Indian Journal of Modern Research and Reviews

This Journal is a member of the *'Committee on Publication Ethics'*

Online ISSN:2584-184X

Research Paper

RAPD-PCR and Phylogenetic Tree Analysis of *Pantoea ananatis* Isolated from Burn

Najat Mohammed Flyyih[1*]

[1]College of Health and Medical Techniques, Furat Al-Awsat Technical University, Kufa, Iraq

Corresponding Author: * Najat Mohammed Flyyih

DOI: https://doi.org/10.5281/zenodo.14548124

ABSTRACT

The study involved 112 clinical samples collected from burn patients with infections at Al-Sadder Educational Medical Hospital and Al-Hakeem General Hospital. The specimens were obtained from skin during the study period from May 2023 to March 31, 2024. The results showed positive bacterial growth in 79 samples, while 33 samples were classified as negative. This study looked to find *Pantoea ananatis* through the application of the Utilization of Random Amplified Polymorphic DNA (RAPD) methodologies in the investigation of genetic diversity. Of the 112 samples examined, 12 (10.71%) tested positive for *Pantoea* based on morphological characteristics, microscopic examination, and biochemical assays. The biochemical test indicated oxidase negativity, catalase positivity, and positivity for methyl red, indole, and urease. The phylogenetic tree derived from RAPD-PCR products was analyzed using 1% agarose gel electrophoresis. The total number of fragments (bands) from two primers is 67. OPX-18 primer shows superior performance concerning the number of fragments (36), primer efficiency (53.73%), and discrimination power (54.54). RAPD typing is regarded as helpful in scenarios involving many isolates due to its cost-effectiveness and simplicity. It is employed across a diverse array of applications within various biological disciplines.

Manuscript Info.

✓ **ISSN No:** 2584-184X
✓ **Received:** 12-09-2024
✓ **Accepted:** 21-11-2024
✓ **Published:** 23-12-2024
✓ **MRR:2(12):**2024;10-15

✓ **Peer Review Process:** Yes
✓ **Plagiarism Checked:** Yes

How To Cite

Najat Mohammed Flyyih. RAPD-PCR and Phylogenetic Tree Analysis of *Pantoea ananatis* Isolated from Burn. Indian Journal of Modern Research and Reviews: 2024; 2(12):10-15.

KEYWORDS: Burn infection, *Pantoea ananatis*, RAPD-PCR, Phylogenetic Analysis

1. INTRODUCTION

Pantoea sp. members of the Enterobacteriaceae family are gram-negative facultatively anaerobic, rod-shaped. *Pantoea*sp. is ordinarily found in environmental niches such as water, soil, sewage, seeds, and vegetables [1]. Dutkiewicz *et al*., (2016) [2] described Pantoea *sp. as* a mysterious bacterium of evil and good. *Pantoea sp*. is a particularly uncommon microorganism related to burn infection [3] *Pantoea* rarely infect humans, but in recent years some pathological cases including meningitis, bacteremia, pneumonia, urinary tract infections, osteomyelitis, joint infection, and Corneal infiltration. the infection belongs to multiple *Pantoea* species that are known to act as opportunistic pathogens including *P. agglomerans, P. ananatis, P. calida, P. dispersal, P. brenneri, P. septica* [4]. The initial adherence and aggregation of microorganisms onto many layers precede biofilm formation, which also has outer membrane proteins that are crucial in disease and significantly contribute to morbidity and mortality [5]. Elucidate that extracellular proteins are instrumental in microbial adhesion and colonization. The pathogen can induce infection due to its virulence factors,

including genes associated with pathogenesis and enhanced virulence within the host [6]. Following the injection of effector proteins, these proteins, produced by the genes and sent into the host's cells via the Type III secretion system (T3SS), inhibit the host's immune response and regulate cellular functions, ultimately altering host defenses to benefit the pathogenic bacteria. Additional virulence. Genes associated with exopolysaccharide (EPS) synthesis were identified, facilitating bacterial adhesion, in conjunction with enzymes involved in bacterial pathogenesis, such as cellulase, which degrades the robust cellulose polymer walls of plant cells, thereby augmenting microbial invasion and elevating the virulence of pathogenic bacteria within host cells[7,8]. Bacterial illnesses that were once managed may reappear due to antibiotic resistance or vaccination evasion. Pathogenic bacterial species exhibit extensive genotypic and phenotypic variation. Biodiversity pertains to the diversity, evolution, and epidemiology of bacterial pathogens, as well as the correlations between the genotypic and phenotypic diversity (ecology, colonization, transmission, virulence, antibiotic resistance, immune response) of strains within certain species[9].

2. MATERIALS & METHODS

Isolation of Microorganisms:

Microorganisms were isolated from the burn infection from Al-Sadder Educational Medical Hospital, Al-Hakeem General Hospital. The microorganisms were isolated on MacConkey agar medium and blood agar at 37°C for 18-24 hours. lactose-fermented colonies were picked up and re-inoculated on a new MacConkey agar plate. The found of *Pantoea* isolates were confirmed with the automated VITEK-2 compact system using GN-ID cards. GN well content in (Appendix 1). The samples were achieved according to manufacturing instructions as follows:

1. **Suspension Preparation:** A sterile swab is utilized to transfer an adequate number of colonies from a pure culture and to suspend the microorganisms in 3.0 ml of sterile saline within a 12 x 75 mm transparent polystyrene test tube. The turbidity was calibrated to correspond with a McFarland No. 0.5 utilizing a turbidity meter referred to as Densi Chek.

2. **Inoculation:** Identification cards are embedded with microorganisms and have an integrated Hoover system. A test tube containing the microorganism suspension was placed on a specialized rack (cassette), with the identification card positioned in the adjacent slot and the transfer tube inserted into the appropriate suspension tube. The filled cassette was manually placed into a vacuum chamber station. Following

3. **Card sealing and Incubation**: Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator.
4. **Optical system:** An optical transmittance system eases the analysis of test reactions via various wavelengths within the visible spectrum.
5. **Test reactions:** Calculations are executed on raw data and juxtaposed with thresholds to fin **.**dresponses for each test.

These cultures were then used for further investigation like DNA extraction, PCR, and RAPD genetic diversity.

Bacterial Density Determination:

Bacteria were cultured in 5 mL of LB medium (1% tryptone, 1% NaCl, and 0.5% yeast extract) and several dilutions were plated on LB medium (LB with 1.5% agar) for estimation of the number of colonies forming units (CFU). Colony counts were made after 24-48 hours of incubation at 37ºC.

PCR Assay:

The whole genomic DNA purification kit is designed for the separation of DNA from Gram-negative bacteria using an extraction kit from Promega (USA). Gel electrophoresis was employed for DNA detection and visualized using a UV transilluminator**.** The DNA concentration was quantified spectrophotometrically using a Nanodrop by assessing its optical density at 260 nm (the extinction coefficient of dsDNA is 50 µg/ml at this wavelength), with concentrations recorded in the ng/ml absorbance range of 0.1-1. The purity of a DNA solution is decided by the OD260/OD280 ratio, which should range from 1.8 to 2.0 for pure DNA [10]**.**

RAPD analysis was conducted in a 25µl reaction consisting of 10µl of 2x master mix, 5µl of DNA samples, 5µl of primer, and 5µl of deionized water. The thermal cycling protocol consisted of a first denaturation for 4 minutes at 92°C, followed by 40 cycles of 1 minute at 92°C, 2 minutes at 37°C, and 3 minutes at 72°C, concluding with a final extension at 72°C for 3 minutes. PCR products in 1% agarose were analyzed using gels stained with ethidium bromide. The primers used were OPZ04 AGGCTGTGCT 3, OP U-17 ACCTGGGGAG, and OPX-18 (5'-TGG CAA GGC A-3'), provided by Operon Technologies (Alameda, CA, USA) **.**

Data Scoring and Dendrogram Analysis:

An arbitrary primer (OPZ04 and OPX-18) was used for the RAPD-PCR assay of *Pantoea sp.* The final volume of the reaction was 25µl consisting of 10µl of master mix 2x, 5µl of DNA samples, 5µl of primer, and 5µl of Deionized water. PCR thermocycler condition was controlled as in Table (3-5). Later, the amplicon resulted from PCR amplification was electrophoresis using 1% agarose, and the bands were visualized using UVI B and software (version 12.14) Data were analyzed considering the presence (1) or absence (0) of bands for every isolate by Past3 program (Hammer *et al.*,2001). also, determent Primer efficiency, polymorphic, percentage polymorphic bands, and Discrimination power

were calculated for each primer using the following equations as described by Hunter and Gaston, (1988) and by Graham and Mcnicol, (1995) [11,12].

3. RESULTS & DISSECTIONS

Source of Bacterial Isolates

During the study period from May 2023 to 31 March 2024, 112 burn specimens were obtained from the skin. The results clarified that the positive results for bacterial growth were (79) and (33), while the rest of the samples were considered negative results, as shown in Table (1-1).

Table 1.1: Distribution of samples of burns infection patients

Sample	*Pantoea* sp.	Gram-negative	Non-growth
Burn	12(10.71%)	67(59.82%)	33(29.46%)

Out of 112 samples investigated 12 (10.71%) were positive for *Pantoea* depending on morphology, microscopic examination, and biochemical. The morphology examination of *Pantoea spp.* colonies on MacConkey agar showed round mid-sized, smooth, punctuate, and pink lactose fermenting, during incubation on blood agar *Pantoea sp*. generates smooth, convex, spherical colonies that are typically non-hemolytic and either non-pigmented or yellow. Colonies ranging from pale yellow to white. Microscopic analysis using oil immersion revealed gram-negative rod-shaped bacteria. The biochemical testing for the sample is presented in Table 1-2. The isolates exhibit common characteristics, being oxidase-negative and catalase-positive. The isolates fermented sugar on TSI agar, resulting in an alkaline or acidic reaction on the slant and an acidic reaction at the bottom, without forming H_2S. The biochemical test findings presented in Table 1 identified *Pantoea sp*. as a supplement to the primary identification of the isolates. The isolates exhibited general characteristics characterized as oxidase-negative and catalase-positive. They demonstrated motility, were positive for methyl red, and tested positive for gas and indole synthesis. The isolates successfully fermented glucose on Kligler iron agar. (KIA), resulting in an alkaline red hue on the slant and a yellow acid with gas at the bottom, but no H2S was formed; moreover, they yielded negative results for Voges-Proskauer and citrate use.

Table 1.2: Biochemical test results of suspected isolates *Pantoea* sp.

No.	Test	Result	No	Test	Result
1	Oxidase	-	6-	Methyl Red	-/+
2	Catalase	+	7-	Vogus-Proskuar	variable
3	Indole	+/-	8-	Motility	+
4	Citrate Utilization	variable	9-	H2S	-
5	Gas	+/-	10-	TSI	A/A, K/A

Identification was validated utilizing the automated VITEK-2 compact system with GN-ID cards, achieving a message confidence level classified as particularly good to outstanding (probability percentage range from 93% to 98%). *Pantoea sp*. constituted 12 instances (10.71%) as depicted in figure (1-1), out of a total of 79 Gram-negative bacterial growths on MacConkey agar (59.82%). *Pantoea ananatis* constituted 5 (41.66%), whilst other Pantoea species accounted for 58.33%. *P. ananatis* is the sole species that yielded favorable findings for indole and is further distinguished from other species by its exclusive ability to ferment D-Arabitol. Data were obtained from the source (13). illustrated in Figure (1-2):

bioMérieux Customer: Microbiology Chart Report Printed January 4, 2024 9:44:16 AM CST

Patient Name: m, mahabad Patient ID: 3120231
Location: Physician:
Lab ID: 3120231 Isolate Number: 1

Organism Quantity:
Selected Organism : Pantoea spp

Source: **Collected:**

Comments:

Identification Information	**Analysis Time:** 5.93 hours	**Status:** Final
Selected Organism	95% Probability **Pantoea spp**	
	Bionumber: 4627630550130210	
ID Analysis Messages		

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	+	5	lARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	lLATk	+	41	AGLU	-	42	SUCT	-	43	NAGA	+	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Fig. 1.1: Final identification results of *Pantoea spp*. isolates with VITEK-2-compact system

Table 1.2: Carbohydrate fermentation for identification of *P. ananatis*

Carbohydrate	Result
Lactose	+
Sucrose	+
Maltose	+
Glucose	+
Xylose	+
D-Sorbitol	+
Lactose	+
Sucrose	+
Maltose	+
Glucose	+
Xylose	+
D-Sorbitol	+
Lactose	+

Genotyping of *Pantoea* sp. by RAPD analysis

Randomly amplified polymorphic DNA (RAPD) analysis was performed using two arbitrary primers (OPZ04 and OPX-18) and a PCR technique. The RAPD-PCR products were resolved by 1% agarose gel electrophoresis. The Total number of fragments (bands) of Two primers (67). OPX-18 primer is the most competent where the number of fragments, Primer efficiency (53.73) %, and Discrimination power. RAPD markers have diverse uses in gene mapping, population genetics, molecular evolutionary genetics, and the breeding of plants and animals. This is mainly due to the speed, cost, and efficiency of the technology to manufacture huge numbers of markers in a short period compared with other molecular technologies.

Table 1.3: Information about four primers

	OPZ04	**OPX-18**	**Total**	**Mean**
Total number of fragments	31	36	67	34
Fragments	4-8	5-9		
Molecular weight Range of fragments	(250-1130) bp	(168-1322) bp		
Polymorphic bands	30	36	66	33
Percentage of Polymorphic bands	96.77	100	196.77	98.385
Primer efficiency %	46.26	53.73		
Discrimination power	45.45	54.54		

A dendrogram illustrating the genetic relationships among five *Pantoea ananatis* isolates, generated using RAPD analysis using two primers, reveals significant diversity in the genetic material (DNA) of the *Pantoea* isolates, as seen by the varying number of amplified bands and their corresponding molecular weights. There are certain similarities to the primers of *Morganella* morganii [14]. The variation in the number of amplified bands and molecular weight observed with these primers may reflect differences in the distance between loci on the template DNA of bacteria in different samples, to which the primers' complementary nucleotide sequences bind [15]. The discriminatory capabilities of primers were assessed based on the observed banding patterns that facilitated the genotyping of these isolates. The selection of primers for RAPD analysis is the most crucial factor, and utilizing multiple primers enhances the diversity of banding patterns. Consequently, some strains isolated from various sources exhibit greater differentiation, thus employing more than one primer is advantageous [16]. The degree of similarity % among isolates is presented in Table (1-4). The highest percentage of similarity (1) was seen between *P. ananatis* 3 and *P. ananatis* 4, followed by 0.83 for *P. ananatis* 5. Differences in the degree of similarity may arise from variations in strains, sub-strains, and geographical distribution of bacterial isolates [17].

Table 1.4: The Percentage of similarity degree between 5 *P. ananatis*

	1	**2**	**3**	**4**	**5**
1	1	0.58	0.64	0.64	0.64
2	0.58	1	0.58	0.58	0.58
3	0.64	0.58	1	1	0.83
4	0.64	0.58	1	1	0.83
5	0.64	0.58	0.83	0.83	1

A dendrogram was produced using the data from two primers to create a phylogenetic tree (Fig 1-2). One primary cluster was seen between P. ananatis 3 and *P. ananatis* 4, with a similarity of 0.83 to *P. ananatis*. Phylogenetic analysis seeks to elucidate the evolutionary relationships among a set of sequences. A conventional phylogenetic tree or cladogram categorizes species into a diagram illustrating their relative evolutionary divergence. Molecular sequence alignment data may be utilized to construct a phylogenetic tree for a certain gene sequence family. The bifurcations in phylogenetic trees signify evolutionary distance either from sequence similarity scores or from information-theoretic models quantifying the mutational steps necessary to transform one sequence into another [18].

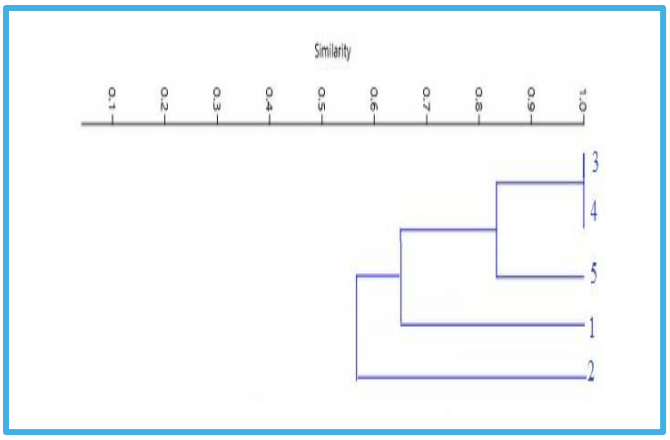

Fig. 1.2: Dendrograms showing the genomic relationship between 5 *P. ananatis* isolates as found by RAPD analysis with 2 different arbitrary primers.

Michelim *et al.* (2008) demonstrated that RAPD exhibits high discriminatory power, rendering it one of the most appropriate methods for local Proteus investigations (19). Additionally, RAPD typing is particularly advantageous in scenarios involving numerous isolates, and due to its simplicity and cost-effectiveness, the RAPD technique is employed across various domains of Biology. The RAPD test may, in certain situations, identify single-base alterations in genomic DNA. Most single nucleotide alterations in a primer sequence result in a complete alteration of the amplified DNA sequence pattern [20].

4. CONCLUSION

1. Isolated *P. ananatis* from burn infection which 5(41.66%)
2. Primers (OPZ04 and OPX-18) used show phylogenetic tree and similarity degree between *P. ananatis*.
3. RAPD analysis is the faster and simpler method used for typing of *Pantoea ananatis* by using a specific primer.

5. RECOMMENDATION

1. Further research would be necessary to determine the mechanisms that are involved in the process of virulence in *Pantoea ananatis.*
2. Antibiotic therapy must be restricted and controlled legally because the misuse may cause the spread of resistance.
3. Study virulence factor phenotypic and genotypic to understand how *Pantoea ananatis* prevalent to clinical bacteria.

REFERENCE

1. Achbani EH, Mazouz H, Benbouazza A, Sadik S. First detection of *Pantoea ananatis*, the causal agent of bacterial center rot of onion in Morocco. *J Crop Prot*. 2016;5(1):11–7.
2. Dutkiewicz J, Mackiewicz B, Lemieszek MK, Golec M, Milanowski J. *Pantoea agglomerans*: a mysterious bacterium of evil and good. Part III. Deleterious effects: infections of humans, animals and plants. *Ann Agric Environ Med*. 2016;23(2).
3. Kara SS, Aslan MH, Albayrak Y. A rare pathogen in a burn patient: *Pantoea agglomerans*. *Med Sci*. 2016;5(3):878–83.
4. Venincasa VD, Callegan M, Astley RA, Siatkowski RM. Contact lens-related polymicrobial keratitis from *Pantoea agglomerans* and *Escherichia vulneris*. *Am J Ophthalmol Case Rep*. 2016;1:5–7.
5. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol*. 2004;2(2):95–108.
6. Oosthuizen MC, Steyn B, Lindsay D, Brözel VS, von Holy A. Novel method for the proteomic investigation of a dairy-associated *Bacillus cereus* biofilm. *FEMS Microbiol Lett*. 2001;194(1):47–51.
7. Abdulamir A, Al-baaj A, Al-Ramahy SK. Determination of some virulence genes (*avrxacE2, avrxacEl, hrpG, acrAB*) in *Pantoea spp.* isolated from hospitals of Al-Diwaniyah city, Iraq. *J Phys Conf Ser*. 2020;12113.
8. ALbaaj AAA, Al-Ramahy SK. Molecular study of bacteria *Pantoea spp.* in Diwaniyah city, Iraq. *J Phys Conf Ser*. 2019;62060.
9. Yao X-Y, Liu H, Sun J, Zhang Y-Q, Lv Z-H, Zhang X-L, et al. Epidemiology and genetic diversity of *Bartonella* in rodents in urban areas of Guangzhou, Southern China. *Front Microbiol*. 2022;13:942587.
10. Seidman LA. *Basic laboratory calculations for biotechnology*. CRC Press; 2021.
11. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol*. 1988;26(11):2465–6.
12. Graham J, McNicol RJ. An examination of the ability of RAPD markers to determine the relationships within and between *Rubus* species. *Theor Appl Genet*. 1995;90:1128–32.
13. Mantelin S, Saux MF-L, Zakhia F, Béna G, Bonneau S, Jeder H, et al. Emended description of the genus *Phyllobacterium* and description of four novel species associated with plant roots: *Phyllobacterium bourgognense* sp. nov., *Phyllobacterium ifriqiyense* sp. nov., *Phyllobacterium leguminum* sp. nov., and *Phyllobacterium brassicacearum* sp. nov. *Int J Syst Evol Microbiol*. 2006;56(4):827–39.
14. Flyyih NM, Abdul-Hassan LS, BasimaBasim MA, Ali S. Determination of genetic diversity among antibiotic-resistant *Morganella morganii* strains using RAPD molecular marks.
15. Nadarasah G, Stavrinides J. Quantitative evaluation of the host-colonizing capabilities of the enteric bacterium *Pantoea* using plant and insect hosts. *Microbiology*. 2014;160(3):602–15.
16. Mullally CA, Fahriani M, Mowlaboccus S, Coombs GW. Non-*faecium* non-*faecalis* enterococci: a review of clinical manifestations, virulence factors, and

antimicrobial resistance. *Clin Microbiol Rev*. 2024;e00121-23.
17. Loncaric I, Heigl H, Licek E, Moosbeckhofer R, Busse H-J, Rosengarten R. Typing of *Pantoea agglomerans* isolated from colonies of honey bees (*Apis mellifera*) and culturability of selected strains from honey. *Apidologie*. 2009;40(1):40–54.
18. Gibas C, Jambeck P. *Developing bioinformatics computer skills*. O'Reilly Media, Inc.; 2001.
19. Michelim L, Muller G, Zacaria J, Delamare APL, Costa SOP da, Echeverrigaray S. Comparison of PCR-based molecular markers for the characterization of *Proteus mirabilis* clinical isolates. *Braz J Infect Dis*. 2008;12:423–9.
20. Wong H-C, Lin C-H. Evaluation of typing of *Vibrio parahaemolyticus* by three PCR methods using specific primers. *J Clin Microbiol*. 2001;39(12):4233–40.