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RESEARCH ARTICLE

Different Molecular Methods to Determine Genetic Diversity in *Pseudomonas aeruginosa* Clinical Isolates

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Abstract

A total of (210) clinical specimens were collected from patients suffering from different infections such as burns, wounds, ear infections, diabetic foot, and urinary infections, cultured on differential media. All clinical specimens were cultured on different media. The results indicated that 22 (10 %) of the isolates were belong to *P. aeruginosa*. It was found that six isolates (27.27%) were isolated from wounds, five isolates (22.72%) from burns, six isolates (27.27%) from ear infections, two isolate (9%) from urine and three isolates (13.63%) from diabetic foot infections. In this study, used RAPD, BOX, and ERIC-PCR for the detection of phylogenetic diversity of *pseudomonas aeruginosa* isolated from different samples, In general, 18 isolates characterized using the three molecular techniques had comparable number of bands with some degree of polymorphism. *P. aeruginosa* isolates from the same source were clustered in to different groups. The three molecular techniques generated 2 main clusters, and the results of dendrogram of these techniques reveals that two isolates number(11,12) were similarity 100% in three methods techniques this suggest that this give the more discrimination power. While the pattern of others recorded some difference in the distribution.

Keywords: *Genotyping, Phenotyping, Pseudomonas aeruginosa, Molecular detection, RAPD, BOX, ERIC.*

Introduction

Pseudomonas aeruginosa is a common gram negative, aerobic, rod shaped bacterium that is widely spread in the soil, water, plants, animals, and humans, where it is exceptionally encountered as part of the normal temporary flora. This opportunistic pathogen of humans causes serious nosocomial infections in burns, wounds, and cystic fibrosis and contributes significantly to morbidity and mortality [1]. It has multiple pathogenicity factors that are used to overcome host defenses. Pathogenesis in *P. aeruginosa* facilitates adhesion, modulate or disrupt host cell pathways, and target the extracellular matrix. *P. aeruginosa* can form biofilms to protects it from antibiotics and the host immune system [2].

The genome of *P. aeruginosa* is large (> 6 Mbp) and exhibits a mosaic structure composed of a large core genome into which accessory genes are inserted *en bloc* at specific sites, called region of genomic plasticity (RGP) [3]. This universal distribution of Pseudomonas members suggests an amazing degree of genetic diversity and adaptability [4, 5]. Several typing methods have been developed to survey the genetic diversity and the spread of *P. aeruginosa* [6]. Each method has its own advantages and disadvantages. Due to the plasticity of the *P. aeruginosa* additional genome [7].Random Amplified Polymorphic DNA (RAPD) -PCR technique as a useful tool for investigation of the genetic variation among *P. aeruginosa* strains. RAPD-PCR has received considerable attention in recent years for epidemiological studies, due to its simplicity, rapidity, sensitivity, reproducibility, low cost [8]. BOX elements are mosaic repetitive elements comprised of different combinations of three subunit sequences.

These three subunit sequences are *box A*, *box B*, and *box C* which are 59, 45, and 50 nucleotides long, respectively. Meanwhile, ERIC sequences are 126 bp long with a highly conserved central inverted repeat. It is situated in noncoding transcribed regions of

the chromosome. Briefly, the BOX primer anneals on the *boxA* subunit of BOX elements whereas the ERIC primer synthesizes DNA sequences outward from inverted repeats [9]. Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR is a proper technique for DNA typing characterized as high type ability, stability, and rapid reversal reproducibility with low complication and price, which can be used to screen, discriminate, and determine genetic relatedness among the strains with the same accuracy of pulsed-field gel electrophoresis PFGE. This method is a common technique in which the intergenic regions of target repetitive non-coding sequences in the genome of bacteria are represented [10].

Materials and Methods

Clinical Specimens

The specimens were obtained from different sites of infections (burns, wounds, ear, diabetic foot, and urine); each swab was taken carefully from the sites of infections and transfer to the laboratory of microbiology /college of medicine. Urine (mid-stream urine) was collected from patients suffering from UTIs in sterile screw-cap container. Swabs from burn, wound, ear were collected from patients before they take any antibiotics or cleaning and swab from diabetic foot patients who diagnosis depended on physician then the swab collect before cleaning. Each specimen was inoculated on selective media and identified by biochemical reaction according to the diagnostic procedures recommended in [11].

DNA Extraction

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company (Gene aid, UK).

Primer Sequences

The primer sequences and PCR conditions that were used are listed in Table (1).

Table 1: The primer sequences and PCR conditions

Genes Name	Primer sequence (5´- 3´)	product Size bp	PCR condition	Reference
ERIC-1	Sense – **CAC TTA GGG GTC CTC GAA TGT A** Antisense – **AAG TAA GTG ACT GGG GTG AGC G**	**110-1535 bp**	95 C^0,3 min. 94 C^0, 1 min. 52 C^0, 1 min. 72 C^0, 2 min. 35 cycles 72 C^0 , 5 min.	[12]
BOX	Sense – **CTA CGG CAA GGC GAC GCT GAC G**	**200-1550 bp**	94 C^0, 1 min. 94 C^0, 5 min. 48 C^0, 2 min. 72 C^0, 2 min. 35 cycles 72 C^0 , 5 min.	[13]
RAPD	Sense – **AGC AGG CCA A**	**500-1500 bp**	94 C^0, 4 min. 94 C^0, 1 min. 36 C^0, 1 min. 72 C^0, 2 min. 30 cycles 72 C^0 , 10 min.	[14]

Results

Isolation of *P. aeruginosa* from Clinical Samples

A total of 22 strains of *P. aeruginosa*, were originally isolated from a variety of clinical specimens: urine (2), wound swab (6), ear swab (6), swab from burn (5) and from diabetic foot (3). The strains were identified as *P. aeruginosa* on the basis of typical morphology by gram-negative staining, a positive oxidase and catalase reaction, growth at 42 C^0, growth on Cetrimide agar and other conventional biochemical tests.

Detection of Phylogenetic Diversity of *Pseudomonas aeruginosa* Isolated from different Samples by RAPD-PCR

A total of 22 amplified DNA fragments ranging in size from 300-2000 bp detect by

using one random primer (primer 272). The primer reveals (0-10) fragment with size ranging from (300-2000 bp) among the isolates, it was found that two isolates formed no band, three isolates provide 6 bands, two isolates provided 7 bands, three isolates formed 10 bands, four isolates formed 8 bands, and three isolates formed 9 bands as show in Figure (1).

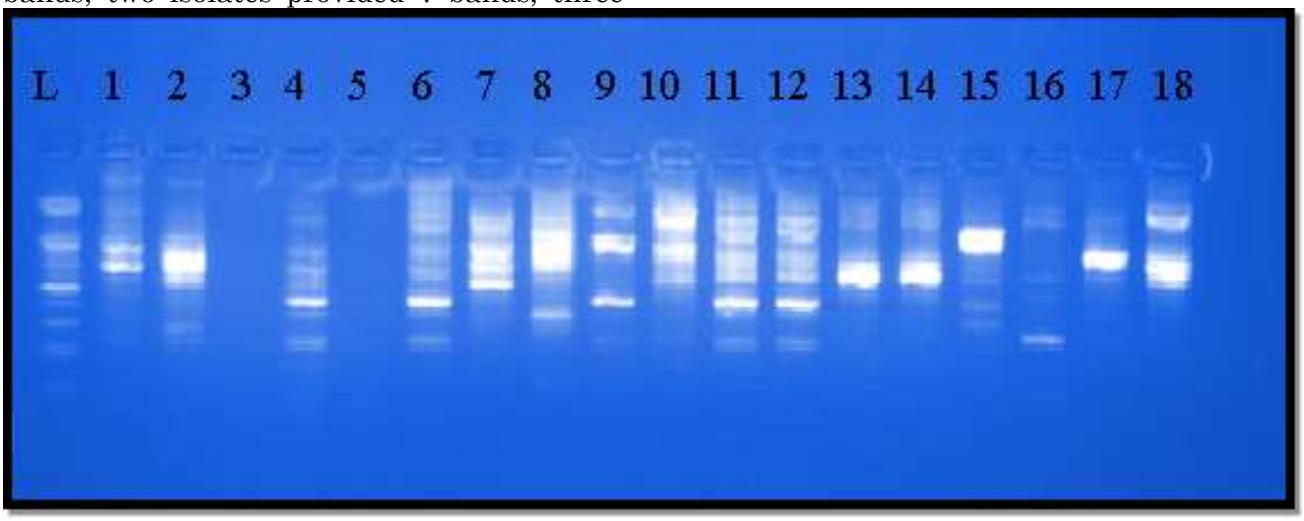

Figure 1: Agarose gel electrophoresis at 70 volt for 50 min. for RAPD gene in *P.aeruginosa* . PCR product visualized under U.V light at 280nm. After staining with ethidium bromide. The primer reveals (0-10) fragment with size ranging from (300-2000 bp)

The results of RAPD analysis revealed that the amplified products of most isolates vary in the molecular size patterns even with equal total fragment. Dendrogram analysis was constructed on the basis of the averaged similarity with the use of (UPGMA) has been used to show the phylogeny occurrence of strains. According to the dendrogram, the (18) strains of *p. aeruginosa* were distributed into two cluster as (A and B), where cluster A included two sub clusters and contain (16) isolates which divided into two branches, the first branch contain (15) isolates in which isolates (11 and 12), and isolates (13, 14) show 100% similarity. The second branches contain one isolate. While the cluster (B) contain two isolates, isolates number (3,5) show 100% similarity, as shown in Figure (2). In this study, the RAPD primer showed DNA polymorphism among *Pseudomonas aeruginosa* isolates from different clinical sample, either in the occurrence of amplified fragment or in the variable genetic similarities of each isolates with the other. The diversity could be due to the fact that they all were obtained from different sources, or due to the genetic instability of pseudomonas.

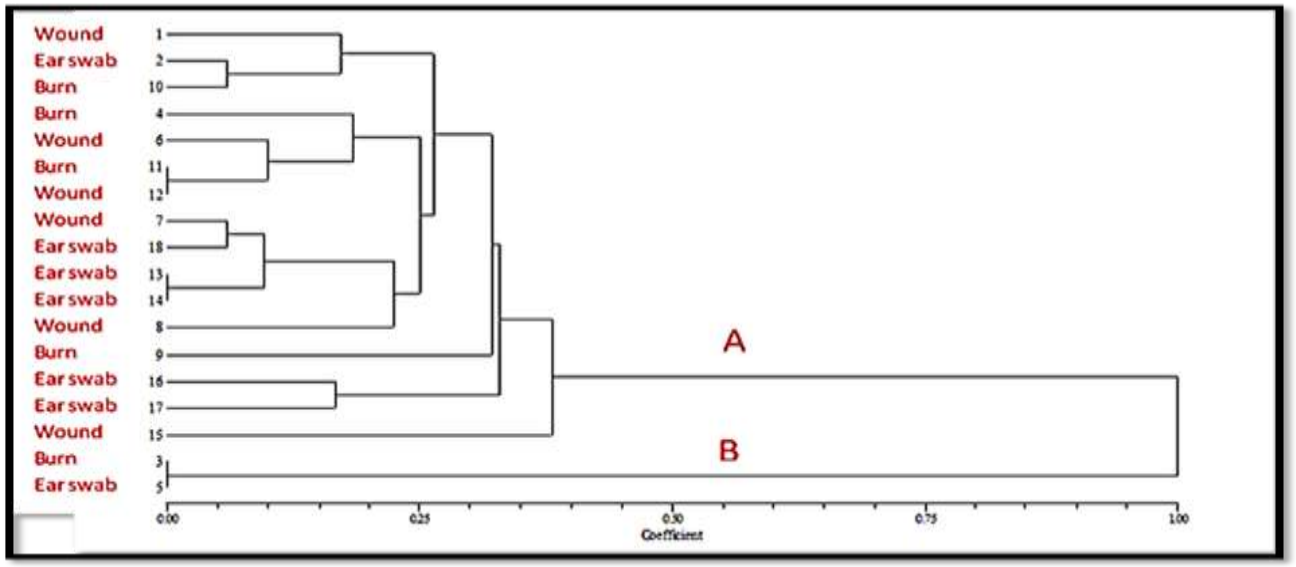

Figure 2: Dendrogram of Pseudomonas aeruginosa fingerprint by cluster analysis based on (SPSS) cluster analysis program using (RAPD) primer

Pethannan *et al* [15]. Refer that the use of molecular markers to study genetic diversity will help in characterizing the pseudomonas spp. Isolated from clinical samples, the cluster analysis and phylogenetic tree depending on genetic distance reveal the genetics relationship between the *P. aeruginosa* isolates. Mahmmudi *et al* [16] shows that RAPD-PCR markers are powerful and effective techniques in identify and initial screening of samples, examining the differences among species and identify at the level of strain as well as compared with biochemical methods need to spend less time and lower coast.

Detection of Box-element Fingerprint in *P. aeruginosa*

The Box genomic fingerprinting methods, was assessed for differentiation of 18 clinical Pseudomonas aeruginosa isolates under study.

Based on the results of cluster and discriminate function analysis, the length, sequence and position of these sequences in the genome are variable and often unique for each isolates. By using Box primer for Pseudomonas aeruginosa performed different DNA patterns generated with 9 amplicons that gave a polymorphic bands varied in size from 650-2000 bp. The result also shows that the DNA patterns from bands ranges from (0-9) band, and one isolate form no bands, and the isolates number (11,12) and (7, 17) have the same number and molecular size patterns, while sources of isolation was different (isolate 11 from burn and isolate 12 from wound) (isolate 7 from wound while 17 from ear swab). As shown in Figure (3).

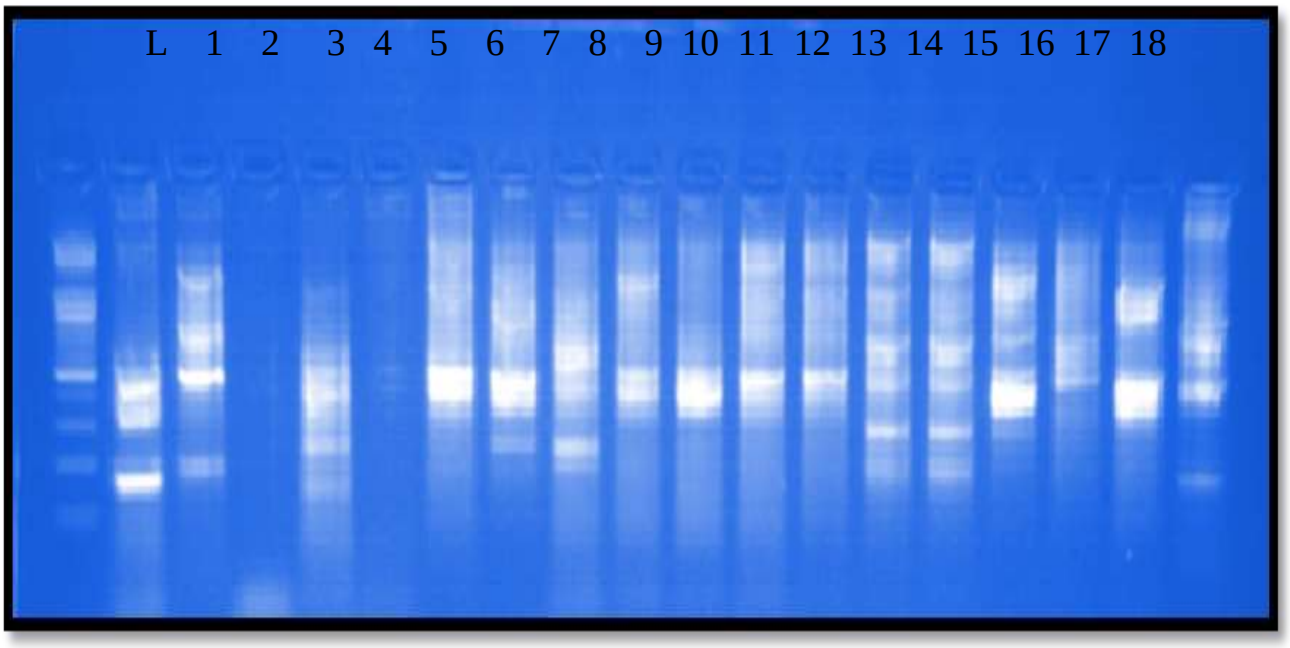

Figure 3: Agarose gel electrophoresis at 70 volt for 50 min. for Box gene in *P.aeruginosa* . PCR product visualized under U.V light at 280nm. After staining with ethidium bromide. The primer reveals (0-9) fragment with size ranging from (650-2000) bp

The data from Box Dendrogram of *p. aeruginosa* isolates were grouped into two main groups; Clusters (A and B) group A contain (17) isolates, while Cluster B contain one isolate. As shown in Figure (4) and it was showed that 100% similarity were observed in isolates (11 and 12) and (7 and 17).The isolate from the same isolated site over slightly genetic variation. As shown in Cluster A which subs grouped into six genotypes. However, the detection of Box in *p. aeruginosa* appears in different pattern of distribution as conserved sequences in bacterial genome associated with gene recombination at specific loci. Wolska *et al* [17]. Found that Box-PCR finger printing revealed 38 genetic patterns, among them 7 main genotypes, ranging from (200-400 bp). Most of the Box sequences were encounter in close proximity to genes, suggesting then potential role as a regulatory elements controlling coordinate virulence or competence- related gene expression, Box elements are key element in adaptive bacterial evolution [18].

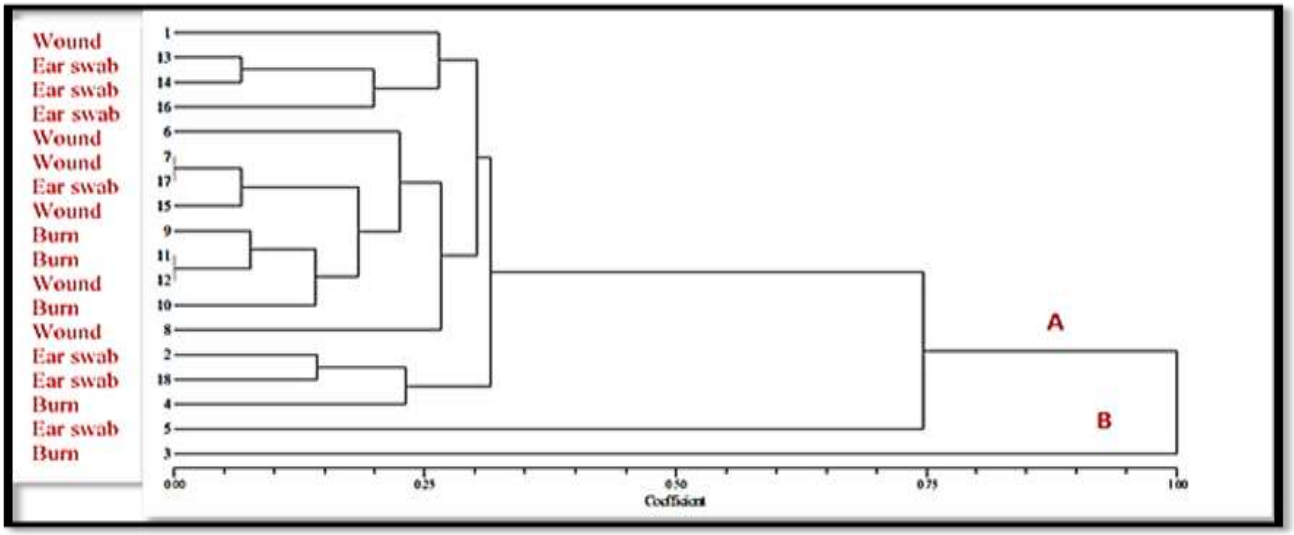

Figure 4: Dendrogram of *Pseudomonas aeruginosa* fingerprint by cluster analysis based on (SPSS) cluster analysis program using (BOX) primer

Enterobacterial Repetitive Intergenic Consensus (ERIC) DNA fingerprint Analysis

ERIC-PCR fingerprinting revealed (18) genetic patterns Figure (5).

The clusters were shown in (0-7) band between (400-2000) bp. The most characteristic products of PCR for *p. aeruginosa* were (1600-1200-1000-825), one isolate have no bands.

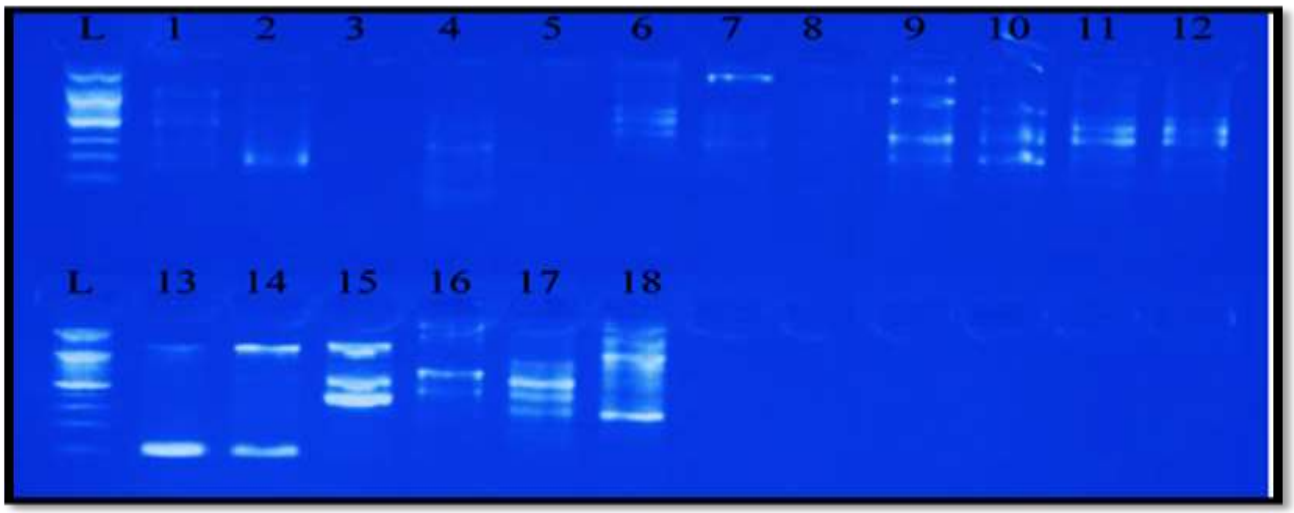

Figure 5: Agarose gel electrophoresis at 70 volt for 50 min for ERIC gene in *P.aeruginosa*. PCR product visualized under U.V light at 280nm. After staining with ethidium bromide. The clusters were shown in (0-7) band between (400-2000) bp.

ERIC-PCR typing revealed 13 main genotypes. The dendrogram results show to main clusters (A and B), were cluster A contain three isolates which are all identical 100% isolates number (3, 5, 8) as shown in Figure (6) while cluster B contain two sub cluster, one sub cluster contain two isolates which are given similarity 100% isolates number (13,14). Other sub cluster contain (13) isolates where isolates number (6, 16) are similar 100% and isolates number (11, 12) also give 100%. It was also observed that the same genetic types of *P. aeruginosa* were isolated from different sites of infection, so this was possible to postulate that the same clone was present in the environment which probably epidemiologically are related [19]. Referred that the isolates of *P. aeruginosa* revealed (11) genotypes containing 2 to 8 isolates. Also demonstrate that the sufficient discriminating power of ERIC-PCR for the investigation of clinical *P. aeruginosa* isolates. Also ERIC-PCR. Can provide more discriminative DNA patterns of bacterial source and was able to show species specific comparison for other type methods [20].Observed that ERIC-PCR typing for *P. aeruginosa* revealed (17) groups of genotypes with (25-92%) similarity and the PCR product were between 100-1200 bp, and found that two isolates were non typed by ERIC-PCR.

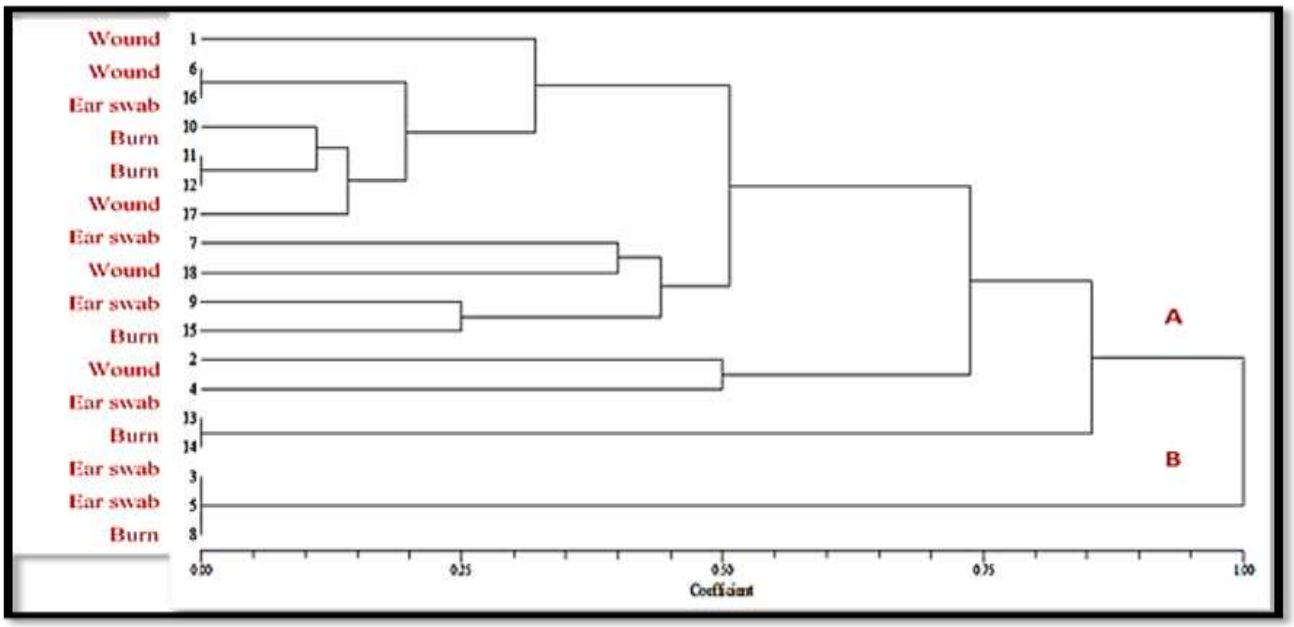

Figure 6: Dendrogram of *Pseudomonas aeruginosa* fingerprint by cluster analysis based on (SPSS) cluster analysis program using (ERIC) primer

Conclusions

From this study, we can concluded that RAPD, BOX, ERIC analysis appears to be a valuable tool in epidemiological, surveillance and for investigation the distribution of types in environment and human. It was observed that high degree of heterogeneity existed among *P. aeruginosa* isolates. Among all currently used methods, ERIC-PCR turned out to be powerful tool for the study of clinical *P. aeruginosa* isolates diversity.

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