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Molecular Study of Persistence of *Nocardia asteroides* and *Nocardia otitidiscaviarum* Strains in Patients with Long-Term Nocardiosis

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Three *Nocardia asteroides* and five *Nocardia otitidiscaviarum* isolates recovered from three patients with long-term nocardiosis were compared by random amplification of polymorphic DNA fingerprinting, antibiotic susceptibility testing, and enzymatic characterization. Results obtained with primer DKU49 (P. Palittapongarnpim, S. Chomyc, A. Fanning, and D. Kunimoto, J. Infect. Dis. 167:975–978, 1993) provide evidence that patient A was infected by two *N. asteroides* strains during a single episode of nocardiosis and that patients B and C remained infected by the same strain, respectively. Resistance to minocycline that was present in the first isolate recovered from patient B reverted to intermediate resistance in the second isolate and reverted to susceptibility in the third isolate. Resistance to penicillin G and β-lactams was acquired by the second isolate obtained from patient C.

Nocardia spp. are gram-positive, aerobic actinomycetes with rudimentary to extensively branched vegetative hyphae which fragment into bacteroid, rod-shaped to coccoid elements. Nocardiae have a worldwide distribution in soil, and at least six species are pathogenic for humans and animals. They may enter the body via inhalation of contaminated dust particles or via wounds contaminated with dust or soil (14).

Nocardiosis is an indolent process, most frequently characterized by primary pulmonary lesions that may be typically subacute or chronic. The disease tends to be more acute in immunodepressed patients. Remissions and exacerbations lasting for days or weeks are characteristic. The disease may spread hematogenously to other organs and has a predilection for the central nervous system. In the skin, several types of lesions may be seen, including mycetoma. The mycetoma is a slowly progressive process that may take several years to evolve (2).

No report describes long-term persistent nocardiosis in which several isolates from the same patient were obtained and genetically compared. Similarly, comparison of relapse isolates with original strains in recurrences of *Nocardia* infection after repeated, prolonged, and apparently successful chemotherapy are generally not performed. However, a recurrent *Nocardia* pneumonia was diagnosed in a patient with chronic granulomatous disease over a 2.5-year interval on the basis of plasmid analysis and antibiotic susceptibility data (11).

Accurate methods for strain identification are essential for comparison of clinical isolates. Conventional phenotypic methods based on serotyping (19), biotyping (22), and typing with a yeast killer system (17) have been applied to nocardiae. New molecular genotyping methods based on analysis of chromosomal DNA provide more reproducible data and avoid dependence on potentially variable phenotypic traits (21). Plasmid profiling could be a reliable method (11), but only 25 and 50% of *Nocardia asteroides* and *Nocardia farcinica* isolates, respectively, carry plasmids (20). Moreover, plasmid acquisition or loss cannot be excluded. Although ribosomal DNA gene profiling (ribotyping) is of value in typing numerous bacterial pathogens (3), this technique did not allow the differentiation of reference strains from strains isolated during a limited outbreak (9). Moreover, ribotyping is time-consuming and is not well suited to routine use. However, this method could allow species diagnosis (13). A PCR method based on the random amplification of polymorphic DNA (RAPD) fragments by using a single primer with an arbitrary sequence has been described previously (25) and has been applied to the typing of various pathogenic microorganisms (24).

In the present study, we evaluated the application of RAPD fingerprinting with 10- and 12-mer primers of arbitrary sequence for comparison of *N. asteroides* and *Nocardia otitidiscaviarum* strains isolated from patients with long-term persistent nocardiosis.

MATERIALS AND METHODS

Organisms and growth conditions. Eight *Nocardia* isolates from three patients with long-term persistent nocardiosis and six epidemiologically unrelated isolates (four *N. asteroides* isolates [D to G] and two *N. otitidiscaviarum* isolates [H and I]) from human sources obtained from the National Reference Center for Human Mycosis and Antifungal Agents (Institut Pasteur, Paris, France) were studied. The microbiological characteristics of persistent isolates and clinical data from the corresponding patients are presented in Table 1. Patients A, B, and C remained infected with *Nocardia* strains for 5, 18, and 28 months, respectively. The isolates were identified by conventional methods (6). All bacteria were maintained on Bennett's agar medium at room temperature before being treated simultaneously. Isolated colonies from fresh cultures were subcultured for 3 to 5 days at 34°C on a sterile cellulose acetate membrane (Millipore, Bedford, Mass.) deposited on blood agar.

Primer selection and synthesis. Several 10- and 12-mer oligonucleotides were synthesized on an automated synthesizer (Milligen model; Millipore) by the phosphoramidite method. Primer DKU49 (CCGCCGACCGAG; G+C content, 83%) (18) was found to produce informative DNA patterns and was therefore used throughout the study.

DNA extraction, PCR amplification, and detection of PCR products. A colony was resuspended in 650 μl of reaction mixture containing 500 μl of sterile pyrolyzed water and 150 μl of Chelex solution (15% [wt/vol] Chelex 100 resin 0.1% [wt/vol] sodium dodecyl sulfate, 1% [vol/vol] Nonidet P-40, 1% [vol/vol] Tween 80) and boiled for 30 min at 100°C (7). The samples were then centrifuged for 8 min at 5,000 × *g*, and 35 μl of the supernatant was used in a 50-μl PCR mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM (each) deoxynucleoside triphosphate, 2.5 U of Hi-*Taq* DNA polymerase (Bioprobe Systems), and 1 μM primer. After preliminary trials with different annealing temperatures and times and various concentrations of *Nocardia* DNA, amplification was run for 45 cycles of denaturation for 1 min at

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TABLE 1. Microbiological and clinical data from isolates recovered from patients with persistent nocardiosis

Isolate designation	Date of isolation (mo/day/yr)	Biological sample	Species identification	Patient characteristic			
				Site of nocardiosis	Underlying condition	Treatment[a]	Apparent outcome
A.1	6/10/94	Cutaneous abscess puncture	*N. asteroides*	Cutaneous	HIV[b]	AMX + GM, then TMP + DO, then GM + TMP + DO	Unchanged
A.2	9/12/94	Adenopathy puncture	*N. asteroides*	Cutaneous	HIV	GM + TMP + DO	Unchanged
A.3	11/18/94	Adenopathy puncture	*N. asteroides*	Cutaneous	HIV	IPM + AN + DO	Improved
B.1	4/22/91	Subcutaneous biopsy	*N. otitidiscaviarum*	Inguinal area		SXT-TMP	Unchanged
B.2	6/12/91	Subcutaneous biopsy	*N. otitidiscaviarum*	Inguinal area		SXT-TMP	Unchanged
B.3	10/27/92	Subcutaneous biopsy	*N. otitidiscaviarum*	Inguinal area		SXT-TMP	Cured
C.1	10/26/89	Subcutaneous biopsy	*N. otitidiscaviarum*	Left foot		GM + MNO	Unchanged
C.2	2/25/92	Subcutaneous biopsy	*N. otitidiscaviarum*	Left foot		MNO	Improved

[a] AMX, amoxicillin; AN, amikacin; DO, doxycycline; GM, gentamicin; IMP, imipenem; MNO, minocycline; SXT-TMP, sulfamethoxazole-trimethoprim.
[b] HIV, human immunodeficiency virus.

94°C, annealing for 1 min at 42°C, and elongation for 2 min at 72°C. A predenaturing incubation at 94°C for 5 min and a final elongation step at 72°C for 5 min were included with each amplification. Amplification was performed in a DNA thermal cycler (Omnigene; Hybaid, Teddington, United Kingdom). After amplification, 12-μl samples were analyzed by electrophoresis for 5 h at 80 V in horizontal TAE (40 mM Tris-acetate, 2 mM $Na_2EDTA \cdot 2H_2O$) gels containing 2% agarose (Ultrapure; Bio-Rad) and 0.5 μg of ethidium bromide per ml. The gels were then exposed to UV light to visualize the amplified products and photographed. The reproducibility of the reaction was tested at intervals of different numbers of days with freshly extracted DNA.

Plasmid screening, antibiotic susceptibility testing, and enzymatic characterization. For plasmid screening, the extraction procedure of Kado and Liu (12) was used. Antibiotic in vitro susceptibility testing was performed on Mueller-Hinton agar as described previously (4). The strains were scored as susceptible, intermediate, or resistant according to the recommendations of the Comité de l'Antibiogramme of the French Society for Microbiology (1). β-Lactamase production was detected with Cefinase (Becton Dickinson and Co., Paramus, N.J.). Enzymatic profiles of isolates were determined with the API ZYM system (API System S.A., La Balme les Grottes, France) as described previously (5).

RESULTS

Of the 12 primers tested, 11 produced no detectable products or no reproducible results. Primer DKU49 yielded reproducible DNA patterns after several PCR assays separated by 7-day intervals. Amplification of genomic DNA from the *Nocardia* strains with the primer resulted in RAPD patterns consisting of 5 to 15 distinct DNA fragments ranging in size from 200 to ~2,000 bp (Fig. 1). The patterns of isolates from each patient were found to be similar, but they were found to differ among patients. The patterns of all control strains were found to be different from those of clinical isolates and from each other.

RAPD with the DKU49 primer provided reproducible differences among *Nocardia* species and among *N. asteroides* and *N. otitidiscaviarum* isolates. This result confirms the findings of Exmelin et al. (9). Although it has been suggested that the discriminatory power of RAPD could be increased by using more than one primer (15), our study confirmed that good results can be obtained with a single selected primer.

The RAPD patterns were considered to be identical on the basis of similar numbers and matching positions of all bands. Isolates recovered from the second or eventually the third sample from the same patient were of the same genotype as the first isolate, except for those from patient A. Two distinct genotypes were identified among the three isolates recovered from this patient, the third being indistinguishable from the first one.

According to a rapid alkaline extraction procedure, none of the isolates appeared to contain plasmids. The antibiotic susceptibility patterns of the three isolates recovered from patient B and the first and third isolates from patient A were similar (Table 2). A single antibiotic resistance difference was observed among the three isolates from patient B: the first isolate was resistant to minocycline, whereas the second and third isolates were intermediate and susceptible, respectively. Different in vitro antimicrobial susceptibility patterns were also observed between strains isolated from patient C and between the second and the two remaining isolates from patient A.

The enzymatic profiles of all isolates differed from one patient to another (Table 3). The enzymatic profiles of isolates from patient B were identical, as were those of isolates from patient C. The first and third isolates from patient A had identical enzymatic profiles, whereas the second isolates exhibited strongly different profiles. These data show that enzymatic profiles have limited value for classification of isolates. Differences in enzymatic profile clearly distinguish different isolates, but the opposite is not systematically true, since two unrelated isolates can exhibit the same profile (e.g., isolates A.1 and A.3 and isolate G).

DISCUSSION

Coinfection with *N. asteroides* and other organisms (e.g., *Mycobacterium* spp. and *Aspergillus* spp.) has been reported (10, 16). As far as we are aware, the present work is the first study indicating that nocardiosis can be caused by different strains in the same patient. The RAPD and enzymatic patterns obtained with the first and last isolates were significantly dif-

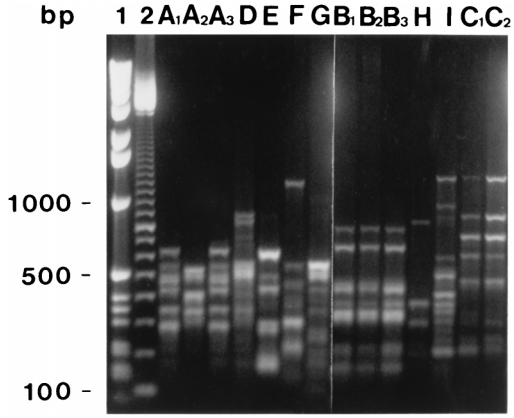

FIG. 1. RAPD profiles of isolates after amplification with primer DKU49. Lanes: 1, size reference marker (1-kb scale; Gibco BRL, Life Technologies, Cergy-Pontoise, France); 2, size reference marker (100-bp ladder; Pharmacia Biotech, Uppsala, Sweden). The designation numbers of the strains are indicated at the top (where, e.g., A_1 is A.1 from the text).

TABLE 2. In vitro antimicrobial susceptibility patterns of isolates

Antibiotic (disk content)	Resistance pattern of isolate from[a]:							
	Patient A			Patient B			Patient C	
	A.1	A.2	A.3	B.1	B.2	B.3	C.1	C.2
Penicillin G (10 IU)	R	R	R	R	R	R	I[b]	R[b]
Ampicillin (10 μg)	S	R[b]	S	R	R	R	S[b]	R[b]
Amoxicillin (25 μg)	I	R[b]	I	R	R	R	S[b]	R[b]
Amoxicillin (20 μg) + clavulanic acid (10 μg)	S	S	S	S	S	S	S[b]	R[b]
Cefamandole (30 μg)	S	I[b]	S	I	I	I	R	R
Imipenem (10 μg)	S	S	S	R	R	R	S[b]	R[b]
Carbenicillin (100 μg)	S	R[b]	S	R	R	R	S[b]	R[b]
Cefotaxime (30 μg)	S	S	S	R	R	R	R	R
Ceftriaxone (30 μg)	S	R[b]	S	R	R	R	R	R
Tetracycline (30 IU)	R	R	R	R	R	R	R	R
Minocycline (30 IU)	S	R[b]	S	R	I[b]	S[b]	S	S
Kanamycin (30 IU)	R	R	R	R	R	R	S	S
Tobramycin (10 μg)	S	R[b]	S	R	R	R	S	S
Amikacin (30 μg)	S	S	S	S	S	S	S	S
Gentamicin (10 IU)	S	R[b]	S	R	R	R	S	S
Sulfamethoxazole (200 μg)	S	S	S	R	R	R	S	S
Sulfamethoxazole (23.75 μg) + trimethoprim (1.25 μg)	S	S	S	R	R	R	S	S
Trimethoprim (5 μg)	R	R	R	R	R	R	R	R
Erythromycin (15 IU)	R	R	R	R	R	R	R	R
Vancomycin (30 μg)	R	I[b]	R	R	R	R	R	R

[a] R, resistant; I, intermediate; S, susceptible.
[b] Variance from initial isolate.

ferent from the profiles of the second isolate recovered from patient A, which provides evidence that this patient was infected with more than one *N. asteroides* strain during a single episode of nocardiosis. This was confirmed by correlation with antimicrobial susceptibility patterns (Table 2). It is possible that patient A was initially infected with two strains of *N. asteroides* and that this was not reflected in the single-colony isolate tested from the primary isolation plates. If two strains were present at various levels of relative abundance, the strain present in a lower level of abundance might not have been detected if only a few colonies had been sampled. Given the relative simplicity of RAPD, we recommend amplification of DNA from several independent colonies recovered on primary isolation. On the other hand, repeated sampling at timed intervals could allow the detection of a new strain or a strain not previously isolated. Involvement of a new strain could elucidate some aspects of clinical resistance.

The similarity of the RAPD and enzymatic patterns obtained for the three isolates recovered from patient B suggests that this patient remained infected by the same strain of *N. otitidiscaviarum* throughout the course of infection. This assumption was supported by antimicrobial susceptibility profiles. However, during the 18-month infection period, resistance to minocycline, which was present in the first isolate, reverted to intermediate resistance in the second isolate and to susceptibility in the third isolate. Tetracycline resistance is due to the presence of a resistance gene (*tet*) that specifies a protein that mediates active efflux of antibiotic from the cell (23). Resistance to tetracycline in the absence of resistance to minocycline has already been observed in several gram-positive bacterial genera, including staphylococci and propionibacteria (8). Hypothetically, acquired susceptibility to minocycline in *Nocardia* species could be due to mutation of *tet* genes coding for minocycline resistance.

Although the antimicrobial susceptibility patterns of the two isolates recovered from patient C were different, the similarity between the RAPD and enzymatic patterns indicated that the patient remained infected by the same clone. However, resistance to penicillin G, ampicillin, amoxicillin, ticarcillin, imipenem, and carbenicillin was acquired, while susceptibility to other antibiotics remained unchanged. Resistance to penicillin G and β-lactams could result from production of a β-lactamase, since resistance was found in isolate C.2, whereas it was not found in isolate C.1 (Table 3).

TABLE 3. Enzymatic profile detected by API ZYM and β-lactamase production detected by Cefinase

Enzymatic activity	Profile of isolate from[a]:													
	Patient A			Patient B			Patient C		Control patient					
	A.1	A.2	A.3	B.1	B.2	B.3	C.1	C.2	D	E	F	G	H	I
Alkaline phosphatase	+	0[b]	+	+	+	+	+	+	+	±	+	+	+	+
Butyrate esterase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Caprylate esterase	±	+[b]	±	+	+	+	+	+	+	±	+	+	+	+
Myristate lipase	0	0	0	0	0	0	0	0	0	0	±	0	+	±
Leucine arylamidase	+	+	+	+	+	+	+	+	+	±	+	+	+	+
Valine arylamidase	0	±[b]	0	0	0	0	0	0	0	0	0	0	0	0
Cystine arylamidase	0	±[b]	0	0	0	0	0	0	0	0	0	0	0	0
Trypsin	0	±[b]	0	0	0	0	±	±	+	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acid phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phosphohydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0
β-Galactosidase	0	0	0	+	+	+	±	±	0	0	0	0	+	+
β-Glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-Glucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β-Glucosidase	+	+	+	+	+	+	+	+	+	0	+	+	+	+
N-Acetyl-β-glucosaminidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-Mannosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-Fucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0
β-Lactamase	0	+	0	0	0	0	0	+	ND	ND	ND	ND	ND	ND

[a] +, positive activity; ±, weak activity; 0, no activity; ND, not determined.
[b] Variance from initial isolate.

Although the number of patients observed here is small, the findings of our study have demonstrated that cutaneous nocardiosis due to *N. asteroides* may be curable with a short course of imipenem and amikacin, an alternative therapy that was selected on the basis of in vitro susceptibility data (patient A). Furthermore, cutaneous or subcutaneous nocardiosis due to *N. otitidiscaviarum* can be cured by prolonged treatment with sulfamethoxazole-trimethoprim, although this drug combination would not be considered effective in vitro or in vivo against *N. otitidiscaviarum* isolates (patient B). Minocycline appeared to be a good substitute for sulfonamides in patient C.

In conclusion, the RAPD assay developed here can be used to establish whether *Nocardia* isolates collected at intervals of time from the same patient are similar. Thus, the persistence and recurrence of *Nocardia* infection can be traced more accurately. Moreover, this technique could be used in order to identify possible sources of transmission to humans.

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