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Molecular Identification of *Nocardia* Strains from the Soil by *hsp65* Gene: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (RFLP) and Analysis of Sequence 16S rRNA Gene

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Abstract

Background: *Nocardia* species are nocardiosis agents particularly in immune disorder patients with the clinical manifestations including fatigue, malaise, weight loss, cough, and dyspnoea. Due to time - consuming bacterial culture and insufficient phenotypic tests alone, rapid molecular assays for the diagnosis of *Nocardia* are essential. The primary objective of this study was the analysis of the polymerase chain reaction - restriction fragment length polymorphism (PCR - RFLP) and PCR sequencing of 16S rRNA gene for the identification and differentiation of *Nocardia* species.

Methods: 32 *Nocardia* isolates were identified by single digestion of 441 - nt fragments of the 65 - heat - shock protein gene with endonucleases *BstEII*, *Msp*I, and *Hin*fI and sequencing of 16S rRNA gene. These isolates were collected from the soil of various geographical regions of Iran by paraffin baiting technique in our previous study.

Results: Some of the isolates had new patterns that were not identified by PCR - RFLP of *hsp65* gene in literature; therefore, they were identified by sequence analysis of 16S rRNA gene. Six strains of *Nocardia* with new DNA banding patterns in PCR - RFLP of *hsp65* (A and B) could not be identified by their *hsp65* restriction - endonucleases fragment patterns. Strain En49 after analysis of sequencing data was recognized as *Nocardia coubleae* , which its PCR - RFLP profile of *hsp65* has not been reported in the literature so far.

Conclusions: The PCR - RFLP - *hsp65* is faster than other sequencing-based techniques, but species accurate identification in strains with new PCR - RFLP profiles is not possible. The 16S rRNA gene sequencing is a suitable method to determine the percent similarity and phylogenetic relationships of *Nocardia* species.

Keywords: *Nocardia*, RFLP, Soil, *hsp65*, 16S rRNA

1. Background

The genus *Nocardia* includes gram - positive, partially acid - fast, strictly aerobic, and filamentous bacteria of the family *Nocardia*ceae in the order of Actinomycetales (1-5). Members of the genus *Nocardia* comprise several species that can be found worldwide as saprophytic soil microorganisms (1, 2, 5). *Nocardia* species are the causes of a variety of infections in humans that include cutaneous, pulmonary, ocular, cerebral, and systemic nocardiosis (5) and human is infected through aerosols or scratch (5). Since the growth of *Nocardia* is very slow and it may be covered by normal microflora and other rapidly growing bacteria, the isolation can be difficult (6). Antibacterial therapy of *Nocardia* infections depends on the organ or site and correct diagnosis of *Nocardia* species (7, 8). However, regarding the numerous reports about *Nocardia* in Iran, rapid and accurate identification of *Nocardia* spp. is of importance. One hundred twenty - seven clinical isolates of *Nocardia* from different major cities of Iran were studied between 2009 and 2015. *Nocardia asteroides* was the most frequently recovered, followed by *Nocardia farcinica* and *Nocardia cyriacigeorgica* (9). Other reports have shown *N. cyriacigeorgica*, *N. asteroides* complex, *Nocardia nova* complex, *Nocardia otitidiscaviarum*, and *Nocardia transvalensis* in Iran (10-13). The prevalence of *Nocardia*l infections in another study was 5.28% (6). Recognition of *Nocardia* to species

level is important for the four reasons: definitive and accurate identification, predicting antimicrobial susceptibility, epidemiological purposes, and effective treatment (14-16). The identification of *Nocardia* used to be conducted via traditional methods (colonial and microscopic morphology and biochemical tests) (15, 17). The routine identification of *Nocardia* species is difficult (18). Due to time - consuming bacterial culture and insufficient phenotypic, the use of rapid, sensitive, and accurate molecular methods for accurate diagnosis of infections and bacterial species seems necessary (5, 14). Today, molecular techniques developed for accurate identification include PCR sequencing alone or with other molecular analyses such as PRA (PCR restriction enzyme pattern analysis) that is one of the first molecular techniques for the identification of *Nocardia* species (1, 19). The RFLP method is a PCR reaction based on restriction enzyme digestion. The use of molecular approaches such as PCR - RFLP has been the focus of recent investigations to distinguish the newer species of *Nocardia* isolates from other actinomycetes genera or *Mycobacteria* species (14, 20). In the current study, *hsp65* gene was used for the identification purpose by the RFLP technique. There are molecular methods relying on sequencing of one of the most important genes to determine the species. The 16S rRNA gene is valuable for the identification and taxonomic classification because this gene is a housekeeping and conserved gene that is in all bacteria. The fragment of 16S rRNA gene (1500bp) is a conserved and variable area that is used for taxonomic comparisons. The use of 16S rRNA gene sequencing in the clinical laboratory is beneficial for identifying unknown bacteria (21). In the current study, the polymerase chain reaction - restriction fragment length polymorphism (PCR - RFLP) of *hsp65* gene and sequencing of 16S rRNA gene analyses were performed for a definitive identification of *Nocardia* species of soil.

2. Methods

According to our previous study, soil samples were randomly collected from 4 cm deep from various geographical regions of Iran. The samples in sterile plates were transferred to actinomycetes lab within 24 - 48 hours. Strains of *Nocardia* were isolated by paraffin baiting technique. All isolates were identified to the species level by biochemical tests in our previous study (17, 22). Then, PCR - RFLP of the *hsp65* gene and sequencing of 16S rRNA gene were done for accurate identification of *Nocardia* isolates.

2.1. DNA Extraction

DNA extraction was done by boiling in STET solution. STET buffer contained Tris - HCl (10 mM), NaCl (0.1 M), EDTA (1 Mm), and Triton X100 (%5 (v/v)) for 100 ml STET "pH = 8". Briefly, the isolates of *Nocardia* were cultured on nutrient agar medium and incubated at 35°C for 5 days. A loop full of the pure colony of bacteria was suspended in 200 μl (microliters) of STET buffer and boiled at 100°C for 30 min and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred into a sterile micro tube, added by 500 ml cold ethanol, and incubated for 60 min at -20°C. After this stage, the microtube was centrifuged for 10 min at 13,000 rpm and the supernatant was discarded. DNA pellets were dried and dissolved in 50μl (microliters) sterile distilled water and stored at -20°C (23).

2.2. PCR - RFLP (Polymerase chain reaction - restriction fragment length polymorphism)

2.2.1. Amplification of a Portion of the hsp Gene

After DNA extraction, a 440 - bp fragment of the *hsp65* gene encoding the 65 - kDa heat shock protein from *Nocardia* strains was amplified by using two primers described by Telenti et al. (24). The following primers were used: a forward primer with the sequence 5' - ACCAACGATGGTGTG TCCAT - 3' (TB11); a reverse primer with the sequence 5' -CTTGTCGAACCG CATACCCT - 3' (TB12) (24). Reaction mixtures consisted of 2.5 U of Taq polymerase, 1.5 mM MgCl2 (50mM), 0.3mM each primer, 0.2 mM each deoxynucleoside, 1 × PCR buffer and 5ml of DNA extract. The PCR amplification was performed in a total volume of 50 ml. The temperature cycling for amplification was performed in the initial denaturation step (94°C for 5 min), 40 cycles of amplification (94°C, 55°C, and 75°C for 1 min at each temperature), and the final extension step (72°C for 10 min). The amplicons were electrophoresed on 1.5% agarose gel with ethidium bromide. We considered H37RV strain of *Mycobacterium tuberculosis* (manufacturer of the 441bp fragment) as a positive control at PCR reaction.

2.3. Restriction Fragment Length Polymorphism (RFLP)

After amplification, the PCR products were digested with *Hin*fI (Biolabs Co., England), *Bst*EII, and *Msp*I (Fermentas Co.) restriction enzymes separately (14, 18). Enzymatic digestion of PCR products was performed under appropriate conditions (according to the enzyme manufacturer instructions). All the three digest enzymes showed the highest activity at 37 °C. RFLP products were electrophoresed in 3% agarose gel (Invitrogen, USA) in 0.5 × TBE buffer (Tris - Borate - EDTA) at 50 V for 6 h, followed by staining with ethidium bromide. For determining the size of the fragments generated by PCR - RFLP, we used a 50bp molecular marker (Geneon - Germany).

2.4. Amplification of a Portion of the 16S rRNA Gene

The 1500 - bp fragment of the 16S rRNA gene was amplified using two universal primers including a primer with the sequence 5' - AGAGTTTGATCMTGGCTCAG - 3' (27F) and a primer with the sequence 5' - AAGGAGGTGWTCCARCC - 3' (1525R) (2). Reaction mixtures consisted of 2.5 U of Taq polymerase, 1.5 mM MgCl2 (50mM), 0.3mM each primer, 0.2 mM each deoxynucleoside, 1 × PCR buffer, and 5ml of extracted DNA. The temperature cycling for amplification was performed in the initial denaturation step (94°C for 5 min), 32 cycles of amplification (94°C for 5 min; 55°C for 1 min, and 75°C for 90 sec), and the final extension step (72°C for 10 min). PCR products were electrophoresed in 1.5% agarose gel with ethidium bromide.

2.5. Sequencing of the 1500 - Bp Fragment of the 16S rRNA Gene

The agarose gel containing 16S rRNA gene of band 1500 bp was cut and sent to Bioneer company (Korea) for sequencing.

2.6. Sequence Analysis of 16S rRNA Gene

The 16S rRNA gene sequences of *Nocardia* isolates were aligned with the sequences of *Nocardia* species (retrieved from GenBankTM database) using the jPhydit software according to instructions. The sequences of strains were analyzed through BLAST search whit the sequences of bacteria available in databanks of NCBI (25). The phylogenetic tree was plotted using MEGA 5 software (26).

3. Results

We present an analysis of 32 *Nocardia* isolates from soil in Iran using banding patterns produced by single digestions of 440 - nt fragments of the *hsp65* gene with endonucleases *Bst*EII, *Msp*I, and HinfI. The restriction digest is then separated and visualized by agarose gel electrophoresis (Figure 1). After PCR - RFLP analysis of the *hsp65* gene, four different RFLP profiles were revealed based on the fragments sizes, which were numbered from A to D. The restriction profiles of each isolate for each used enzyme are shown in Table 1. The RFLP profiles obtained in this study were compared with the previously known patterns by Rodriguez et al. (14). Thus, the profile *Bst*EII, 440; *Msp*I, 120/140/180; and *Hin*fI, 440 obtained in this study (pattern C in the table) determined a group of *Nocardia* spp. including *Nocardia abscessus*, *Nocardia araoensis*, *Nocardia asiatica*, *Nocardia asteroides* type IV, *N. asteroides* type IVATCC 14759, *Nocardia brevicatena*, *N. cyriacigeorgica*, *Nocardia higoensis*, *Nocardia neocaledoniensis*, *Nocardia paucivorans*, *Nocardia puris*, and *Nocardia vinacea* according to Rodriguez et al. (14). Since some of the *Nocardia* species have similar PCR-RFLP patterns, we performed PCR - RFLP analysis to reveal biochemical and morphological characteristics (17). After RFLP - PCR analysis, 9 isolates (En1, En2, En3, En5, En6, En20, En22, En35, and En57) as *N. asteroides* drug pattern type VI, and 14 isolates (En4, En9, En11, En19, En21, En23, En24, En26, En38, En40, En42, En48, En51, and En53) as *N. cyriacigeorgica*, and 1 isolate (En8) as *N. otitidiscaviarum* were identified. The results of PCR - RFLP of *hsp65* gene in all isolates were similar to the results of phenotypic tests and sequencing of 16S rRNA gene. The results of RFLP and sequence analysis of these isolates are presented in Table 2. Isolate En56 by sequence analysis of 16S rRNA was determined as *N. neocaledoniensis* while, based on its *hsp65* - RFLP pattern, it was identified as *N. asteroides* (14). In some strains, 440 bp fragment was not digested by *Bst*EII enzyme, whereas others were digested into two fragments with 130bp and 310bp size range (Figure 2A); these fragments have not been reported so far. It is noteworthy that after PCR - RFLP of PCR products, only the enzyme *Bst*EII created new fragments. Finally, in these isolates, two PCR - RFLP profiles (new pattern A and B) were detected by a combination of *Bst*EII, *Msp*I, and *Hin*fI digest analysis. A new pattern A (*Bst*EII, 130/310; *Msp*I, 120/140/180; *Hin*fI, 190/250) in isolate 28 and a new pattern B (*Bst*EII, 130/310; *Msp*I, 70/110/120/140; *Hin*fI, 190/250) in isolates En39, En43, En46, En47, and En49 were reported. For accurate identification, these isolates underwent 16S rRNA gene sequencing. These isolates after sequence analysis of 16S rRNA gene were diagnosed as *N. otitidiscaviarum*, *N. soli, Nocardia cummidelens*, and *N. coubleae* (See Table 2). The new restriction patterns were added to the previously published patterns. The phylogenetic tree of the 16S rRNA gene of our isolates was constructed using the neighbor - joining algorithm with bootstrap analysis for 1000 replicates in the MEGA 5 software (Figure 3).

4. Discussion

The use of molecular methods for identification of *Nocardia* is based on restriction endonuclease digestion, nucleic acid amplification, or nucleotide sequencing techniques. For accurate diagnosis of *Nocardia* spp. due to their diversity, we cannot rely on using phenotypic or genotypic methods alone (27). A combination of molecular and phenotypic methods is efficient in detection and identification of *Nocardia* species. To date, the *Nocardia* genus comprises 113 species (28). The present study was done to identify *Nocardia* species based on profiles obtained from PCR - RFLP of the *hsp65* gene and sequencing of the 16S rRNA gene. The most commonly used enzymes in *Nocardia* are *Msp*I, *Hin*fI, *Bst*EII, and *Bsa*HI (14, 18). PCR - RFLP analysis of the *groEl* gene has been done to distinguish *Nocardia* isolates

Table 1. RFLP Fragment Patterns Obtained After Digestion of *hsp65* Gene Whit Three *Bst*EII, *MSPI*, and *Hin*fI Restriction Enzymes[a]

Fragment Sizes (Bp) Obtained by:			Pattern Name[b]	Identification of *Nocardia* Spp.	The En Strains Exhibiting Each of the Four *hsp65* Gene Banding Patterns (A - D)
BstEII	***Msp*I**	***Hin*fI**			
130/310	120/140/180	190/250	A[c]	Unkouwn	En28
	70/110/120/140	190/250	B[d]	Unkouwn	En39, En43, En46, En47 and En49
440	120/140/180	440	C[e]	*N. abscessus, N. araoensis, N. asiatica, N. asteroides* type IV, *N. asteroides* type IVATCC 14759, *N. brevicatena, N. cyriacigeorgica, N. higoensis, N. neocaledoniensis, N. paucivorans, N. puris, N. vinacea*	En1, En2, En3, En5, En6, En12, En20, En22, En35, En57, En4, En9, En11, En19, En21, En23, En24, En26, En38, En40, En42, En48, En51, En53 and En56
		110/330	D[e]	*N. brasiliensis, N. inohanesis, N. niigatensis, N. otitidiscaviarum, N. shimofusensis, N. yamanashiensis*	En8

[a] We identified *Nocardia* spp. based on the 16S rRNA gene sequencing; please, refer to Table 2 for Genbank accession number on the 16S rRNA gene sequence.
[b] Four patterns (A to D) were observed for *hsp65* gene digested by three restrictions *Bst*EII, *Msp*I, and *Hin*fI with comparison of fragment sizes from restriction in this study.
[c] "A" and "B" are new RFLP patterns. Therefore, the identification of these isolates to the species level based on this method alone was not possible.
[d] "A" and "B" are new RFLP patterns. Therefore, the identification of these isolates to the species level based on this method alone was not possible.
[e] The RFLP patterns "C" and "D" according to the database published by Rodríguez et al. (2006) belonged to 12 species; therefore, the identification of these strains based on RFLP pattern was performed with respect to the phenotypic features (in Table 2).

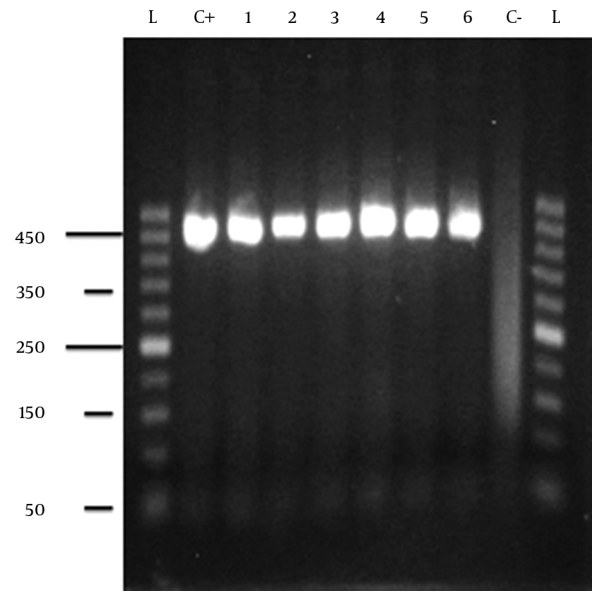

Figure 1. Agarose gel electrophoresis of PCR products amplified from *hsp65* gene of *Nocardia* isolates with Telenti primers. Lanes labeled from left to right: L: generuler 50 bp ladder; C+, positive control (H37Rv); lanes 1 through 6, isolates number; C-,negative control (no DNA). The numbers are molecular sizes in kilobases.

from the genus *Mycobacterium* (29). Steingrube et al. reported 12 species and taxa of *Nocardia* by a PRA method using the *Msp*I and *Bsa*HI enzymes (30). Thus, the PRA (PCR restriction enzyme pattern analysis) allowed the identification of the drug pattern types in *Nocardia* spp. In addition, PCR - RFLP of the *hsp65* gene can lead to a rapid, presumptive species identification (14). Here, we have earned the pattern profiles of the isolates of *Nocardia*. In the present study, the results obtained from biochemical tests of the strains of *Nocardia* from soil (17) were compared with the results obtained from PCR - based RFLP assay. It appears that PCR - based RFLP analysis is a useful tool to detect and distinguish *Nocardia* spp. from the soil and clinical specimens and distinguish *Nocardia* from another similar genus such as *Mycobacterium, Rhodococcus*, *Dietzia*, etc. (20, 31). In a study, biochemical tests, amplification, and REA (Restriction Endonuclease Analysis) of portions of the 16S rRNA gene (digested by *Hin*PII and *Dpn*II) and *hsp65* gene (digested by *Msp*I, *Hin*fI, and *Bsa*HI) were done to detect 28 isolates of *Nocardia* and one isolate was not identifiable by its HSP gene RFLP pattern (18). In our study, seven strains (En12, En28, En39, En43, En46, E47, and En49) produced *hsp65* restriction-endonuclease fragment patterns that were not in agreement with their identification by biochemical/phenotypic tests (Table 2). RFLP patterns in these strains, except strain En12, were different from other known PCR - RFLP profiles. In this study, 25 isolates showed PCR - RFLP patterns and sequencing of 16S rRNA gene results similar to biochemical tests (Table 2). Significantly, each of these approaches has its advantages and disadvantages to identify species. On the other hand, any of them are usually not sufficient to identify all strains of *Nocardia* alone. For instance, one of the advantages of the PRA technique (PCR - RFLP analysis targeting *hsp65* gene) is differentiation of the genus *Mycobacterium* of *Nocardia* species (32). Another study using the *hsp65* PCR - RFLP method and restriction enzymes *Msp*I, *Hin*fI, *Bsa*HI, *Hae*III, and *Bst*EII iden-

Table 2. Results of PCR - RFLP of the *hsp65* Gene and Sequencing of 16S rRna Gene (Genus of *Nocardia* Studied)

Strain(S) (Isolate [No. of Isolates])	Phenotypic Test Results[a]	RFLP Pattern of *hsp65* Gene[b]	RFLP Results (Species)	Sequencing Results of 16s rRNA(Species Designation of Strain)	Genbank Accession Number on the 16S rRna Gene Sequence
En28	*N. africana*	A	Not identified	*N. ignorata*	KP137541
En39	*N. otitidiscaviarum*	B	Not identified	*N. otitidiscaviarum*	KP137543
En43	*N. otitidiscaviarum*	B	Not identified	*N. soli*	KP137544
En46	*N. coubleae*	B	Not identified	*N. cummidelens*	KP137545
En47	*N. asteroides*	B	Not identified	*N. cummidelens*	KP137546
En49	*N. asteroides* complex	B	Not identified	*N. coubleae*	KP137547
En56	*N. asteroides*	C	*N. asteroides*	*N. neocaledoniensis*	KP137548
En12	*N. africana*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137529
En1	*N. asteroides*	C	*N. asteroides*	*N. asteroides*	KP137517
En2	*N. asteroides*	C	*N. asteroides*	*N. asteroides*	KP137518
En3	*N. asteroides*	C	*N. asteroides*	*N. asteroides*	KP137519
En5	*N. asteroides*	C	*N. asteroides*	*N. asteroides*	KP137524
En6	*N. asteroides*	C	*N. asteroides*	*N. asteroides*	KP137525
En20	*N. asteroides*	C	*N. asteroides*	*N. asteroides*	KP137520
En22	*N. asteroides*	C	*N. asteroides*	*N. asteroides*	KP137521
En35	*N. asteroides*	C	*N. asteroides*	*N. asteroides*	KP137522
En57	*N. asteroides*	C	*N. asteroides*	*N. asteroides*	KP137523
En4	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137526
En9	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137527
En11	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137528
En19	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137530
En21	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137538
En23	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137531
En24	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137532
En26	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137533
En38	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137534
En40	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137535
En42	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137536
En48	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137537
En51	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137539
En53	*N. cyriacigeorgica*	C	*N. cyriacigeorgica*	*N. cyriacigeorgica*	KP137540
En8	*N. otitidiscaviarum*	D	*N. otitidiscaviarum*	*N. otitidiscaviarum*	KP137542

[a] Refer to reference (17).
[b] Refer to Table 1. See the PCR - RFLP band patterns of *hsp65* gene.

tified nine isolates to species and biotype levels. In addition, three isolates were identified to species level and two isolates to genus level (20). Rodriguez - Nava et al. focused on PRA of the *hsp65* gene using *Bst*EII, *Msp*I, and *Hin*fI to differentiate *Nocardia* species and reported the restriction patterns of 36 species of *Nocardia* many of which had the same restriction patterns (14). Some of *Nocardia* species have similar biochemical and morphological features and PCR - RFLP profiles. It seems further testing for species detection probabilities such as DNA sequencing is essential especially for new species (18). The 16S rRNA gene sequence plays an important role in species identification

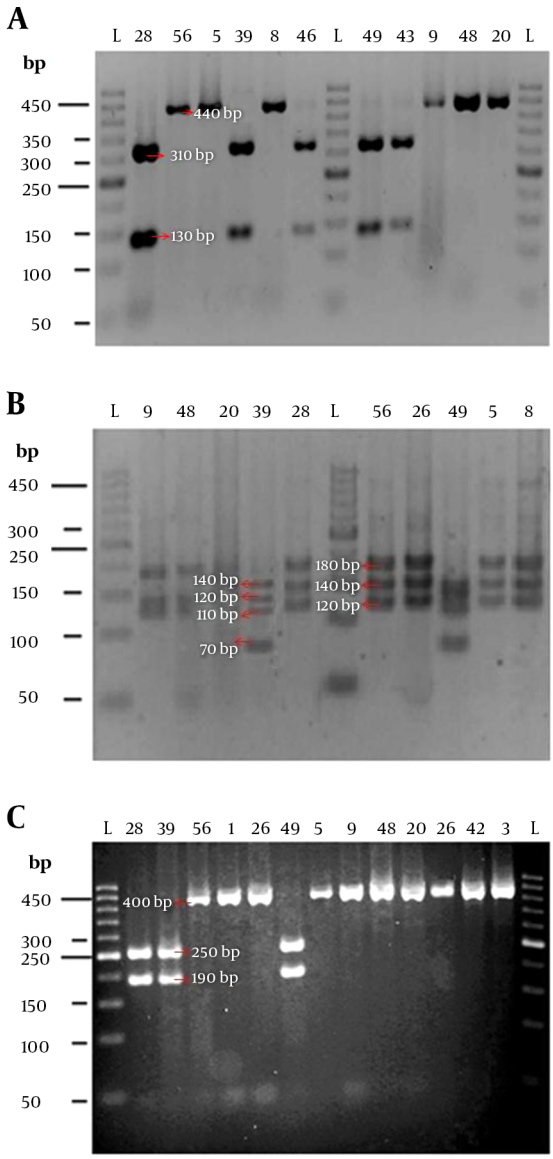

Figure 2. *hsp65* RFLP patterns observed with *Bst*EII (A), *Msp*I (B), and *Hin*fI (C) for the randomly selected *Nocardia* isolates on 3% agarose gels. L, GeneRuler 50 bp ladder; numbers from left to right, isolates number; bp, base pairs. The expected sizes of the products are indicated in Table 1.

and taxonomy. This gene is used for phylogenetic studies (31, 33). By using *Bst*EII digests alone, unique RFLP patterns (130+ 310 bp bands) were obtained for 6 strains of *Nocardia* (En28, En39, En43, En46, En47, and En49). In these strains, the *Msp*I restriction enzyme produced restriction patterns consisting of three bands (three strains) or four bands (four strains) and *Hin*fI enzymes produced restriction patterns consisting of two bands in these 6 isolates (Table 1; Figure 2). Eventually, after analyzing the RFLP band patterns generated by each of the three enzymes, we detected new RFLP patterns "A" and "B". We performed the 16S rRNA gene sequences (1500bp) for all the isolates of *Nocardia*, especially for strains that gave the new RFLP patterns (See Table 1). The gene Bank accession numbers of the 16S rRNA gene for *Nocardia* isolates in our study are given in Table 2. Based on phenotypic test results in our previous report (17), isolate En28 was identified as *N. africana*. After the analysis of PCR - RFLP of the *hsp65* gene, we observed a new pattern "A" (*Bst*EII, 130/310; *Msp*I, 120/140/180; *Hin*fI, 190/250). This isolate after conducting the sequence analysis of 16S rRNA gene was 100% similar to *N.ignorata* DSM 44496. The RFLP profile of the *hsp65* gene for *N. ignorata* according to the report of Rodriguez - Nava et al. was *Bst*EII, 80/320; *Msp*I, 130/120/115/70; *Hin*fI, 190/250 (14). After performing the BLAST comparison, isolates En46 and En47 (whit RFLP pattern B) showed the greatest similarity to *N. cummidelens* HBUM174688 (En 46; similarity 99.66% with a difference in 5 nucleotides, En47; similarity 99.93% with a difference in 1 nucleotide), when compared to the NCBI nucleotide sequence (25). Isolates En39 and En43 had a similar RFLP pattern "B" (*Bst*EII, 130/310; *Msp*I, 70/110/120/140; *Hin*fI, 190/250), which, after sequencing of 16S rRNA gene, were identified as *N. otitidiscaviarum* and *N. soli*, respectively (See Table 1). Steingrube et al. (1995) reported various patterns for *N. asteroides* and *N. otitidiscaviarum* by *Msp*I for initial digestion and then restriction with *Bsa*HI for an amplified 439 - bp segment of the 65 - kDa heat shock protein gene (DNA amplification and restriction) (30). According to previous studies, the RFLP pattern of the *hsp65* gene was reported for *N. ignorata* as *Bst*EII, 80/320; *Msp*I, 130/120/115/70; *Hin*fI, 190/250, *N. soli* and *N. cummidelens* as *Bst*EII, 80/320; *Msp*I, 130/120/115/70; *Hin*fI, 190/250, *N. asteroids* type IV, *N. cyriacigeorgica*, and *N. neocaledoniensis* as *Bst*EII, 440; *Msp*I, 180/145-130/120 - 115; *Hin*fI, 440 (14). In this study, we obtained new RFLP patterns for *N. cummidelens*, *N. ignorata*, and *N. soli* (See Table 2). It is possible that some *Nocardia* spp. contained two different *hsp65* restriction - endonuclease fragment patterns (or more than two). Therefore, the *hsp65* restriction - endonuclease fragment pattern results for *N. ignorata*, *N. soli*, and *N. otitidiscaviarum* differed from those of other research (14). The 16S rRNA gene sequencing showed that strains En8 and En39 both belonged to *N. otitidiscaviarum*, while strain En8 had the expected *hsp65* restriction - endonuclease fragment pattern D and strain En39 had a new pattern B. The RFLP pattern obtained in the isolate En49 (RFLP pattern B:*Bst*EII, 130/310; *Msp*I, 70/110/120/140; *Hin*fI, 190/250) due to the sequence similarity of the 16S rRNA gene (Figure 3, similarity 99.06% to *N. coubleae* OFN N11) was considered as *N. coubleae* (See Table 2). It is noteworthy that, any pattern for the RFLP of *hsp65* gene was not identified for *N. coubleae* until now. Hence, determining

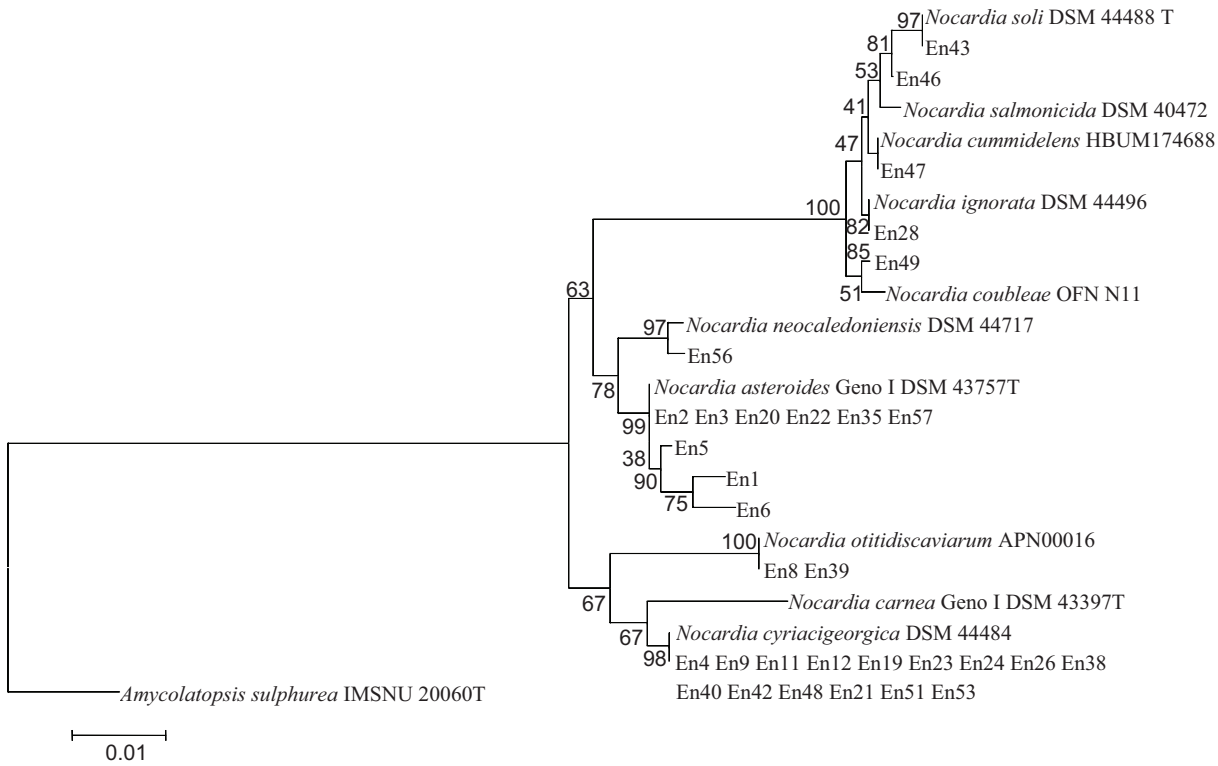

Figure 3. A 1500 - bp fragment of the 16S rRNA gene sequence - based phylogenetic tree of *Nocardia* isolates with those of closely related species computed by the Neighbor - joining (NJ) analyses and Kimura 2 - parameter (K2P) model. The support of each branch determined from 1000 bootstrap samples. Bar 0.01 indicates one nucleotide su*B*stitution per 100 nucleotides.

the *hsp65* gene patterns in various species of *Nocardia* can be helpful to get a more accurate diagnosis. In addition to the identification of *Nocardia* spp., the PCR - RFLP and sequencing techniques could be used for detection and differentiation of unusual species of *Nocardia* and distinguish *Nocardia* and *Mycobacterium* species from each other (30). Based on the findings of previous published papers and the results of the present study, the same RFLP pattern of *hsp65* gene could be present in several different *Nocardia* species. Similarly, the phenotypic properties may be different even in *Nocardia* isolates that belong to the same species. PCR - RFLP analysis targeting *hsp65* is a rapid method for identification of *Nocardia* species if the pattern of all species was determined in databanks of NCBI. Therefore, it is recommended that RFLP - *hsp65* profiles be determined to create a comprehensive database for all discovered species of *Nocardia*.

4.1. Conclusions

We obtained two new PCR - RFLP profiles of *hsp65* gene for *Nocardia* isolates from soil. Strains that exhibited new RFLP profiles should be further analyzed by sequence - based methods such as full sequences of 16S rRNA gene.

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Footnote

Conflicts of interest: There is no conflict of interest

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