Identification of Aerobic Actinomycetes Species Based on HSP-65 Gene Polymorphism Analysis by PCR-RFLP

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Abstract: Interest in the identification and taxonomy of aerobic actinomycetes, has been increasing as a result of the increasing number of immunocompromised individuals in the population who are at greater risk for actinomycoses, especially those with advanced human immunodeficiency virus disease. The occurrence of clinical isolates of aerobic actinomycetes that are inherently resistant to specific antimicrobials increases the significance of timely and accurate species and taxon recognition. Eighty-eight clinical actinomycete strains, previously identified to the genus level, were studied for restriction fragment length polymorphism (RFLP) by using an amplified 439-bp segment of the 65-kDa heat shock protein gene (HSP-65). Digestion with MspI followed by digestion with Hinfl produced RFLP band patterns which lead to the identification of all 40 strains of Nocardia into N. asteroids, N. nova, N. transvalensis N. farcinica, N. brasiliensis and N. otitidiscaviarum. In case of Streptomyces, restriction patterns produced by MspI could differentiate the included 32 strains of Streptomyces into three main species, Streptomyces somaliensis, Streptomyces albus and Streptomyces griseus. The eight genera of Actinomadura included in this study were identified as Actinomadura madurae while the amplicons from all eight isolates of Gordona sp. exhibited MspI recognition sites and a two-band RFLP pattern with Hinfl that was unique for the genus Gordona. MspI separated them into G. sputi and G. bronchialis. These studies extended the feasibility of using PCR-RFLP analysis as a rapid method for the identification of all clinically significant species and taxa of aerobic actinomycetes.

Key words: Actinomycetes, identification, PCR, Restriction fragment length polymorphism (RFLP).

INTRODUCTION

Molecular systematics, which includes both classification and identification, has its origin in the early nucleic acid hybridization studies, but has achieved a new status following the introduction of nucleic acid sequencing techniques[1]. Significance of phylogenetic studies based on 16S rDNA sequences is increasing in the systematics of bacteria and actinomycetes[2]. Sequences of 16S ribosomal DNA have provided actinomycetologists with a phylogenetic tree that allows the investigation of evolution of actinomycetes and also provides the basis for identification. Analysis of the 16S rDNA begins by isolating DNA[3] and amplifying the gene coding for 16S rRNA using the polymerase chain reaction[4]. The purified DNA fragments are directly sequenced. The sequencing reactions are performed using DNA sequencer in order to determine the order in which the bases are arranged within the length of sample[5] and a computer is then used for studying the sequence for identification using phylogenetic analysis procedures. However, analysis of 16S rDNA generally allows us to identify the organisms up to the genus level only. The use of PCR coupled with restriction endonuclease analysis of PCR products has been the focus of recent interest for the separation of mycobacteria from nocardiae[6] as well as for the recognition of species within the genera Mycobacterium and Nocardia[6,7,8,9,10,11,12].

The occurrence of clinical isolates of aerobic actinomycetes that are inherently resistant to specific antimicrobials increases the significance of timely and accurate species and taxon recognition[12,13,14,15]. Successful application of molecular biological methodology to the development of protocols for rapid differentiation of mycobacterial species was demonstrated by Telenti et al.[11]. These authors used PCR-restriction enzyme pattern analysis (PRA) of an amplified 439-bp segment of the 65-kDa heat shock protein (hsp-65) gene and introduced the abbreviation PRA for this method that has now gained wide acceptance[10]. Application of this methodology has since been expanded to include 50 commonly encountered pathogenic species and taxa of aerobic actinomycetes comprising the genera Mycobacterium[10,11], Nocardia[12,14,15] and Actinomadura, Gordona, Rhodococcus, Streptomyces, and...
in silico restriction endonuclease digestions. Fragment patterns were compared with those from the containing ethidium bromide (10 μg ml). The restriction

**MATERIALS AND METHODS**

**Isolates and Culture Media:** The present study included 88 clinical strains (previously identified by Muharram et al.[17] to the genus level) comprising four taxonomic groups of aerobic actinomycetes.

**DNA Extraction:** Actinomycete strains were grown in 10 ml International Streptomycetes Project Medium 1 (ISP 1)[18] with agitation at 30°C for 18–24 h and examined by Gram stain. Cells (4 ml) were harvested by centrifugation (7500 g for 2 min), washed once with 500 ml of 10 mM Tris-HCl/1 mM EDTA (TE) buffer (pH 7.7) and resuspended in 500 ml TE buffer (pH 7.7). The samples were heated in boiling water for 10 min, allowed to cool for 5 min and centrifuged (7500 g for 3 min). The supernatant (300 ml) was transferred to a clean tube and stored at 4°C. If melanin or other pigments were produced during growth in ISP-1, cultures were grown in Middlebrook 7H9 broth, as these pigments interfered with the PCR.

**PCR Amplification:** PCR was carried out in 50 μl volumes containing 2 mM MgCl2, 2U Taq polymerase, 150 mM of each dNTP, 0.5 μM of each primer and 2 μl template DNA. Primer AZF1 (5’-AGCAACCAACGATGTTGTCTCAT-3’) and AZF2 (5’-CAGTTGTCGAAAAACCCGATC-3’). The PCR programme used was an initial denaturation (96°C for 2 min), 30 cycles of denaturation (96°C for 45 s), annealing (56°C for 30 s) and extension (72°C for 2 min), and a final extension (72°C for 5 min). The PCR products were electrophoresed on 1% agarose gels, containing ethidium bromide (10 μg ml), to ensure that a fragment of the correct size had been amplified.

**PRA Analysis:** Commercially available restriction endonucleases (New England Biolabs, Beverly, Mass., and Promega, Madison, Wis.) were screened for their optimal production of species-specific restriction fragment pattern polymorphism (RFLP) band patterns. In the PCR amplification, satisfactory amplicons were readily obtained from all test strains where a DNA band of 439 bp was developed. In previous studies[10,14,19] a practical schematic diagram for the identification of species of Nocardia, Streptomyces, Actinomadura, Gordonia, by restriction endonuclease analysis of PCR-amplified 65-kDa HSP gene sequences was made. In this diagram RFLP band values are expressed as the number of nucleotide base pairs. In this study we tried to evaluate this molecular method to complete the identification of a previously identified genera[17] to the species level. Screening of restriction endonucleases resulted in the selection of MspI for initial digestion and then digestion with BsaHI, and HinfI, a procedure which produced complete separation of all of the included actinomycete genera in this study.

In case of *Nocardia*, restriction patterns produced by *MspI* ranged from one DNA band to three bands. A single DNA band of about 439 bp was developed in case of *Nocardia farcinica* (Fig. 1c, lanes 1 and 2) and three DNA bands with base pair size ranged from 70 bp to 305 bp were developed in case of *Nocardia nova* (Fig. 1b, lanes: 2, 3, 4, 10 and 11), *N. brasiliensis* and *N. otitidiscaviarum* (Fig. 1b lanes 5 to 9 and 12) and in case of *N. transvalensis* (Fig. 1c, lanes 3, 4 and 5).

*BsaHI* produced restriction patterns ranged between two and four DNA fragments. In case of *N. asteroides* two DNA fragments of base pair size ranged from 65 bp and 335 bp were developed (Fig. 2a, lanes 1 to 3). Three DNA fragments pattern appeared with *BsaHI* in two taxons of *Nocardia*. The first one in case of *Nocardia nova*, where DNA fragments developed as 60 bp, 70 bp and 310 bp (Fig. 2b. Lanes 1 to 5). The second pattern appeared with *N. farcinica* in which the fragment size developed as 65, 75 and 270 base pairs (Fig. 2b. lanes 9 and 10).

However, a restriction pattern of four DNA fragments produced with *BsaHI* in two other *Nocardia* taxons. The first one appeared as 60, 70, 80 and 185 base pairs in case of *N. brasiliensis* (Fig. 2b. lanes: 6 to 8) while the second pattern developed as 70, 80, 100 and 185 in case of *N. otitidiscaviarum* (Fig. 2b. lanes 11 and 12).

The eight genera of *Actinomadura* included in this study were identified as *Actinomadura madurae* where they developed a RFLP pattern of 180 and 265 base pairs with *Mspl* (Fig. 3b, lanes 1 and 2) and a single DNA band of 439 with *HinfI* (Fig. 3b. lanes 3 and 4).
Fig. 1: RFLP band patterns from *MspI* digests of PCR-amplified sequences of the *Nocardia* 65-kDa HSP gene. (A) Lanes: 1 to 3, *N. asteroides*; lanes 4 to 6, *N. transvalensis*; lane M, ladder marker (100-bp). (B) Lanes: 1, 5 to 9 and 12 *N. brasiliensis* and *otitidiscaviarum*; lanes: 2 to 4, 10 and 11, *N. nova*. (C) Lanes: 1 and 2, *N. farcinica*; lanes: 3 to 5, *N. transvalensis*.

Amplicons from all eight isolates of *Gordona* sp. exhibited *MspI* recognition sites and a two-band RFLP pattern of 245 and 150 bp with *Hinfl* (Fig. 3b, lanes 1 and 2) that was unique for the genus *Gordona*. *MspI* and *Hinfl* RFLP band patterns readily separated *G. sputi* and *G. bronchialis*.

As shown in Fig. (3) *Streptomyces* isolates produced RFLP patterns that matched species or taxon-specific patterns in the PRA database and resulted in an identification that correlated with the identification by traditional methods. In this figure, three different amplicons were produced that contained *MspI* recognition sites.

In case of *Streptomyces*, restriction patterns produced by *MspI* ranged from two DNA bands to three bands. A double DNA band pattern of 105-335 base pairs was developed in case of *Streptomyces griseus* upon restriction of the amplified sequences of the 65-kDa HSP gene (Fig. 4a, lanes 7 to 9). However, a pattern of three DNA bands with base pair size raged of 75, 110 and 205 bp were developed in case of *Streptomyces somaliensis* (Fig. 4a lanes: 1 to 3,) and in case of *Streptomyces albus* but with a base pair size of 110-130 and 200 bp (Fig. 4a lanes 4 to 6).

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**Fig. 3(A):** RFLP band patterns from *MspI* (lanes: 1 nd 2) and *Hinfl* (lanes: 3 and 4) digests of PCR-amplified 65-kDa HSP gene of *Actinomadura madurae*. (B). RFLP band patterns from *MspI* (lanes: 1 and 2) and *Hinfl* (lanes: 3and 4) digests of PCR-amplified 65-kDa HSP gene of *Gordonia bronchialis*. M. Ladder marker (100bp)

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**Fig. 4:** RFLP band patterns from *MspI* and *Hinfl* digests of PCR-amplified sequences of the 65-kDa HSP gene of *Streptomyces*. (A) *MspI* digests. Lanes 1 to 3, *Streptomyces somaliensis*;lanes 4 to 6, *S. albus*; lanes: 7 to 9, *S.griseus*. (B) *Hinfl* digests. Lanes 1 to 3, *Streptomyces somaliensis*; lanes 4 to 6, *S. albus*; lanes: 7 to 9, *S. griseus*. M. Ladder marker (100bp).
Organisms[20,21,22]. Identification to the genus level is all diagnosed by culture and identification of the central nervous system, and cutaneous infections that diseases in humans and animals, including pulmonary, described species. Many of these can cause clinical Streptomyces albus Discussion:

The genus Nocardia forms a group of 30 validly described species. Many of these can cause clinical diseases in humans and animals, including pulmonary, central nervous system, and cutaneous infections that are all diagnosed by culture and identification of the organisms[20,21,22]. Identification to the genus level is well established based on chemotaxonomic characteristics, mainly on the presence of a major amount of mesodiaminopimelic acid, arabinose, galactose, and mycolic acids with 46 to 60 carbons in cell walls[23,24].

As the number of species is increasing, it is becoming evident that taxonomical complexity causes ambiguities in the interpretation of individually described phenotypic markers and that early identification strategies employed over the past 15 years have to be revised[25]. Thus, albeit identification of Nocardia isolates to the species level is important for estimation of pathogenicity and prediction of antimicrobial susceptibility (and ultimately for prognosis), conventional species identification remains difficult, inaccurate, and time consuming.

Because of the slow growth of Nocardia, PCR assay and restriction endonucleases cleavage can be completed within 2 days. PCR, which allows the specific and sensitive amplification of a preselected DNA region, has been intensively applied to the examination of the molecular genetics of organisms, especially the identification of the DNA sequences that are specific for the organism tested. The target regions usually are pathogenicity or virulence genes. Polymerase chain reaction coupled with Restriction fragment pattern polymorphism PCR-RFLP protocol with the hsp gene was used previously in the identification of Mycobacterium species[10,11,14] and restriction of the 439 bp of the hsp gene by MspI and BsaHI provide nearly complete differentiation of all species and taxa of Nocardia[10,14].

PCR amplification enabled amplification of a 440-bp fragment coding for a part of a 65-kDa heat shock protein. This fragment was digested with restriction endonuclease analysis. The results allowed establishment of a practical identification scheme according to the numbers and sizes of the bands generated with each enzyme. It has been used for the identification of different species of aerobic actinomycetes, including the Nocardia species, with an accuracy of 98.69[15,19,31].

However, since the scheme described for the identification of actinomycete species presented several subgroups (several profiles with several bands for which sizes were not easy to estimate), the interpretation of the result for a clinical strain was difficult within the actinomycete group. On the contrary, if we knew in advance whether the strains belonged to the Nocardia genus, use of interpretation by the technique of Steingrube et al.[19] made identification of the different species easier. Therefore, the technique described here complements the technique of Steingrube et al.[19]. The genus- and species-specific techniques could be combined to accomplish the identification of Nocardia clinical isolates within 48-72 h after receipt of cultures in the reference laboratories.

The lack of BstEII recognition sites in the amplified 65-kDa HSP gene sequence from all 216 isolates of aerobic actinomycetes in this study provided a rapid molecular technique for differentiating them from mycobacteria. As reported previously, amplicons from the majority of Mycobacterium species, including all pathogenic rapidly growing species and taxa, have one or more BstEII recognition sites[10,11,14,32].

In the present study differentiation of other aerobic actinomycete species and taxa as well as some Nocardia taxa with these two endonucleases was found to be incomplete. Additionally, intraspecies polymorphism was a problem with BsaHI-derived RFLP patterns. BsaHI produced intraspecies polymorphism with 10 of 18 taxa of Nocardia[19]. Multiple RFLP band patterns from several species in the differentiation schema tended to complicate species identification. The substitution of Hinfl for BsaHI produced an identification system that completely differentiated the taxa in this study. Limited use of BsaHI in the present identification protocol resulted in intraspecies polymorphism only with isolates of N. otitidiscaviarum. Of the four taxa that were not separated with MspI and Hinfl, BsaHI was effective in differentiating isolates of N. brasiliensis and N. otitidiscaviarum, but separated only 40% of the N. transvalensis isolates from those of the N. asteroides. Biochemical identification of clinical aerobic actinomycete isolates, in addition to being laborious and time-consuming, is not always diagnostic and may produce results that do not match typical criteria or those for designated type strains. Of the 8 Gordona isolates examined in this study, biochemical identification based on the carbohydrate utilization reactions indicated that 4 isolates were G. bronchialis, 2 isolates were G. sputi, and 2 isolates did not match any identifiable pattern and were designated Gordona.
spp. PCR-RFLP analysis with MspI and Hinfl produced RFLP band patterns for three of these four isolates that were identical to those exhibited by the five G.sputi isolates, thereby providing a conclusive identification that was not otherwise possible by biochemical methods MspI produced multiple RFLP band patterns from amplicons of G. bronchialis, the N. asteroides, N. ottidiscaviarum, N. transvalensis, and the genus Streptomyces, while Hinfl did so only with the genus Streptomyces. Intraspecies polymorphism with MspI was limited to two to three patterns, as opposed to those with BsaHI and HaelII, which often produced up to five and six different patterns within individual species. RFLP patterns derived from MspI alone were sufficient for separating 74% (24 out of 32) of the Streptomyces isolates from all other aerobic actinomycetes. A more detailed study of a larger collection of clinical isolates of Streptomyces will be required in order to determine the degree to which the observed RFLP pattern heterogeneity is related to species differences or intraspecies polymorphism. Hinfl produced very large fragments of 335 to 395 bp from 18 of 19 isolates of Streptomyces that further facilitated differentiation. These very large RFLP bands were unique to Streptomyces isolates and readily distinguished them from isolates of all other aerobic actinomycete species and taxa.

In short, this study showed that the PCR amplification of 439-bp segment (amplicon) of the 65-kDa heat shock protein gene combined with restriction analysis can be applied to the identification of strains of the has been used for the differentiation of commonly encountered pathogenic species and taxa of aerobic actinomycetes comprising the genera Nocardiad, Actinomadura, Gordona, and Streptomyces. This PCR-RFLP methodology distinguished clinical isolates of aerobic actinomycetes with 96.8% accuracy and can be readily and economically implemented for routine clinical use. Moreover, it will facilitate epidemiological studies of the human.

REFERENCES


