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Typing of Some Clinically Significant *Nocardia* Strains Using a Digoxigenin-Labelled rDNA Gene Probe

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Abstract: In this study, ribotyping (restriction polymorphism ribosomal RNA analyses) was applied as a taxonomic tool for bacteria belonging to the genus *Nocardia*. A total of 21 *Nocardia* strains were clearly distinguished using a combination of *Eco*RV gene restriction endonuclease patterns together with a digoxigenin-labelled *Streptomyces violaceoruber* TK21 rDNA probe. Five distinct ribotypes were identified, and each ribotype contained 4 to 8 restriction fragments ranging in size from 1.2 to 20.7 kb. The evaluation of the results showed that *N. brasiliensis*, *N. farcinica*, *N. otitidiscaviarum* and *N. seriolea* are taxonomically well-established species, except *N. pseudobrasiliensis*. Therefore, it can be concluded that ribotyping technique provide a useful information for the classification of clinically significant *Nocardia*.

Key Words: *Nocardia*, Ribotyping, Taxonomy

Klinik Önemi Olan Bazı *Nocardia* Suşlarının Digoksijeninle İşaretlenmiş Ribozomal DNA Probu Kullanılmasıyla Tiplendirilmesi

Özet: Bu çalışmada ribotyping metodu, *Nocardia* cinsine ait bakterilerin ayırtedilmesinde, taksonomik bir yöntem olarak uygulanmıştır. Toplam 21 *Nocardia* suşu, *Streptomyces violaceoruber* TK21 suşundan elde edilen digoksijeninle işaretlenen ribosomal DNA probu ve *Eco*RV endonukleaz enziminin birlikte kulanılmasıyla açık bir şekilde birbirlerinden ayırtedilebilmişlerdir. Herbiri 1.2 ile 20.7 kb ölçüm değerinde ve 4 ile 8 fragmente sahip birbirinden ayrılan beş ribotip belirlenmiştir. Sonuçların değerlendirilmesi, *N. pseudobrasiliensis* dışında *N. brasiliensis*, *N. farcinica*, *N. otitidiscaviarum* ve *N. seriolea*'nın taksonomik olarak iyi tanımlanmış türler olduğunu gösterdi. Böylelikle, ribotyping tekniğinin klinik önemi olan *Nocardia*'nın sınıflandırılmasında faydalı bilgiler sağladığı sonucuna varılabilmektedir.

Anahtar Sözcükler: *Nocardia*, Ribotyping, Taksonomi

Introduction

The long and turbulent taxonomic history of the genus *Nocardia* will not be considered in detail as it has been the subject of several comprehensive reviews (1-7). This taxon, which was originally described by Trevisan (8) to accommodate five species, subsequently became a dumping ground for nocardioform bacteria, that is, for aerobic actinomycetes which formed a mycelium that fragmented into rods and cocci. It was only with the application of modern taxonomic methods, notably chemotaxonomic, molecular systematic and numerical phenetic procedures, that relationships between members of this heterogeneous group were clarified (9).

Nocardiae cause a variety of suppurative infections in humans and animals. Infection may occur by inhalation, through contaminated wounds and by traumatic implantation. The potential causative agents of nocardiosis are *Nocardia asteroides*, *Nocardia brasiliensis*, *Nocardia farcinica*, *Nocardia nova*, *Nocardia otitidiscaviarum*, *Nocardia pseudobrasiliensis* and *Nocardia transvalensis* (5, 10, 11).

More recently, typing techniques have become increasingly rapid and applicable to identify and differentiate biotechnologically and clinically significant microorganisms (12, 13). Ribotyping has been applied to a wide range of microorganisms, including members of

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the genera *Actinomyces* (14), *Corynebacterium* (15), *Mycobacterium* (16), *Rhodococcus* (17), *Streptomyces* (18), and *Tsukamurella* (19). The method has also been used to clarify taxonomic relationships within the *Nocardia asteroides* complex (20, 21). The results of these studies suggest that ribotyping might be used to clarify taxonomic relationships within and between nocardial species.

The purpose of this study was to evaluate ribotyping as a tool for the taxonomy and identification of clinically significant *Nocardia* strains.

Materials and Methods

Test strains and cultivation

The sources and strain histories of the test organisms are given in the Table. All of the organisms were maintained as glycerol suspensions (20%, v/v) at -20ºC. Single colonies of the test strains were grown on GYEA (22) plates for 7 d at 30ºC and used to inoculate 50 ml amounts of GYE broth held in 100ml conical flasks. The inoculated flasks were shaken at 150 revolutions per minute (rpm) at the respective temperature for up to 7 d when growth was subcultured onto GYEA plates to check that the cultures were pure. Nocardial biomass was harvested by centrifugation at 10,000 rpm for 10 min, washed twice with sterile TE buffer (Tris-HCL pH 8.0, 10mM; EDTA 1mM) and stored at -20ºC. Approximately 100 mg wet weight cells of each organism were used for DNA extraction.

DNA extraction and purification protocols

The guanidine thiocyanate DNA extraction procedure of Pitcher *et al.* (23) was used with specific modifications to optimise the isolation of DNA from the test strains. Pretreatment of nocardial cells with proteinase K (100 μg/ml) and sodium dodecyl sulphate (SDS, final

Table Sources and histories of the organisms included in the ribotyping experiments.

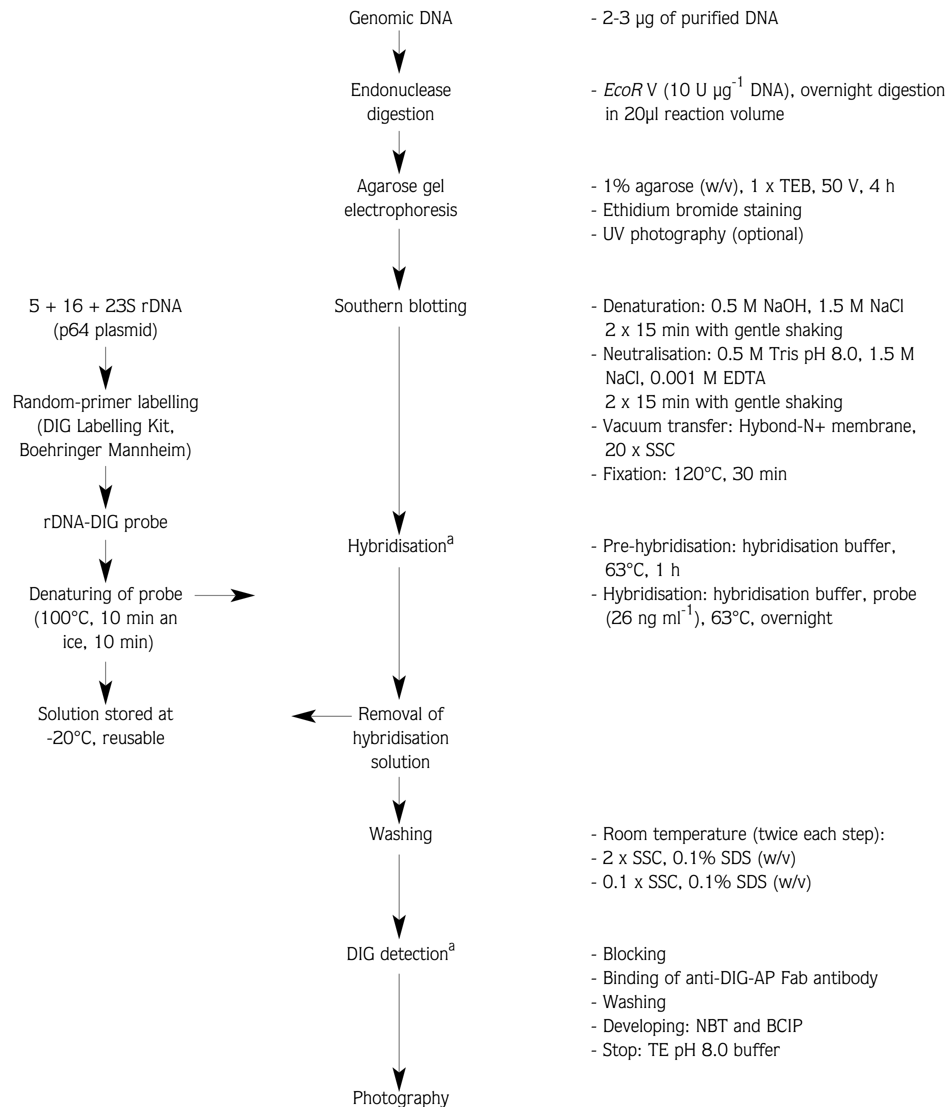
Lab. No.	Species	Source
N 318^T	*N. brasiliensis*	R.E. Gordon, IMRU 845; J.D. Schneidau Jr.,381; A. Batista,631; IP 337 [Goodfellow (1971), cluster 5; Orchard & Goodfellow (1980), subcluster 4B; Hookey (1983), cluster 33 (*N. brasiliensis*)]
N 428	*N. brasiliensis*	R.E. Gordon, IMRU 1336; M.P. Lechevalier,L-36 (*Nocardia* sp.), soil
N 471	*N. brasiliensis*	A.González-Ochoa, Instituto de Salubridad y Enfermedades Tropicales, Mexico City, Mexico; 4115; mycetoma, lower leg
N 475	*N. brasiliensis*	A Gonzáles-Ochoa, 4023; mycetoma, forearm
N 898^T	*N. farcinica*	M. Tsukamura, Chubu Chest Hospital, Obu, Aichi-chen 474, Japan, 23102 (R-3318); ATCC 3318; R.E. Gordon [Orchard & Goodfellow (1980), subcluster 1A; Hookey (1983), cluster 28 (*N. farcinica*)]
N 233	*N. farcinica*	R.J. Olds, Department of Pathology, University of Cambridge, CN 470; *N.asteroides*; cow's milk [(Goodfellow (1971), subgroup 1B; Orchard & Goodfellow (1980), cluster 11)]
N 669	*N. farcinica*	S.G. Bradley, MAC 300 [Orchard & Goodfellow (1980), cluster 9 (*N.asteroides*); Hookey (1983), cluster 28 (*N. farcinica*)]
N 690	*N. farcinica*	K.P. Schaal, N5; strain Karlsruhe [Orchard & Goodfellow (1980), cluster 11]
N 1158^T	*N. otitidiscaviarum*	NCTC 19349; ATCC 14629; R.E.Gordon; infected middle ear of guinea pig
N 231	*N. otitidiscaviarum*	R.J. Olds, CN 749; isolated from a dachshund (K.P. Schaal, N 206)
N 232	*N. otitidiscaviarum*	R.J. Olds, CN 751, isolated from a corgi (K.P. Schaal, N 207)
N 313	*N. otitidiscaviarum*	I.P 751; K.P. Schaal, N 208
N 1237^T	*N. pseudobrasiliensis*	P. Boiron, Unite de Mycologie, Institut Pasteur Paris, France, CIP 104600; leg abscess (ATCC 51512)
N 1234	*N. pseudobrasiliensis*	P. Boiron, N 249; brain abscess
N 1116^T	*N. seriolae*	JCM 3359; K. Hatai, NA 8191; spleen of a yellowtail (*Seriola quinqueradiata*), Nagasaki, Japan
N 1118	*N. seriolae*	JCM 5849; K. Hatai, NA 8231; spleen of a yellowtail (*Seriola quinqueradiata*), Nagasaki, Japan
N 1119	*N. seriolae*	JCM 5850; K. Hatai, NA 8191; N. Matsumoto KRN 8403; kidney of a Japanese flounder, Kagawa, Japan

^{T}, Type strains. Abbreviations: ATCC, American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD., USA; IMRU, Institute of Microbiology, Rutgers State University, New Brunswick, N. J., USA.; IP, Institut Pasteur, Rue du Dr. Roux, Paris, France; JCM, Japan Collection of Microorganisms, Saitama, Japan and NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK.

concentration 2%, w/v) was found to greatly facilitate the susceptibility of cells to the standard digestion and extraction procedure.

Restriction polymorphisms of ribosomal RNA genes

The analysis of the restriction polymorphisms of the ribosomal genes (ribotyping) of the test strains (Table) was performed as outlined in Figure 1. Genomic DNA (*ca.* 2-3 µg) was digested with *EcoR* V (C↓AATTC) restriction endonucleases using 10 units of enzyme per µg of DNA in a 20 µl volume reaction, as recommended by the manufacturer (24). DNA fragments were separated in 20 cm long agarose gels (1%, w/v; 0.3 mm thickness). Electrophoresis was carried out at 50 V for 4 h at room temperature in 1 x TEB buffer (Tris-borate-EDTA; 25). The gels were stained for 20 min with ethidium bromide (0.5 µg ml-1 in water) and destained in water prior to UV photography.



[a] Hybridisation and detection were performed according to the recommendations of the DIG system manufacturer (Boehringer Mannheim Biochemica, 1993). Hybridisation buffer consisted of 5 x SSC, 0.1%, w/v N-laurylsarcosine, 0.02%, w/v SDS and 1%, w/v blocking reagent (Boehringer Mannheim Biochemica, 1994). AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium.

Figure 1. Protocol for ribotyping experiments using digoxigenin labelled rDNA probes.

Results and Discussion

DNA samples prepared from the test strains (Table) were checked to ensure the absence of extensive shearing and degradation prior to digestion with the restriction enzyme and after electrophoresis of the genomic digests. All of the strains were found to have chromosomal DNA which was pure enough for ribotyping. The required amount of DNA needed for each test strain was obtained from about 100 mg of biomass.

A pilot study was carried out to evaluate the ribotype patterns obtained when three restriction endonuclease enzymes, that is, *Eco* RV, *Nco* I and *Pst* I, were used to cut the genomic DNA of seventeen test strains. Ideally, ribotype patterns should consist of several discrete bands spread across gels. It is evident from Figure 2 that *Eco* RV was the most suitable of the three enzymes as clear hybridisation fragments were obtained over a wide size range for most of the test strains. This enzyme, which has one or two restriction sites in the16S rRNA gene of members of *Nocardia* species, was chosen to examine seventeen test strains. In contrast, DNA extracted from the test strains digested with *Nco* I and *Pst* I was either too small to be separated into discrete bands or showed incomplete or no digestion.

The digoxigenin-labelled rDNA probe, which contained fragments of the 5S, 16S and 23S rRNA genes of *Streptomyces (lividans) violaceoruber* strain TK21, hybridised with 4 to 8 fragments of the *EcoR* V-cleaved chromosomal DNA of the test strains (Figure 2). The hybridised fragments, which ranged from around 20.7 to 1.2 kb in size, were well resolved and found to be reproducible in the repeat experiments. Each unique ribosomal DNA restriction profile was designated a ribotype (Figure 3).

Species specific patterns were seen for representatives of *Nocardia brasiliensis*, *Nocardia farcinica*, *Nocardia otitidiscaviarum* and *Nocardia* seriolae. *Nocardia pseudobrasiliensis* gave more than one ribotype pattern (Figure 4).

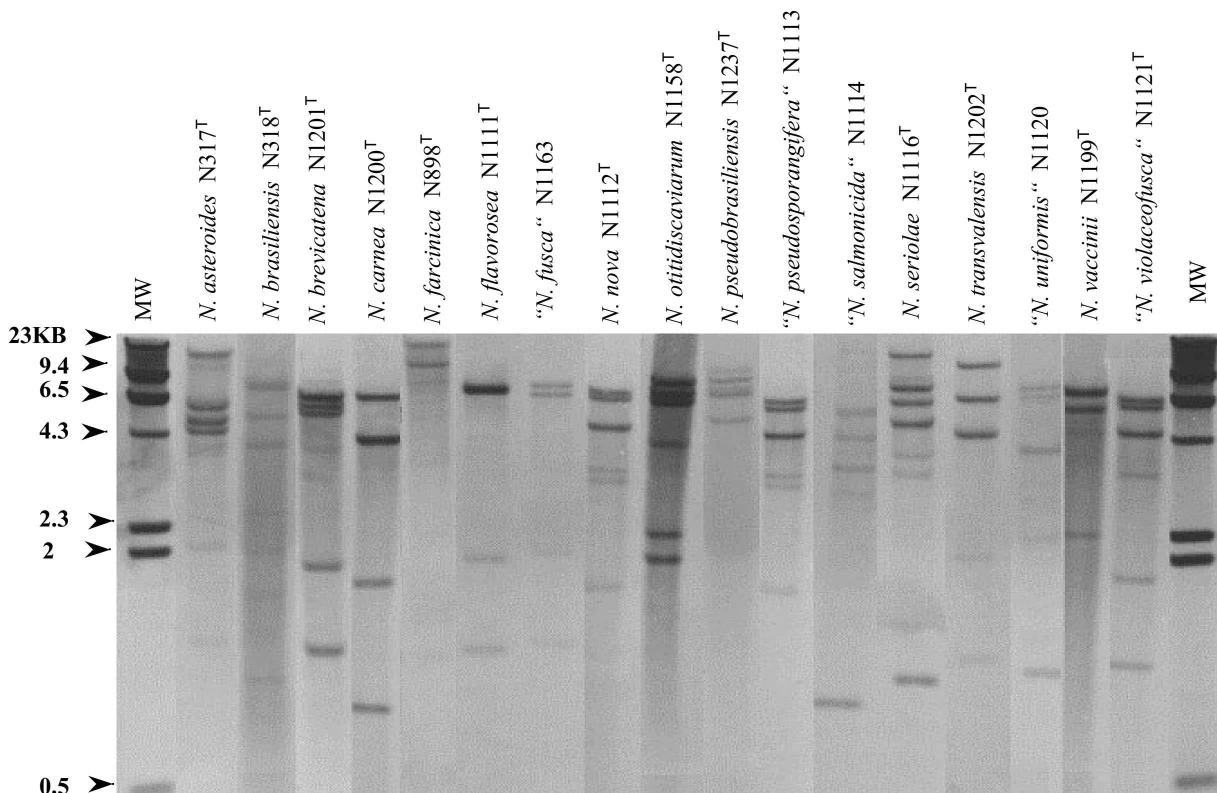


Figure 2. Ribotype patterns of representative nocardiae. The patterns were obtained by electrophoretic separation of *Eco* RV digests of genomic DNA and hybridisation with the digoxigenin-labelled rDNA probe (colorimetric detection). MW, the molecular weight marker was lambda DNA cleaved with *Hind* III.

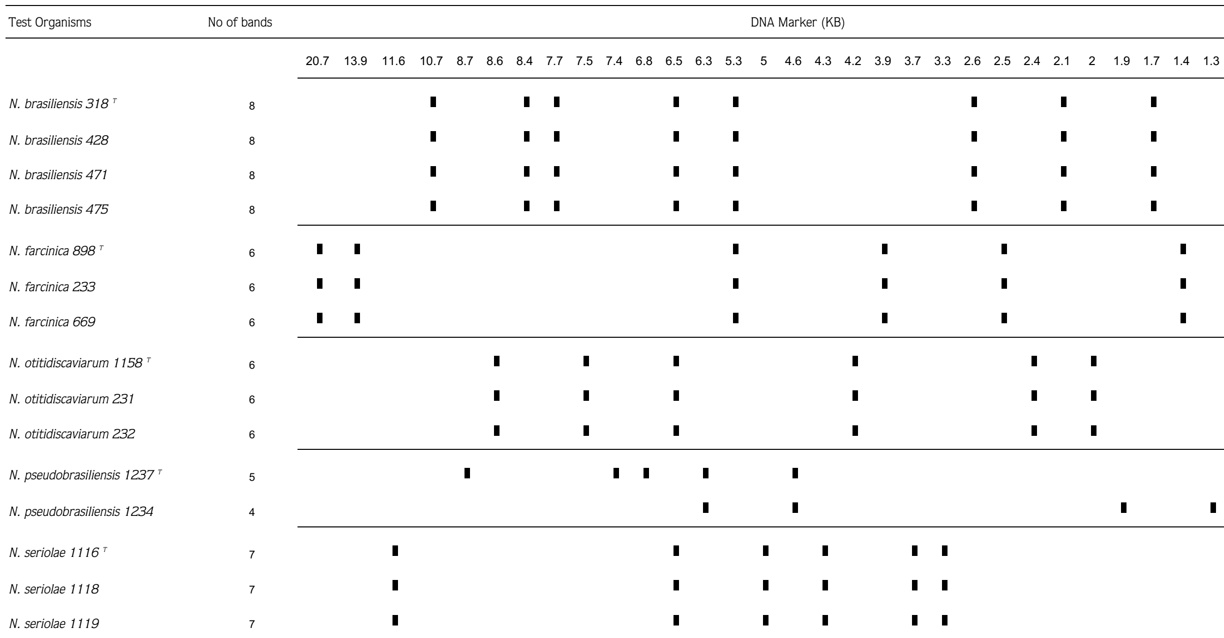


Figure 3. Normalised graph showing the migration patterns of rRNA gene restriction fragments after cleavage with *Eco* RV and hybrisidation with digoxigenin labelled probe.

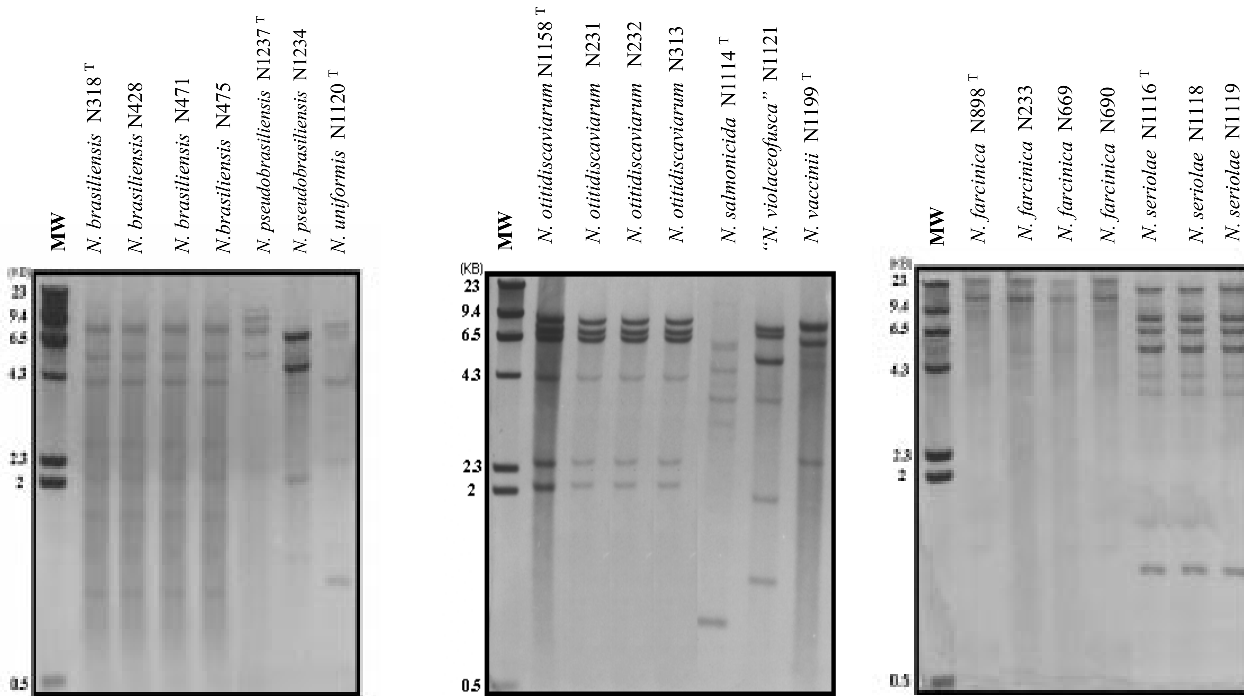


Figure 4. Ribotype patterns of representative strains *N. brasiliensis*, *N. farcinica*, *N. pseudobrasiliensis*, *N. otitidiscaviarum* and *N. seriolae*. The patterns were obtained following electrophoric separation of *Eco* RV digests of genomic DNA and hybrisidation with a digoxigenin-labelled probe (colorimetric detection). MW, the moleculer weight marker was lambda DNA cleaved with *Hind* III.

The ribotype patterns obtained for the representative test strains are in good agreement with corresponding, albeit preliminary, results from earlier studies which suggested that ribotyping might be of value in the classification and identification of nocardiae (20, 21). Similar conclusions have been drawn from studies on members of other actinomycete taxa, including representatives of the genera *Corynebacterium* (26), *Mycobacterium* (27), *Rhodococcus* (17) and *Streptomyces* (18).

It is also clear from the earlier numerical taxonomic studies that *Nocardia brasiliensis* (28-34), *Nocardia farcinica* (28, 31, 33-35), *Nocardia otitidiscaviarum* (28-30, 32, 34, 36, 37) and *Nocardia seriolae* (34) form good taxospecies. *Nocardia pseudobrasiliensis* was proposed by Ruimy *et al.* (38) for organisms previously classified as *Nocardia brasiliensis*. However, comparative molecular systematic work is needed on additional strains of *Nocardia pseudobrasiliensis* to evaluate the internal taxonomic structure of this species.

As a result, bacterial systematics began as a largely intuitive science, but has become increasingly objective due to the development and application of chemotaxonomic, molecular systematic and numerical phenetic methods. The new advances, especially in molecular systematics, lead to the need to compare older and more recent approaches to bacterial classification (39, 40). This exercise promoted the view that bacterial classification at all levels in the taxonomic hierarchy should be based on the integrated use of genotypic and phenotypic data (41). This approach, known as polyphasic taxonomy, was introduced by Colwell (42) to signify successive or simultaneous studies on groups of organisms using a set of taxonomic procedures designed to yield good quality genotypic and phenotypic data (43, 44). Typing systems are becoming increasingly important for characterising microorganisms. They are applied in epidemiologic investigations of disease outbreaks to identify a potential common source and to determine likely mechanisms of disease transmission. The results of these studies suggest that ribotyping might be used to clarify taxonomic relationships within and between clinically significant nocardial species.

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