensive tissue damage ,blood stream invasion and dissemination , many of these extracellular virulence factors are controlled by cell to cell signaling system (Cotar*et al.*, 2010).Because of its speed and versatility PCR has became very rapid reliable tool for molecular biology based diagnosis of

the variety of infectious disease. One of the PCR – based techniques is the random amplifiedpolymorphic DNA (RAPD). its has been widely used for epidemiological investigation and phylogenetics of many microorganisms (Li *et al .,2005*.The aim of this study was suggested and designed to study of genotypic and phenotypic properties of *P.aeuroginosa*, Furthermore, a dendrogram for the estimation of genetic relationship among *P.aeuroginosa*isolates for clustering of genetically similar strains, was also investigated.

METHODOLOGY

sampling

Three hundred samples were collected from clinical and environmental cases from patients suffering from wounds infections , ear infections, urinary tract infection gastrointestinal (diarrhea) , nose infection , throat infection and burn infection. Samples were taken from out and inpatients who admitted to AL-Hilla General Teaching Hospital and Babylon Hospital for Maternity and Pediatric Hospital . Between October 2012 and May2013 .

Phenotypic identification of *P. aeruginosa*

Swabs were enriched in nutrient broth and brain heart infusion broth plated in nutrient agar, MacConkey agar and blood agar ,A single colony was selected and incubated in the selective medium (cetrimide agar).then phenotypic characteristics of *P.aeuroginosa* was described after gram staining including pigments production, after incubation at 37 C Biochemical investigations were done according to Macfaddin ,2000 , which include ;,motility, indole production test, methyl red test, voges-proskauer test, citrate, urease, oxidase, catalase and ability to growth at 42 C .

Table (2) results of phenotypic tests of p.aeuroginosa .

Genotypic identification

DNAExtraction

DNA of *P.aeuroginosa* isolates was extracted and purified using Extraction and purification Kit from Geneaid company (UK)

Primers

Six arbitrary or random primers(OPB-10 ,OPX-01, 272 ,275, 325and RAPD TYPING) obtained from Bioneer, IDTDNA(USA) .Bacterial isolates were tested for single primers for RAPD-PCR technique (table1).

Table 1 .Type and sequence of RAPD primers used for pcr analysis

Primer	Sequence 5----------3
272	5'-AGC GGG CCA A -3'
325	5'-TCA TGA TGC A -3'
OPB-10	5'-CTG CTG GGA C -3'
OPX-01	5'-CTG GGC ACG A -3'
RAPD TYYPING	5'-CAG CCA GC -3'
275	5'-CCG GGC AAG C -3'

RAPD-PCR amplification

Final product of 30µl reaction volumes containing 10 ul of single primer ,12.5 ul of Green Master Mix ,5 ul of Genomic DNA and the volume of reaction was completed up to 30 ul by

adding 2.5 ul of Nuclease free water Amplification was carried out in a thermo-cycler (Eppendorf) programmed for two minutes at 94°C; for 40cycles one minute at 92°C, one minute at 36°C and two minutes at 72°C; and a final extension of ten minutes at 72°C. Amplification products were electrophoresed in 1.8% agarose gels and then visualized by staining with ethidium bromide. Standard molecular markers were also included in each electrophoresis run. Ultraviolet trans-illuminated gels were photographed

RESULTS AND DISCUSSION

Sampling

Clinical samples :- Morphological &biochemical characterization revealed 43 \300(14.3%) isolates of *P.aeuroginosa* from the total samples .Many previous studies pointed out variation in the incidence of *P. aeuroginosa* in different sources area and with a rate of 13.20% in Hilla city (saleh,2012) while(Almasshadani ,2004) in Mousl city found that the rate of *P.aeuroginosa* of clinical and environment samples was 5.7 % .The percentage of *P.aeuroginosa* is avariable in the different studies this may be attributed to drug overuse ,hospitals policy in management of such causes .moreover geographic climate and hygienic factors may also be correlated with the relative variability of results among different areas (Memmel.,et.al,2004) .

The distribution of *P.aeuroginosa* isolates according to site of infection in the present study indicated that *P.aeuroginosa* infections mainly highest in burn infected patients 35% (table 2) .

Table 2 . samples collected from different sources related p.aeuroginosa

Site of infection	Number of samples	Number of *P.aeuroginosa*	*%*
Urine	146	4	2.9
Burn	37	13	35
Ear,nose,phranx	47	12	23.4
Wound	38	6	15.7
Stool,	25	4	16
Environment	7	4	57
Summation	300	43	14.3

The high rate of *P.aeuroginosa* appearance correlated with its ability to utilized of tissue debris ,discharges and tissue fluids that found in burn wounds (Kohanteb*et al* .,2007 ; Mahar, *et al.,* 2010 and Taherzadeh *et al.,* 2011). *P. aeruginosa* is the third most-common pathogen associated with hospital-acquired infections (Moreau-Marquis *et al.,* 2008). The second rate of *P.aeuroginosa* occurrence appear with ENT specimens(ear ,,nose, pharynx) 25% followed by wound infection 7% , stool 16% and urine 2% . Previous studies pointed variations in isolation rate ,the study conducted by Gad etal.,2007 demonstrated a percentage of (72%), (11%), (20%), (20%),(22%),(4.4) for burn ,wound, ear, abscess, ,urine and stool samples respectively .

Phenotypic Characteristics

Conventional diagnostic approaches showed that *P. aeruginosa* produces green pigment and specialized odor on cetrimide agar . Biochemical investigation for *P. aeruginosa* isolates revealed positive results to catalase and oxidase tests, while it was negative for methyl red, VogesProskauer, indole , slowly hydrolyzed urea, and utilized Simmon‟s citrate . The biochemical properties of the organism recorded in this study are the same as obtained by Abro and his coworkers (2009). In general biochemical methods have, to a certain extent, facilitated the identification of *P. aeruginosa*; .However, some of these methods are time consuming . , many other additional morphological, biochemical, and physiological tests are always needed to obtain the exact identification (table3)**.**

Table 3.Phenotypic characteristics of clinical and environmental isolates of *P, aeruginosa* .

Name of test	Results
catalase test	+
oxidase test	+
indole test	_
Hemolysis test	-\+
H2S production	-\+
methel red test	_
voges –proskauer test	_
simmons citrate test	+
motility test	+
pigment production	+
urease test	_
triple suger iron agar	A\K
Biofilm formation	-\+
Gelatinase test	_
Growth at 42 C	+
Gram stain	-

RAPD analysis of *P. aeruginosa.*

Polymorphism assay for *P. aeruginosa* isolates was carried out using four primers . . Random amplification of the DNA of *P.aeruginosa* isolates reveals the efficacy of these selected nucleotides sequences in determination the similarity or variations among all isolates .

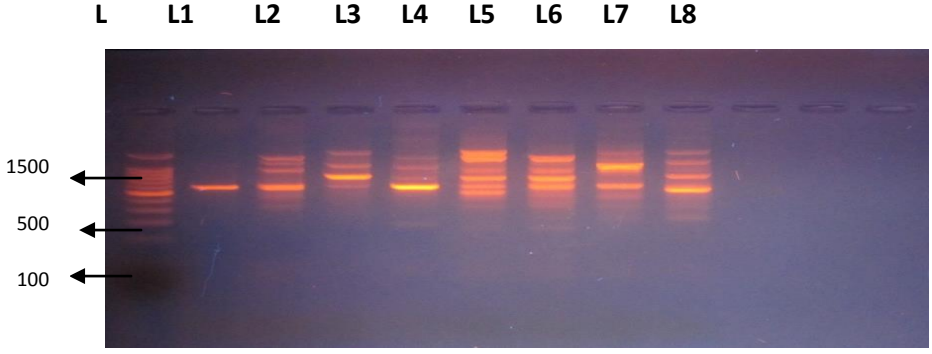

Figure 1. RAPD –PCR using the primer OPB-10.

{1 Line (ladder) , the isolates numbered (1,2,3,4,5,6,7 ,8) were positive for opB-10 primer} amplification of OPB-10 gene determine the similarity of isolates 1,2, , 4 and isolates 4,5 (figure 1) .

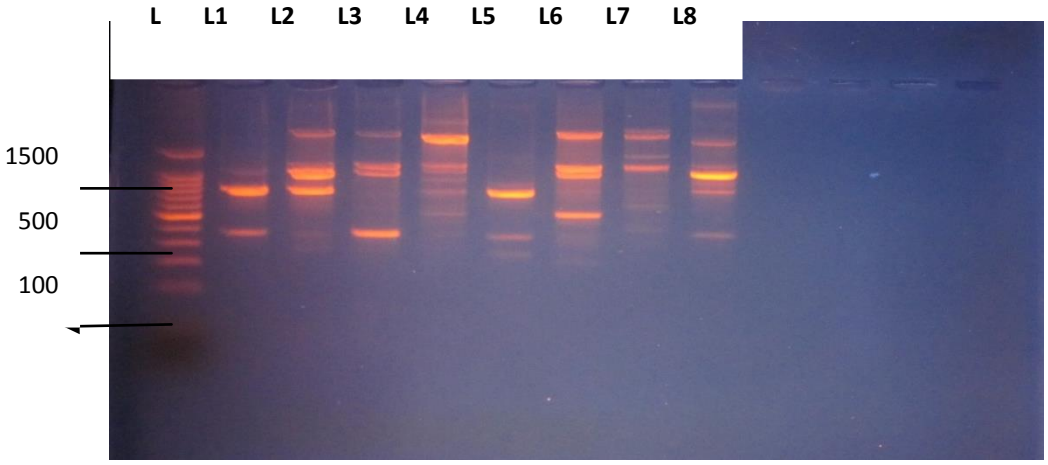

Figure 2 . RAPD –PCR using the primer OPX-01
{1 Line (ladder) , the isolates numbered (1,2,3,4,5,6,7 ,8) were positive for opX-01 primer} amplification of OPB-10 gene determine the similarity of isolates 1,3 and isolates 4,7 and 3, 8 (figure 2).

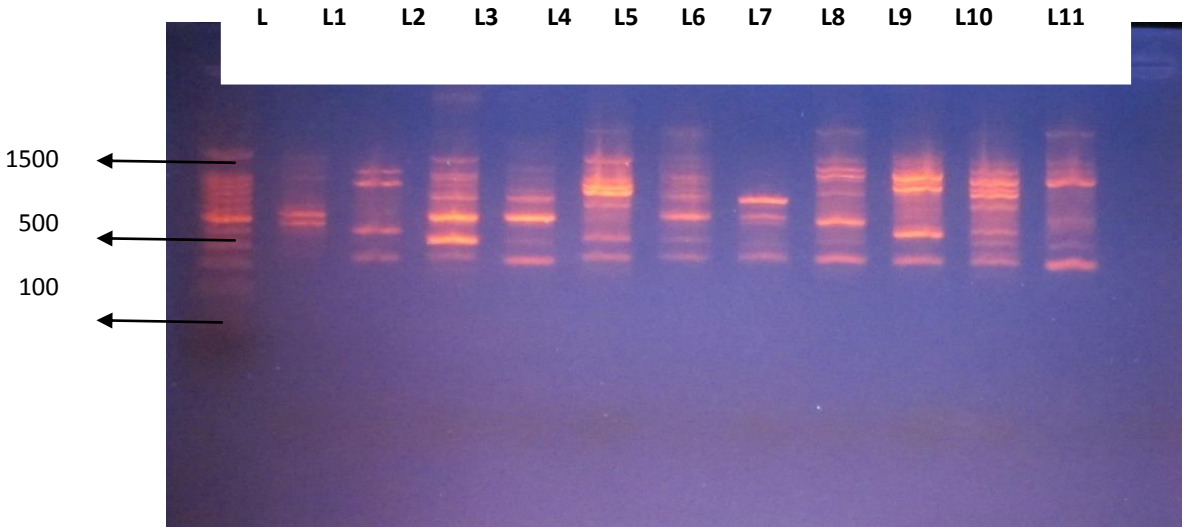

Figure 3 . RAPD –PCR using the primer 272{1 Line (ladder) , the isolates numbered (1,2,3,4,5,6,7 ,8,9,10,11) were positive for 272primer}
Amplification of isolates DNA for the primer 272reveals similarity among 2,5 8 .9 . (figure 3) .

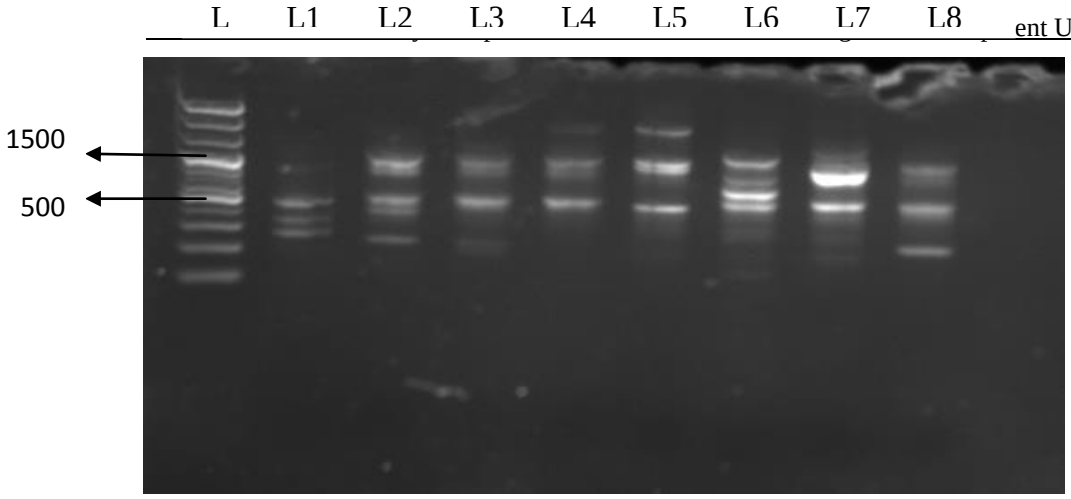

Figure 4 . RAPD –PCR using the primer 275 .{1 Line (ladder) , the isolates numbered (1,2,3,4,5,6,7 ,8) were positive for 275primer}

Genetic characterization of *p.aeuroginosa* isolates by RAPD analysis

Atotal of 34 amplified DNA fragments ranging in size from 150 to 1500pb were observed using four random amplified polymorphic DNA (RAPD) primers (opB-10 ,opX-01 ,272 and 275) where as30 polymorphic and 4 amplified fragments were commonly detected among the 16 *P.aeuroginosa* isolates (table 4) and each of primer give different genetic profiles. Although various methods are variable for genetic characterization of bacterial isolates, RAPD was used successfully for *P.aeuroginosa*and is less costly and time consuming than other methods. The RAPD method is straightforward and does not require previous knowledge of nucleotide sequence of target organism. Furthermore, it is very quick and convenient to perform, since a strain can be typed within 48 hours of harvesting the cells from the agar medium. It is highly sensitive, requires minimum amount of template DNA.The results obtained from this study showed that 272 primer most efficient primer to give amplified band this is agree with (mahenthiralingan *et* al.,1996 ; Akangi *et.al*,2011)).Furthermore, RAPD-PCR is discriminatory because it analyzes the whole genome. However due to its sensitivity and reproducibility, can be affected by small variations in reaction mixture and temperature cycles. Thus, care is needed to standardize the procedure if it is to be used for routine analysis. Positive (type strain template DNA) and negative (no template DNA) should always be included The failure of many primers to amplify DNA may be due to their need to special requirements for amplifications in terms of PCR-reagents or temperature profile, since all of the reaction parameters were identical for all primers. Moreover, differences in banding patterns are likely due to specific requirements of a given primer. The G+C content of the primer may further interfere with PCR yield (Weeden *et al* .,1995 ;Jurallah*et al*., 2005).that the optimal concentration of DNA template differed with the primer used. RAPD analysis would be the most appropriate choice for epidemiologic studies as it is not expensive, smaller amount of DNA are required to provide high amplicons which can be easily interpreted without the need of imaging software (Lin *et al.*,1996).

And finally we can say The current study has shown agreement with pervious study in Canada and Egypt.

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