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ISOLATION AND DISTRIBUTION OF NOCARDIAE IN THE BAS-ZAIRE

by

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Summary — 93 nocardioform bacteria were isolated from the 400 specimens collected in the different ecological areas studied in the Bas-Zaïre region. Sugarplantation yielded the greatest number of bacteria, while the coastal environment of Moanda produced only one strain identified as *N. brasiliensis*. No nocardiae were isolated from sea water. Soil and mud contain the largest number of bacteria whereas water and grass contain only few. The identification tests applied did not allow to determine all the strains isolated. Nineteen strains remained unidentified. The most frequently isolated species is *N. asteroïdes*.

KEYWORDS : Nocardia; Nocardia asteroïdes; Classification; Zaïre Republic.

1. Introduction

Nocardiae are widely distributed in nature. They have been isolated from a wide variety of habitats such as soil, mud, fresh water, sea water etc. (McClung, 1960; Kurup, Randhawa and Sandhu, 1968; Kumar and Mohapatra, 1968). However, literature is poor concerning isolation and distribution of nocardiae in Central Africa.

The purpose of this work concerns nocardiae found in nature in Zaïre, especially in the region of the Bas-Zaïre. Practical schemes easy to perform and applied to a great number of strains, are proposed for identification of the most important species within the genus Nocardia.

2. Material and Methods

2.1. *Origin and nature of the samples*

400 samples were collected from different ecological areas of the Bas-Zaïre :

100 from surroundings of Kinshasa,

50 from a sugarcane plantation (Kwilu-Ngongo),

100 from the savanna between Kinshasa and Matadi,

100 from Mayumbe forest,

50 from coastal environment (Moanda).

Samples of soil, mud, grass and water were collected from these different ecological settings.

In the surroundings of Kinshasa, in the savanna and in Mayumbe forest, 25 samples of soil, mud, grass and water were collected.

In the surgarcane plantation, 25 samples of soil and mud were collected. in Moanda, 25 samples of soil and seawater were collected (table 4).

2.2. *Preliminary treatment of the samples*

2.2.1. Water : 50 ml water were centrifuged during 10 min, at 3.000 r.p.m. The sediment was suspended in 2 ml of sterile A. D.

2.2.2. Grass : samples of 2 to 3 g were cut into small pieces, ground in a mortar with sterile sand and suspended in 2 ml of sterile A. D.

2.2.3. Mud and soil : samples of 0.5 to 1 g did not undergo any preilminary treatment.

2.3. *Isolation of the strains*

The Nocardiae were isolated by the method described by McClung slightly modified, using the paraffin bait technique (McClung, 1960).

Each sample was suspended in a tube containing 5 ml of the sterile carbon free medium. Into each tube a glass rod, coated with sterile paraffin, was introduced. The tubes were then incubated in an incubator at 28 °C and observed weekly until growth appeared on the paraffin near the surface of the liquid medium. Fragments of growth were transferred into bijou bottles containing saline and glass beads, agitated for 2 min on a Vortex type apparatus and a loopful of that suspension was streaked on a plate of nutrient agar. The plates were incubated at 28 °C until colonies with aerial hyphae appeared. Different colonies were then transferred on slants of Löwenstein - Jensen medium in cotton plugged tubes.

When growth on Löwenstein - Jensen medium occurred, this was again streaked on nutrient agar plates to control the purity of the strain.

2.4. *Identification of the strains*

The easiest tests to perform on a great number of strains were chosen, in regard with existing equipment and arduous supply in an african laboratory. In order to include internal controls, as was done previously with identification procedures for mycobacteria (Pattyn and Portaels, 1972), each identification was based on more than 1 test.

2.4.1. *Identification of the genus Nocardia*

The genus Nocardia was recognized by some of the tests proposed by Goodfellow (1973) and Goodfellow *et al.* (1974). They are listed in table 1.

TABLE 1

Identification of the genus Nocardia

	Nocardia	Mycobacterium	« M » rhodochrous
Mycelium (1)	+	−	±
Aerial hyphae (1)	+	−	−
Lysozyme resistance (1)	+	−	−
Carbon substrate :			
Na succinate (1)	+	−	+
Sorbitol (1)	−	±	+
Sucrose (2)	−	±	+

(1) Data from Goodfellow, 1973.
(2) Data from Goodfellow *et al.*, 1974.

2.4.1.1. Morphological tests

Macroscopic morphology was observed after 2 to 3 weeks on Löwenstein. Smears of these cultures were stained by the Ziehl - Neelsen method for examination of microscopic morphology.

Colonial morphology and production of aerial hyphae were observed on nutrient agar plates after 1, 3, 7 and 14 days of incubation at 28 °C.

2.4.1.2. Resistance to lysozyme

Resistance to lysozyme was observed after the method described by Berd (1973) using a solution of 0.1 g lysozyme (Sigma Chemical compagny) in 100 ml 0.01 N-HCl. Five ml of this solution were mixed with 95 ml of sterile glycerol broth and dispensed in 2,5 ml amounts in sterile plugged tubes. These tubes and control tubes of glycerol broth were inoculated with one drop of a culture suspension.

2.4.1.3. Utilisation of sodium succinate, sorbitol and sucrose as single carbon sources

The basal medium described by Gordon and Mihm (1957) was used.

Concentrations of the compounds were sodium succinate 0.1 per cent, w/v, sorbitol 1 per cent, w/v and sucrose 1 per cent, w/v (Goodfellow, 1971, 1973 and Goodfellow *et al.*, 1974).

Two control tubes were included; the first contained the basal medium without any carbon source, the second contained basal medium with glucose (0.1 per cent, w/v). A positive result was recorded if growth in the test tube was equal or greater than that in the control tube plus glucose, and negative when growth was equal or less than that on the control tube without any carbon source.

2.4.2. *Identification of the nocardiae*

Differentiation of Nocardia species was performed using some of the tests proposed by Goodfellow (1971). They are listed in table 2.

2.4.2.1. Hydrolysis of organic compounds

Decomposition of caseine, tyrosine and xanthine were studied by the method described by Gordon *et al.* (1962).

TABLE 2

Identification of Nocardia species

	N. asteroïdes	*N. caviae*	*N. brasiliensis*
Decomposition of :			
Caseine	–	–	+
Tyrosine	–	–	+
Xanthine	–	+	–
Acid from Mannitol	–	+	+
Carbon substrate :			
Testosterone	+	+	–

2.4.2.2. Acid production from mannitol

The medium and method described by Gordon *et al.* (1957, 1959) were used.

2.4.2.3. Utilisation of testosterone as single carbon source

The basal medium described by Gordon and Mihm (1957) was used. The concentration of the testosterone was 0.1 per cent, w/v (Goodfellow, 1971).

3. **Results**

Seventy-nine of the 400 specimens collected from the Bas-Zaïre were positive for nocardioform bacteria, 65 producing a single strain and 14 two different strains.

3.1. *Identification of the strains isolated*

Ninety-two strains were identified, one was lost. All strains produced a substrate mycelium fragmenting into rods and cocci, called « nocardioform » bacteria by Prauser (1967). The colonies of all strains were irregular, bearing sparse to abundant aerial hyphae and had filamentous margins. Among the 92 strains, 83 were resistant to lysozyme and 9 were sensitive to lysozyme.

3.1.1. *Nocardioform bacteria resistant to lysozyme*

Among the 83 strains resistant to lysozyme, one was lost and not tested. All these strains belonged to the genus Nocardia. They used Na succinate as sole carbon source and were unable to use sorbitol. These nocardiae could be subdivided into four groups according to the results of utilisation of testosterone as sole carbon source and production of acid from mannitol. These groups are the followings :

— Group I :

Mannitol : −
Testosterone : +

— Group II :

Mannitol : +
Testosterone : +

— Group III :

Mannitol : +
Testosterone : −

— Group IV :

Mannitol : −
Testosterone : −

3.1.1.1. Nocardiae belonging to group I

Group I (61 strains) corresponded to *N. asteroïdes*. Most strains were weakly acid-fast. Their colour was usually yellow, orange or pink and they carried sparse to abundant white aerial hyphae. Hydrolysis of caseine, tyrosine and xanthine were negative. Most strains (38) were unable to use sucrose as sole carbon source; 5 strains were able to grow on sucrose and 18 were not tested.

3.1.1.2. Nocardiae belonging to group II

The 8 strains belonging to this group were unable to grow on sucrose.

3.1.1.2.1. Three strains were identified as *N. caviae*

They were weakly acid-fast. In young cultures macroscopic appearance was very specific : colour varied from white to cream and colonies had a rough appearance. Frequently aerial hyphae could only be observed with the aid of a microscope.

Xanthine was hydrolysed, caseine and tyrosine were not.

3.1.1.2.2. Five strains belonged to group II, but were different from *N. caviae* strains

Hydrolysis of caseine, tyrosine and xanthine were negative. When young, macroscopic appearance of four of these strains was different from the young *N. caviae* strains : they ressembled much the *N. asteroïdes* strains with an orange to pink colour and abundant white aerial hyphae. The fifth strain differed from the other 4 strains in that it ressembled the young *N. caviae* strains : it was yellow, had a rough appearance and aerial mycelium could only be observed with the aid of a microscope.

3.1.1.3. Nocardia belonging to Group III

Only one strain belonged to this group, identified as *N. brasiliensis*. It was weakly acid-fast. The colony had a brown colour and abundant white aerial

hyphae were produced. On nutrient agar, colonies were slightly different from the other nocardiae isolated. They were extremely dense and the margins showed very short filaments. This strain was unable to grow on sucrose. It attacked caseine and tyrosine but not xanthine.

3.1.1.4.. Nocardiae belonging to group IV

From the 12 strains belonging to this group, 11 strains had the macroscopic appearance of *N. asteroïdes*, were unable to grow on sucrose and did not hydrolyse caseine, tyrosine and xanthine. One strain was very particular. It was brown and aerial hyphae were difficult to observe without a microscope. The colony on nutrient agar had long filamentous margins, short and sparse aerial hyphae. It used sucrose as a carbon source and hydrolysed caseine, tyrosine and xanthine.

3.1.2. *Nocardioform bacteria sensitive to lysozyme*

9 strains were sensitive to lysozyme.

3.1.2.1. Strains belonging to the rhodochrous group

4 strains were identified as *« M » rhodochrous.* They used Na succinate, sorbitol and sucrose as carbon sources and were unable to grow on testosterone. One strain produced acid from mannitol. Caseine, tyrosine and xanthine were not hydrolysed. They were weakly acid-fast. Macroscopically, colonies were smooth; 3 strains were red and produced sparse aerial hyphae, one strain was orange. These strains belonged to « group b » of the « *rhodochrous* complex ». (Goodfellow *et al.*, 1974).

3.1.2.2.

4 strains were very similar to the *Nocardia asteroïdes* group for all the characters studied except lysozyme resistance. We concluded that they were *N. asteroïdes* sensitive to lysozyme.

3.1.2.3.

One strain remained unidentified. It was weakly acid-fast and developed poorly on the culture media used. Colony morphology on nutrient agar was that of nocardiae : yellow, rough colonies with microscopic hyphae. Na succinate and sucrose were used as carbon source, acid was produced from mannitol, and caseine, tyrosine and xanthine were not hydrolysed.

3.1.3. *Overall results of identification*

See table 3.

3.2. *Distribution of the strains in the different ecological areas and in the different samples*

Table 4 indicates the origin of the strains isolated and the number of strains isolated in regard to the number of samples collected.

TABLE 3

Overall results of identification

	Number of strains
Strains resistant to lysozyme.	
group I : *N. asteroïdes*	61
group II : *N. caviae*	3
not *N. caviae*	5
group III : *N. brasiliensis*	1
group IV : N. sp.	12
Unidentified	1
Strains sensitive to lysozyme.	
« *M* » *rhodochrous*	4
N. asteroïdes	4
Unidentified	1
TOTAL	92

TABLE 4

Number of strains isolated (1) **and number of samples collected** (2) **in the different ecological areas**

	Water	Mud	Soil	Grass	Total
Savanna	2/25	10/25	15/25	0/25	27/100
Kwilu-Ngongo			24/25	1/25	25/50
Mayumbe	4/25	13/25	8/25	1/25	26/100
Moanda	0/25		1/25		1/50
Kinshasa	1/25	2/25	10/25	1/25	14/100
Total	7/100	25/75	58/125	3/100	93/400

(1) 1st number in each column.
(2) 2nd number in each column.

Table 5 indicates the origin of the strains according to their identification.

4. **Discussion**

4.1. *Method of isolation*

4.1.1. *The paraffin bait technique*

The paraffin bait technique is very easy to perform. However by this method, only strains able to grow on paraffin as sole carbon source can be isolated. Most nocardiae have the ability to grow on paraffin whereas many

TABLE 5

Origin and identification of the strains

	Strains resistant to lysozyme						Strains sensitive to lysozyme		
	Group I *N. asteroides*	Group II *N. caviae*	not *N. caviae*	Group III *N. brasiliensis*	Group IV N. sp.	Unidentified	« *M* » *rhodochrous*	*N. asteroides*	Unidentified
Savanna :									
water	2								
mud	9						1		
soil	12	3						1	
grass	1								
Kwilu-Ngongo :									
soil	19		4				1		
grass									
Mayumbe :									
water	2						1	1	
mud	5							1	1
soil	2		1	1	6			1	
grass	1				2				
Moanda :									
water									
soil									
Kinshasa :									
water					1		1		
mud	1				1				
soil	6				2	1			
grass	1								
TOTAL	61	3	5	1	12	1	4	4	1

other organisms such as *Actinomadura madurae*, *Actinomadura pelletieri*, (Mariat, 1958; Schniedau *et al.*, 1957; Georg *et al.*, 1973), various fungi and bacteria other than actinomycetales (Mishra *et al.*, 1973) cannot utilise paraffin as sole carbon source. Goodfellow (1971) mentioned that a few strains of *N. caviae* were unable to grow on paraffin and that with his techniques and media all *A. madurae* strains and 90 per cent of the *A. pelletieri* strains were able to utilise paraffin as a sole carbon source. The divergence, concerning *A. madurae* and *A. pelletieri*, between Mariat, Schneidau *et al.*, and Goodfellow may be the result of the differences among techniques and culture media used. The fact remains that the use of the paraffin bait technique introduces by definition a selective factor.

During this investigation by paraffin bait technique, actinomycetales other than nocardiae were isolated : in 50 per cent of the cases, fast growing mycobacteria, and two rough strains belonging to the « *M* » *rhodochrous* group without aerial hyphae (and for this reason not included in chapter 3). No strains belonging to the genus Actinomadura were isolated.

4.1.2. *Use of nutrient agar for isolation of the strains*

The method used led to the isolation of fast growing nocardioform bacterial exclusively. Indeed all colonies of nocardioform bacteria appearing on the nutrient agar plates had to be transferred after 1 to 7 days incubation to avoid overgrowth by various other microorganisms. This again introduces another selective factor.

4.1.3.

The selective factors due to the use of the paraffin bait technique of McClung (1961) and consequently to obtain a rapid transfer from the nutrient agar plates, can partly be avoided by the use of other isolation methods such as those known for the isolation of mycobacteria (Portaels, 1973). But a number of nocardioform bacteria are also killed following decontamination procedures by trisodic phosphate or oxalic acid (Portaels, 1973).

4.2. *Identification of the strains*

4.2.1. *Choice of identification tests*

Two factors determined the choice of the identification tests selected : a) tests had to be applicable to a great number of strains and, b) they had to be adapted to technical limitations typical of an African laboratory, even though more precision could have been obtained with a greater variety of identification tests.

Our results obtained using these identification tests correspond to those obtained by Goodfellow (1971). However, for utilisation of sucrose as sole carbon source, our results differ from those of Goodfellow *et al.*, (1974) : from the 86 strains recognized as Nocardiae (82 strains resistant to lysozyme and 4 *N. asteroïdes* sensitive to lysozyme), 6 strains grew on sucrose.

4.2.2. *Macroscopic morphology and acid fastness*

Acid fastness and macroscopic morphology are two characters very difficult to interpret. They depent on the culture medium used, the age of the culture and the age of the strain since its first isolation (Veldamp, 1970; Berd, 1973). These variations were also observed during the present study and were therefore not taken into consideration for the identification of the strains.

4.2.3. *Identification of nocardiae species*

The study was limited to three species *N. asteroïdes, N. brasiliensis* and *N. caviae* because they are the most important ones, many authors considering the other species as minor with an undetermined systematic position. For these reasons some strains still remain unidentified. However, in this study some comparison is possible between unidentified strains and minor clusters (Goodfellow, 1971) : The 5 strains of group II (other than *N. caviae*), growing on testosterone and producing acid from mannitol may be related to the *N. vaccinii* strains. The 12 strains of group IV unable to grow on testosterone and to produce acid from mannitol may also be related

to minor clusters of Goodfellow (1971); they are perhaps related to *N. hydroxycaroxydans*. Identification tests used did not allow the separation of the *N. asteroïdes* group into different subgroups. The African species *N. farcinica* was not recognized or not present in our samples.

4.3. *Distribution of the strains in the different ecological areas and in the different samples*

4.3.1. *Distribution of the strains in the different ecological areas*

Table 4 shows that the sugarplantation of Kwilu-Ngongo contains the largest number of nocardioform bacteria. The coastal environment, on the other hand, contains the lowest number. This might be due to the high concentration of NaCl in the coastal regions. Nocardioform bacteria are equally distributed in the savanna, the region of Kinshasa and the Mayumbe forest.

Differences observed in the ecological areas studied are statistically significant at the 0.001 level. Other kinds of plantations should also be studied in order to relate the abundance of nocardioform bacteria with the presence of sugarcane.

4.3.2. *Distribution of the strains in the different kinds of samples*

Mud and soil (table 4) contain larger numbers of nocardioform bacteria than grass and water. Differences are statistically significant. The lower abundance of nocardioform bacteria in water and grass could be explained by the techniques used; perhaps the collected specimens were to small. However it is well known that nocardioform bacteria are most frequent in soil.

4.3.3. *Frequency of the nocardia species isolated*

The most frequently isolated species is *N. asteroïdes*. Its distribution in the ecological areas and in the different samples (table 5) was similar to the distribution of the nocardioform bacteria (table 4). The high frequency of isolation of *N. asteroïdes* in regard to other species corresponds to the frequency of *N. asteroïdes* isolated from clinical material (Berd, 1973), although this may be the result of the selective factors introduced by the isolation technique as discussed above.

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Résumé — **Isolement et distribution des Nocardiae au Bas-Zaïre.**

Des 400 échantillons recoltés dans l'environnement du Bas-Zaïre, 93 bactéries nocardioformes ont été isolées. Les plantations de cannes à sucre ont fourni le plus grand nombre de bactéries tandis que l'environnement marin n'en a fourni qu'une seule; cette souche, provenant de sable de la plage, fut identifiée *N. brasiliensis*. Aucune bactérie nocardioforme n'a été isolée de l'eau de mer. Le plus grand nombre de bactéries a été isolé du sol et de la boue, l'eau et les plantes ne fournissant que très peu de bactéries nocardioformes.

Dix-neuf souches sont restées indéterminées, les tests d'identification appliqués n'ayant pas permis l'identification de toutes les souches isolées.

L'epèce la plus fréquemment isolée fut *N. asteroïdes*.

Samenvatting — **Isolering en verspreiding van Nocardiae in Beneden-Zaïre.**

Van de 400 stalen, verzameld in de omgeving van Beneden-Zaïre werden 93 nocardioforme bacteriën afgezonderd.

De suikerrietplantages bevatten het grootste aantal bacteriën, in tegenstelling met de maritieme omgeving die slechts één enkele bakterie opleverde, deze stam die voortkwam uit strandzand werd geïdentificeerd als *N. brasiliensis*. Geen enkele nocardioforme bacterie werd geïsoleerd uit zeewater. Het grootste aantal bacteriën werd geïsoleerd uit de bodem en uit het slijk, het water en de planten leverden slechts weinig nocardioforme bacteriën op.

Negentien stammen bleven niet geïdentificeerd, de toegepaste identificatie tests lieten niet toe alle geïsoleerde stammen te identificeren. De meest frekwent geïsoleerde species is *N. asteroïdes*.

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