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ERIC - PCR is an adequate tool for typing of clinical *Pseudomonas aeruginosa* isolates

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Abstract
In this study, the ERIC - PCR fingerprinting technique was evaluated for the discrimination of clinical Pseudomonas aeruginosa isolates. All isolates except 4 were type able and some isolates showed unique banding patterns. According to our results, ERIC-PCR fingerprinting is applicable for typing of Pseudomonas aeruginosa isolates and can be considered a useful complementary tool for epidemiological studies of members of this genus.
Key words: *Pseudomonas aeruginosa, bacterial diversity, DNA typing method, ERIC -PCR.*

Introduction *Pseudomonas aeruginosa* is an organism commonly occurring in soil, water, plants, animals and humans.Normally, *Pseudomonas aeruginosa* resides in the intestinal tract of a rather small percentage of healthy individuals. It is found sporadically in moist areas of the human skin and in the saliva. It can multiply in almost any moist environment and has minimal nutritional requirements. Moreover, it is tolerant to a wide variety of physical conditions. Consequently, the microorganism can be found frequently in the hospital environment and home reservoirs such as sinks, floors, baths, soapdishes and dishcloths. *Pseudomonasaeruginosa*is considered an opportunistic pathogen as it causes hospital-acquired infection, particularly in immunocompromised hosts, burn victims or in those withfaulty homeostasis mechanisms or metabolic disorders (Hauser and sriram , 2005).
*Pseudomonas aeruginosa*can be internally divided into subgroups by classical methods such as biotyping, serotyping, pyocin typing and phage typing.However, the discriminatory power is much lower than that obtained by molecular typing methods. DNA typing methods have been frequently used forinvestigating the diversity of collections of *Pseudomonas aeruginosa* (Spreet ,2002). These methods include pulsed-field gel electrophoresis (PFGE), (Syrmis*et al*.,2004) Ribotyping (Dawson *et al*., 2002).
Restriction fragment length polymorphicDNA analysis (RFLP) (Dawson *et al*., 2002).
Random amplified polymorphic DNA assay (RAPD) (Speijer,1999).Arbitrary primed PCR (AP-PCR) (Liu *et al*.,1996), amplified fragment length polymorphism (AFLP) (Speijer,1999).and repetitive element based PCR (rep-PCR) (Syrmis*et al*.,2004).Rep-PCR is a method for fingerprinting bacterial genomes, which examines strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes. Three main sets of repetitive elements are used for typing purposes: the repetitive extragenic palindromic (REP) sequence, the enterobacterial repetitive intergenic consensus sequence (ERIC) and the BOX elements. REP-PCR and ERIC-PCR have been shown to be useful for typing *Pseudomonas aeruginosa*isolates (Olive et al., 1999). In this work, we used the ERIC-PCR method for the typing of clinical *Pseudomonas aeruginosa* isolates.

Material and Methods
Bacterial isolates. Sixty isolates of *Pseudomonas aeruginosa* were originally isolated from a variety of clinical specimens:burn (22);wounds (18); sputum (9) ; blood (6); respiratory tract infection (2); and from ear (3). The bacteria were obtained from patients from different wards of the Municipal Hospital, Main Hospital and the outpatients' department in Iraq between July 2013 till Desemper 2013. The strains were identified as *Pseudomonas aeruginosa*according to biochemical patterns in the Api 20NE system (bio Merieux). The control strain NCTC 6749 was also examined. Stock cultures were stored in TSB (tripticase soy broth, Difco) containing 20% glycerol at –80°C.

DNA Extraction: All isolates were grown on nutrient broth for 24 h at 37°C and checked for purity on nutrient agar plates. Approximately two loops worth of biomass were scraped off the agar plates, suspended in 100 µl of sterile distilled water, and boiled for 10 min. After centrifugation at 12,000×*g* for10 min at 4°C, the supernatants were recovered and 5µl was directly used as the template for PCR (Clementino *et al*.,2001).

Application of PCR: In order to confirm the isolates as*P. aeruginosa*, PCR assay that based onhousekeeping gene *(rpsL* gene) sequence with specific primers as described by (Xavier *et al*. 2010) was carried out in 25 µLreaction

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volumes composed from 12.5 µl of GoTaq®Green Master Mix, template DNA 5µl ,forward & reverse primers (1.5 µl for each),and 4.5of Deionized Nuclease–Free water was added to PCR mixture to get final volume of 25 µl. PCR mixture without template DNA was used as a negative control. PCR was run under the following conditions : primary denaturation step at 95°C for 5min, 30 repeated cycles start with denaturation step at 94°C for 30sec, annealing at 57°C for 30sec, and 1 min at 72°C as extension step followed by final extension step at 72°C for 7 min .

DNA amplification by ERIC-PCR: The forward primer (5'-ATGTAAGCTCCTGGGGATTCAC-3')andreverseprimer (5'AAGTAAGTGACTGGGGTGAGCG-3') were used to amplify repetitive sequences present in the chromosomal DNA of *Peudomonasaeruginosa*isolates . ERIC-PCR was carried in 25 µl, volume comprising of 100 ng of *Peudomonasaeruginosa* DNA, 1.5 µl (10 pmol) of each primer and 12.5 µlMastermix . Filtered water was added to the mixture to make a final volume of 25 µl. Reactions were carried out using a programmable thermocycler according to the following thermocycling conditions: 95^{o}C for 2 minutes, 35 cycles of 40 seconds at 95^{o}C, 51^{o}C for 1 minute, 72^{o}C for 8 minutes and final step of 72^{o}C for 16 minutes. Each set of PCR amplification was performed with negative control containing distilled water and a positive control containing *Peudomonasaeruginosa* DNA isolated from a reference strain.

Gel electrophoresis: After PCR, 2 µl aliquot of 6x loading buffer (0.1% bromophenol blue, 50% glycerol) was mixed with 10 µl of each of the PCR solution and the mixtures were resolved by horizontal electrophoresis on a 1.5% agarose gel containing 0.5 µg/µlethidium bromide in 1x TBE buffer (40 mMTris-borate, 1 mM EDTA, pH 8.0). DNA size markers of 100 bp and 1kb were used. The gels were photographed after electrophoresis under UV light to record results. Reproducibility was monitored by comparing the results of repeated ERIC-PCR carried out with the same strains.

Dendrogram construction and genetic relatedness: Dendrogram for cluster analysis of all the isolates were subjected to evaluation. The dendrogram was constructed on the basis of the banding pattern produced by ERIC-PCR. A binary table or a haplotype matrix for each strain was constructed by linearly composing presence (1)/absence (0) data derived from analysis of the gel/ antibiogram was subjected for statistical analysis by Dice coefficient values.

STATISTICA: This enabled the plotting of dendrogram showing the level of genetic similarity among the strains.

Results and Discussion various molecular techniques have been developed for typing strains of *Pseudomonas aeruginosa* (Dawson *et al*, 2002).Pulse field gel electrophoresis (PFGE) is recognizedas the gold standard typing method for *Pseudomonas aeruginosa*. However, it is labor intensive and limited by high cost and extended turnaround time (Syrmis*et al*.,2004). In contrast to PFGE, the highly specific rep-PCR technique is an easy procedure, requiring little time for results, and is characterized by low labor costs (Olive *et al*.,1999).Rep-PCR with primers based on REP and ERIC sequences has been successfully used to differentiate of *Bartonella* spp., *Bacillus subtilis*, *Citrobacte*r *diversus*, *Enterobacteraerogenes*, *Salmonella enteric* Typhi , methicillin - resistant *Staphylococcus aureus* , *Streptococcus pneumonia* , *Acinetobacter baumanii* , *Burkholderia cepacia* , *Burkholderiap seudomallei*, *Pseudomonas aeruginosa* , *Legionella pneumophilia*, *Helicobacter pylori*, *Neisseria gonorrhoeae* and *Neisseriameningitidis*. While REP and ERIC sequences are the most commonly used targets for DNA typing, another repetitive element, the BOX sequence, has been used to differentiate *Streptococcus pneumoniae*(Syrmis*et al*.,2004). Recently, the BOX-PCR method was used for typing strains of different genera of *Pseudomonas* (*Pseudomonasaeruginosa*, *Pseudomonas syringae*, *Pseudomonas putida*and *Pseudomonas fluorescens*) (Marques *et al*.; 2008).In the present study, the ERIC primer sequence was used in PCR to detect differences in the number and distribution of this bacterial repetitive sequence in the clinical isolates *Pseudomonas aeruginosa*genomes. The detection of ERIC sequences by PCR produced 10 DNA fragments ranging from 370 bp to2900 bp with fragments of 370 bp,400bp ,500 bp , 720 bp , 800 bp , 1000 bp ,1800 bp , 2000 bp ,2100bp , 2900 bp found in 31.66 %, 60 %, 35 %, 30 % , 18.33 % ,1.66 % , 11.66 % , 6.66 % , 16.66 % , 41.66 % respectively with 4 isolates untypeable this results partially agree with Mansour *et al*.(2013) who shown*P.aeruginosa* isolates were typeable by ERIC-PCR and yielded 15and 12 ERIC patterns from Egypt and Saudi Arabia respectively, with 4 to 11 bands in Egypt and 3 to 9 bands in Saudi Arabia and the size of amplified DNA bands ranged from 110 bp to 1535 bp of the isolates, respectively.Thedendrogram of similarity obtained by ERIC-PCR demonstrated the existence of two main clusters (A-B) cluster B predominated and contained 44 (73,3%) isolates with (1-3) same bands 36 of the isolates had 100% similarity , forming 10 real clones with 8 unique pattern cluster A contain 16 (26.6%) isolates with (2-5) same bands 11 of the isolates had 100% similarity forming 4 real clones with 5 unique pattern at 100% similarity 14clones generated and 13 unique pattern we also verified that the same patient could either have genomically distinct isolates located in either the same cluster (P23,P24 & P12,P13) or different clusters (P14, P15, P17,P18, P19, P20, P21,P22 , P26 ,P27 ,P30 , P31, P38, P39) observations suggest that some of the patients were colonized by either the same or different isolates along the time , as previously described by Sener *et al*. (2001) . In our study, in majority of cases multiple isolates of *P.aeruginosa*recovered from a patient were more likely to be unrelated than related.This showed the presence of different types in the same patient .This disagree with Ghazi *et al*.(2012) who showed the presence of same clonal types in the same patient while Mansour *et al*.(2013) confirm sharing of certain ERIC types between patient strains may be explained by horizontal transmission from patient to patient ,probably from the hands of health care workers or environmental sources.

The present result confirm the isolates collected from same hospital were more likely to be related than unrelated so transmission of *P. aeruginosa* frompatient-to-patient can lead to spread of clonal strains in other wards of the hospital.Sonosocomial transmission can be prevented with simple hygienic measures,elimination of contaminated material.Care of cleanness and segregation of patients, ERIC-PCR technique represent useful technique for the epidemiological typing of nosocomial bacteria because of their simplicity and speed compared with other molecular technique.The results were similar tothe results of Syrmis*et al*. (2004) suggest that ERIC-PCR are suitable , inexpensive , fast , reproducible and discriminatory DNA typing tools for effective epidemiological surveillance of potential transmissible *P. aeruginosa* isolates between patient .This result comes similar to the mentioned Kidd *et al* . (2011) the ERIC – PCR technique capture a genome - wide perspective on diversity,with differences between strains arising from a potentially wide range of genetic changes, from point mutations through large – scale inter - and intragenomicrecombinational events , although the specific causes of any differences can only be presumed. In the same manner this study depending upon only Eric typing methodwithout synchronize with another typing methods.In this regard,Johnson *et al*. (2007) showed ERIC-PCR and PFGE analyses showed the strongest correlation, suggesting that using both techniques together may be redundant.

Table (1): Molecular weight and percentage value of the bands obtained in ERIC-PCR

Band No.	Molecular weight	Percentage value
ERIC1	370	31.66%
ERIC2	400	60%
ERIC3	500	35%
ERIC4	660	30%
ERIC5	800	18.33%
ERIC6	1000	1.66%
ERIC7	1800	11.66%
ERIC8	2000	6.66%
ERIC9	2100	16.66%
ERIC10	2900	41.66%

In the present study ERIC – PCR based fingerprinting methods were used to investigate the genetic diversity of *P.aeruginosa* isolates from clinical specimens.The isolates were genetically diverse. 14 major ERIC - PCR clonal groups,and 13 unique genotypes were obtained fig (2).The highly heterogeneous strains were isolated from wound. Liu *et al*.(1996) ; Dawson *et al*. (2002) &Syrmis*et al*.(2004) demonstrated sufficient discriminatory power of ERIC PCR for the investigation of clinical *P. aeruginosa*strains.The results of the study of Syrmis*et al*. (2004) showed that ERIC PCR identified six major clonal groups and 58 distinct clonal groups among 163 *P. aeruginsa* strains isolated from patients with cystic fibrosis.while Liu *et al*. (1996) by using three methods RAPD, ERIC PCR and 16S–23S spacer region based RAPD indicated that the 47 isolates of *P.aerugnosa* that had caused bacteraemia in 19 cancer patients were indistinquishable. 17 other isolates that had caused bacteraemia in 10 cancer patients were discrimnated into eight further groups . Twenty four environmental and non-cancer patient isolates were grouped into further distinct group..

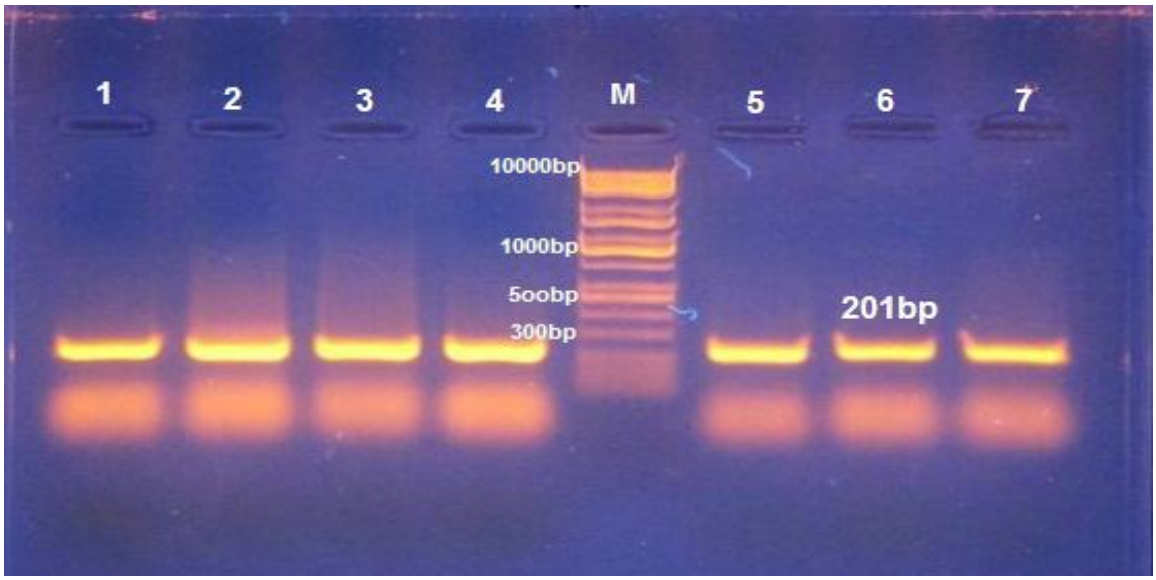

Figure (1): Agarose gel electrophoresis (1%agarose, 7 V/cm^{2}for 60min) of *rpsL*gene (201bp amplicon). Lane M 100bp DNA ladder, lanes(1-7 represent of bands.

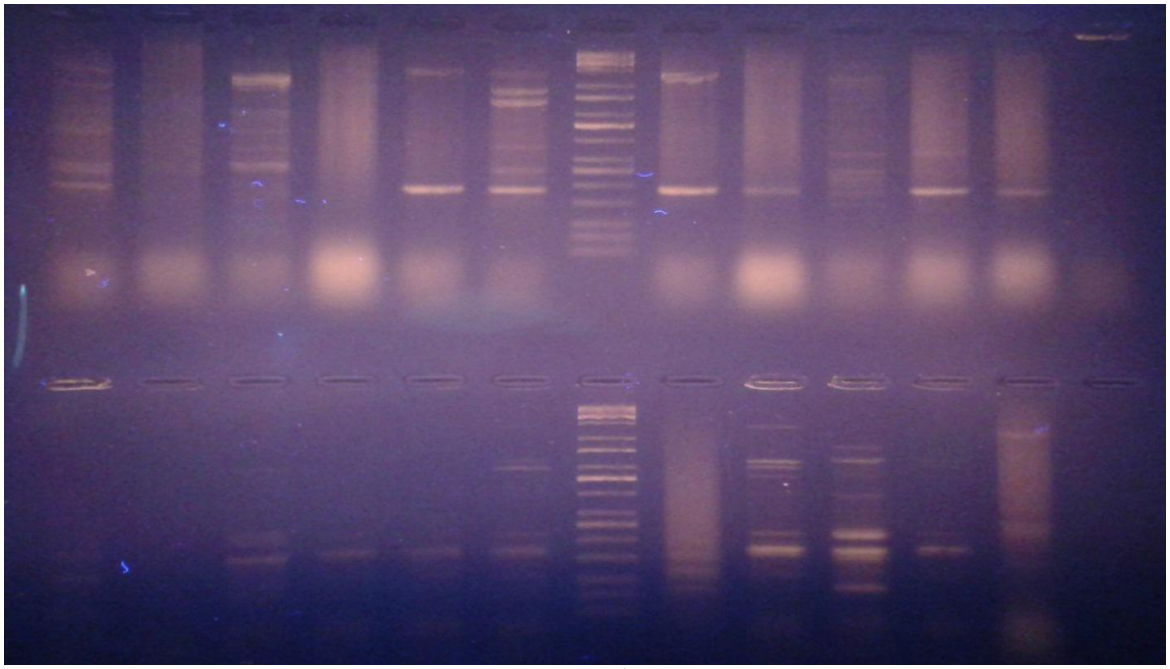

Figure (2):Agarose gel electrophoresis (1% agarose,7 V/cm^2 for 60 min) of ERIC-PCR Lane M 100bp DNA ladder, lanes 1- 12 represent of bands.

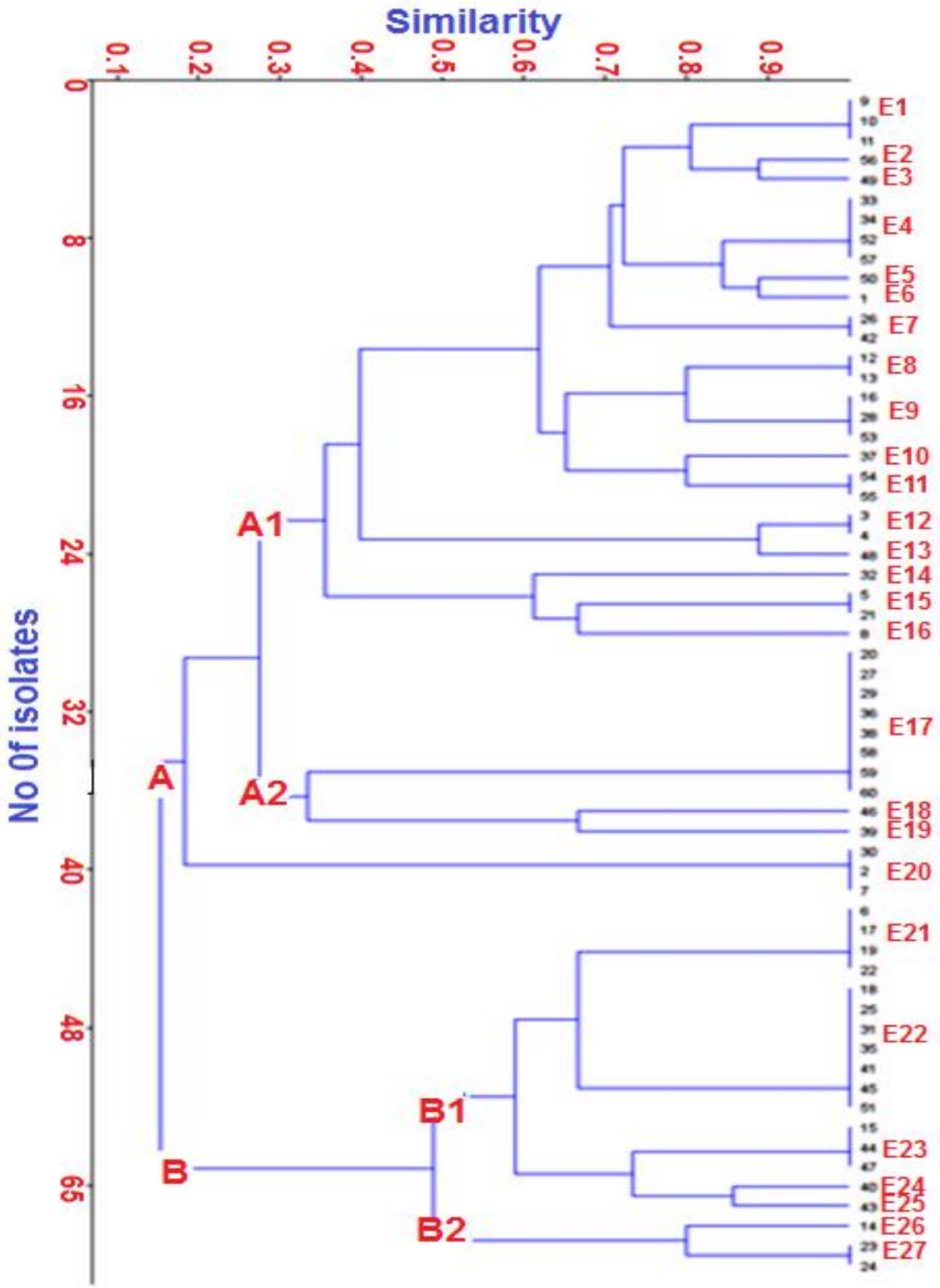

Figure (3-9): Dendrogram obtained from Eric –PCR data

***E is referred to cluster of Eric**

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