

Laboratory Characterization, Identification, and Susceptibility Testing of *Nocardia* Based on Current Molecular Taxonomy

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Isolation and Recovery of Nocardia

- **~76 species of Nocardia currently recognized (2010)**
Approx. 80% described within past 10 years
- **Approximately half pathogenic to humans/animals**
- **Environmental: soil, dust, water, decaying organic matter**

Nocardia

Broad Spectrum of Disease

Localized

Pulmonary

Meningitis

Disseminated

Nocardia/Laboratory Diagnosis

- Macroscopic exam
May produce “Sulfur Granules”, grossly purulent
- Microscopic exam
Stained smears: Gram/Kinyoun Stains
Beaded Gram positive rods, thin,
branching, filamentous, often in clumps
- Usually no other organisms seen on smear;
growth inhibited by other organisms
- Acid fastness not consistent

McNeil and Brown JM., *Man. of Clin. Micro.* 8th edition. 2003.

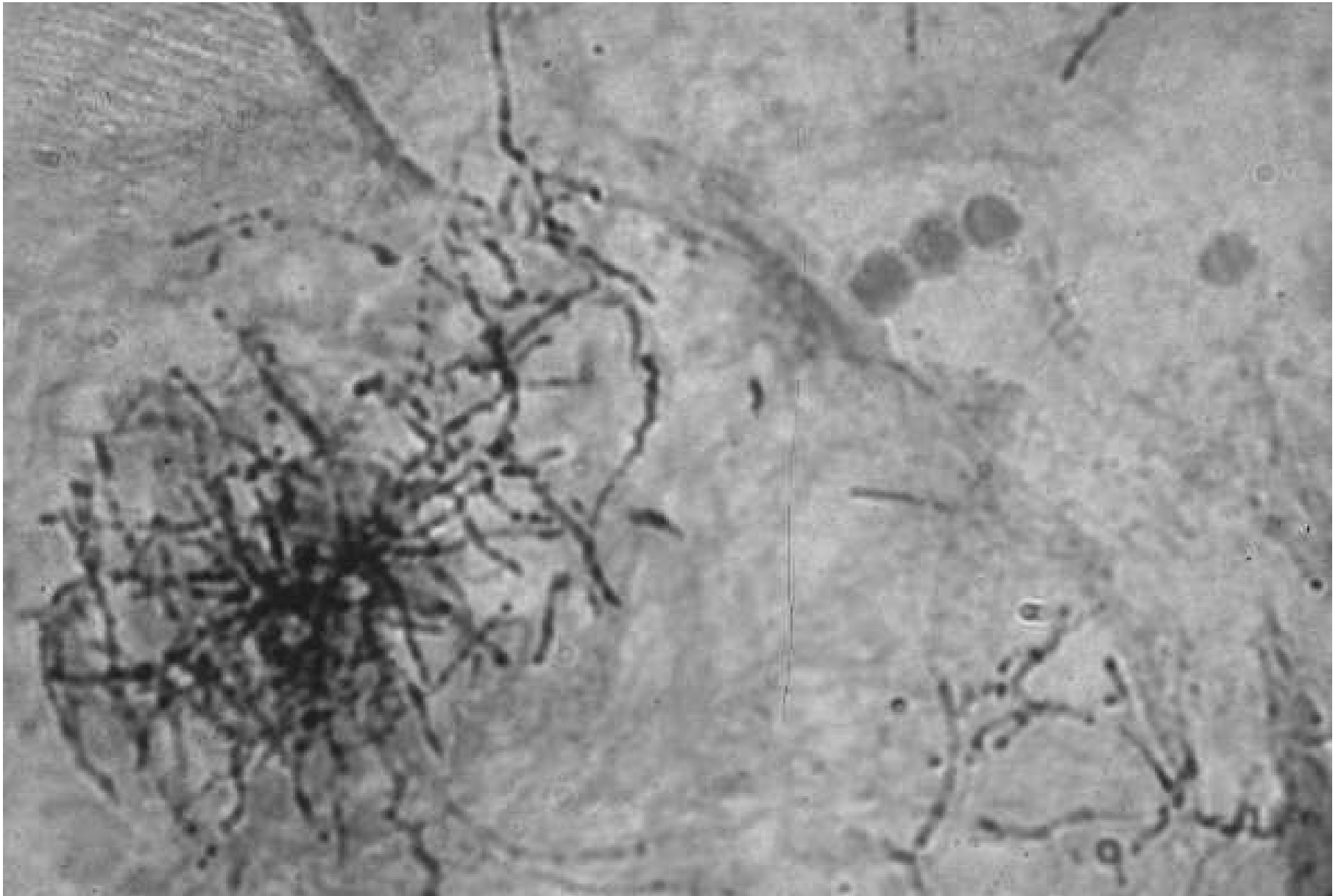
Microscopic Examination (Direct Examination)

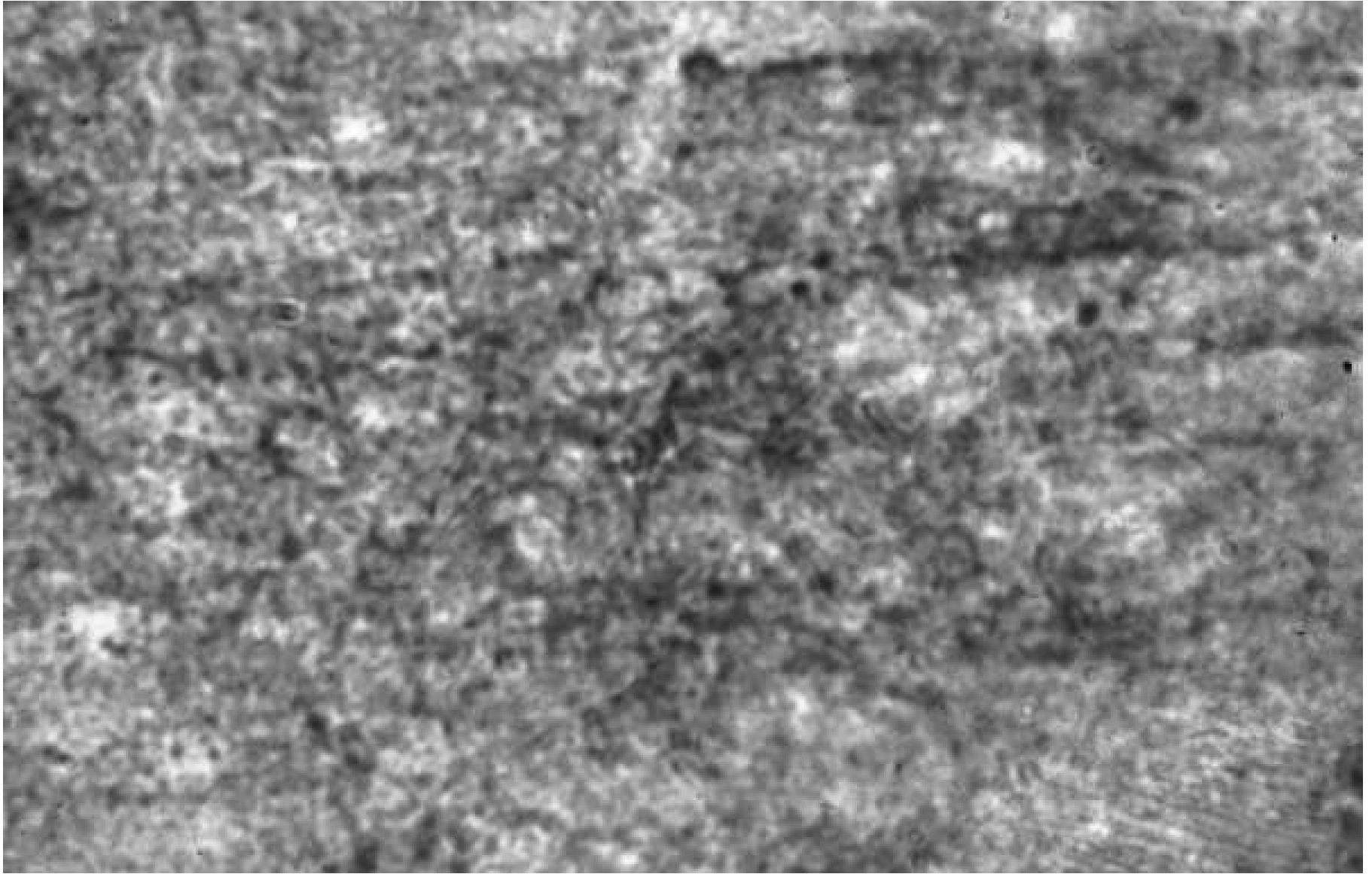
Centrifuge BAL, body fluids

Grind tissue

- ❖ Gram stain (+) beaded, branching thin filamentous rods; background of PMNs.

- ❖ Kinyoun Modified Acid Fast
(1% H₂SO₄)
Partially AF filamentous





Microscopic Examination (Cultured Material)

Results related to:

Type of media

Age of culture

Incubation temperature

Delicate branching filaments to
fragmented filamentous rods,
coccobacilli

Microscopic Examination

(cont'd)

Histopathology

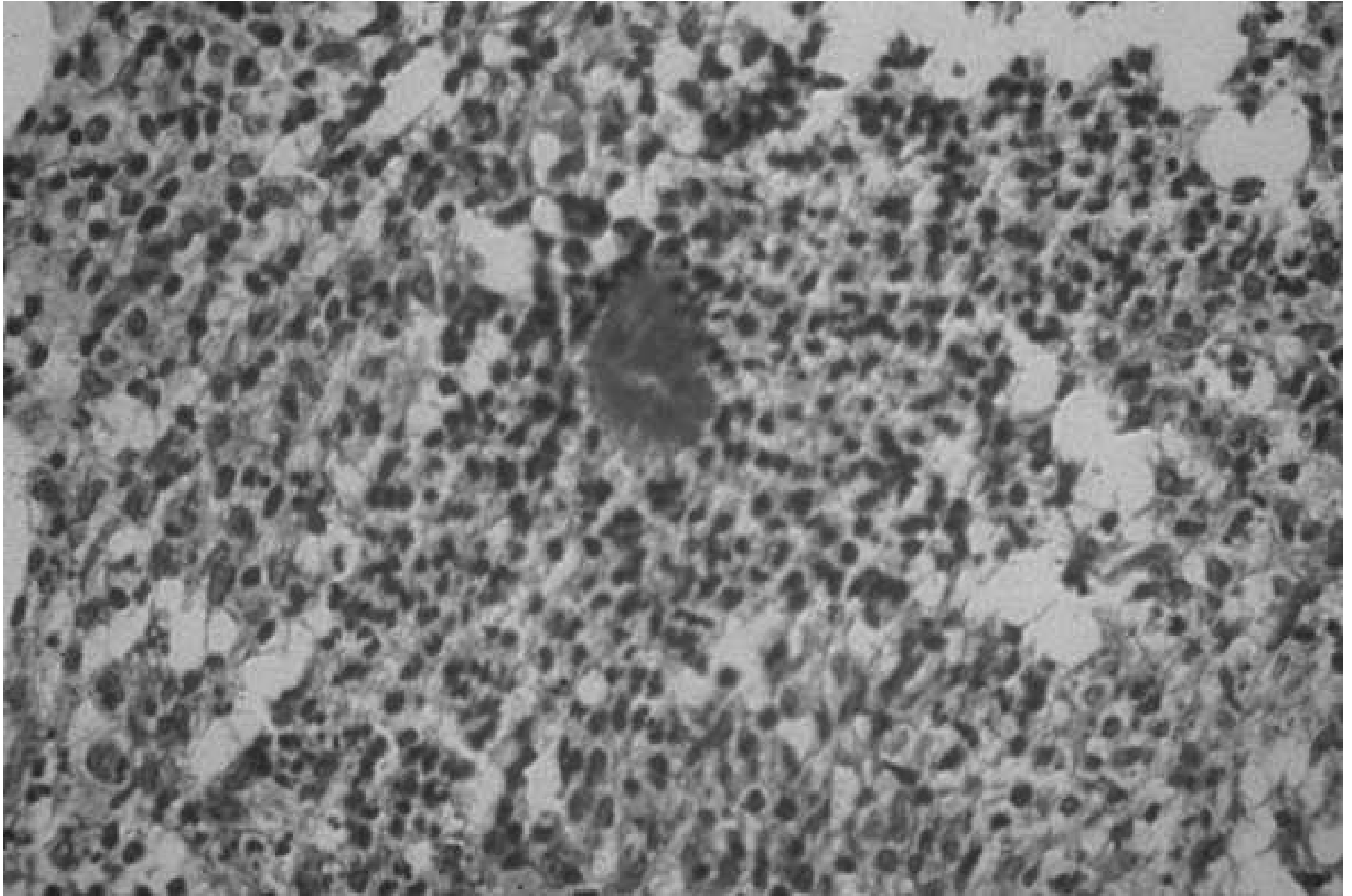
Similar to cultured material

Many PMNs, no granulomas

Sulfur granules (mycetoma)

Common in *N. brasiliensis*; rare in other species

Fite-Faraco Acid Fast Stain in Tissue



Recovery of Nocardia

Decontamination Procedure for AFB

- May decrease yield of Nocardia
 - Lower pH
 - Shorten exposure to decontamination reagents

Recovery of Nocardia

**Incubation – Extended period at least
2 weeks**

25 – 30° / 35° C

Examine daily 1st week, then weekly
(Dissecting scope)

Recovery of Nocardia

Direct Inoculation

Blood, Choc, TSA, BHI, Sab-Dex, LJ,
MB710, 7H11

Buffered charcoal yeast agar (BCYE)

Selective Media: Thayer Martin, BCYE +
Polymyxin B, vancomycin, cefamandole,
SAB-Dex + Chloro may suppress Nocardia

Recovery of Nocardia

(cont'd)

Blood Culture Systems **(approx. 3 – 19 days)**

Conventional

Biphasic

Radiometric / Non-radiometric

Lysis Centrifugation

History of Nocardia

- 1888 – Nocard isolates filamentous aerobic organism from cattle with farcy
- 1888 – Rabe isolates same organism from dog
- 1889 – Trevisan created genus “Nocardia”
N. farcinica (cattle)
- 1890 – Eppinger isolated similar organism from human with fatal infection
- 1898 – 1st human disease “pseudotuberculosis” (pulmonary) in U.S.A.

History of Nocardia (cont'd)

1947 – 1st successful treatment with sulfonamides

1970s – Uncertain taxonomy

Deleted *N. farcinica*

Adopted *N. asteroides* as type species

1980s – Heterogeneity of “*N. asteroides* complex”

1988 – Term “*N. asteroides* complex” introduced to recognize 7 groups characterized by MICs, β -lactamase, biochems, PRA (*hsp 65*)

2000 – Recognition of multiple complexes

History of Nocardia

(cont'd)

Types I, II, VI, VII – No names

Type III – *N. nova*

Type IV – *N. transvalensis* complex

Type V - *N. farcinica*

Wallace, et al., Antimicrob. Agents Chemother. 1988; 32:1776.

Steingrube, et al., J. Clin. Microbiol. 1995; 33:3096

Conventional ID of Nocardia

- Morphological/Physiological Characteristics
- Smooth to chalky appearance; aerial hyphae
- Growth 48-72 hrs
- Meso-diaminopimelic acid
- Short chained mycolic acids, fatty acids
- Phospholipids, Arabinose, Galactose
- Tuberculostearic acid
- DNA G + C content 64 – 72%

Molecular ID of Nocardia

Conventional techniques no longer adequate for definitive identification

Recognition of Nocardia complexes

When to call it a species or a complex?

Species – Gene sequencing unambiguously identifies an isolate as a distinct species

Complex – Gene sequencing and/or other testing (PRA/susceptibility) indicates it is closely related, or has similar characteristics to a previously identified complex.

Important to communicate what species are included in a complex.

Species vs. Complex

- Species – Isolate is identical or nearly identical to the type strain of a validly-described species.
- Sequence similarity to the type strain is dependent on the gene sequenced.
- Guidelines for species similarity varies by genus.

FORMERLY

“N. asteroides complex”

<u>Drug Pattern</u>	<u>Molecular ID</u>	<u>%</u>
Type I	<i>N. abscessus</i>	20
Type II	“Related to: <i>N. brevicatena</i> , <i>N. paucivorans</i> , <i>N. carnea</i> , <i>N. sienata</i> , <i>N. testacea</i> ”	<1
Type III	<i>N. nova</i> complex	18
Type IV	<i>N. transvalensis</i> complex (<i>N. wallacei</i>)	5
Type V	<i>N. farcinica</i>	17
Type VI	<i>N. cyriacigeorgica</i>	35
Type VII	Misc. (ATCC 19247T)	5

Wallace, et al., *Antimicrob. Agents Chemo.* 32: 1776, 1988.
Steingrube, et. al., *J. Clin. Micro.* 33:3096, 1995.

16S rRNA Gene Sequencing

- Also called 16S rDNA
- Most common sequencing tool
- Present in all bacteria
- Approximately 1550 bases in length
- Contains conserved and variable regions
- Participates in a critical cellular function
- Minimal changes over time so it's a good measure of phylogenetic relationships

16S rRNA Gene Sequencing for *Nocardia* species

- Standard universal primers can be used for amplification
- Many conserved regions allow for effective primer selection
- Variable regions allow discrimination of many species

500-bp Sequencing of the 16S rRNA Gene

- Based on the sequence variability of the first 500 bases of the gene
- Popular technique because only one primer set is needed
- Care must be exercised because of the possibility of misidentifications of some species

Nocardia species that cannot be differentiated by 500-bp sequencing

N. abscessus – *N. asiatica* – *N. arthritidis*

N. africana – *N. elegans* – *N. veterana*

N. exalbida – *N. gamkensis**

N. higoensis – *N. shimofusensis*

*Not described as a human pathogen

12 Species More Frequently Cause Human Disease

N. abscessus

N. otitidiscaviarum

N. brasiliensis

N. paucivorans

N. brevicatena

N. pseudobrasiliensis

N. cyriacigeorgica

N. transvalensis

N. farcinica

N. veterana

N. nova

N. wallacei

SCIENTIFIC /SCIENTIFIQUE

Identification of Nocardia species by Partial Sequencing of the 16S rRNA gene: The Sun Sets on *Nocardia asteroides*

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Nocardia species are opportunistic pathogens that cause a variety of human infections. Recent descriptions of new species from environmental sources and human infections make maintaining a comprehensive biochemical test panel database difficult and 16S rRNA gene sequencing has been proposed as an alternate means of identification. We acquired 16S rRNA gene sequences for 49 valid and six putative *Nocardia* species and 23 taxonomic groups from GenBank and used these sequences to identify 100 clinical and 88 reference *Nocardia* isolates. Our database identified 98% of the clinical *Nocardia* isolates with *N. farcinica* (24 isolates) being the most frequently identified species followed by *N. cyriacigeorgica* (23 isolates) and *N. nova* (22 isolates) representing 69% of all *Nocardia* species sent for identification. *Nocardia* species validly described since the year 2000, including six isolates of *N. beijingensis* which is the first report of this species from human clinical material, comprised 42% of all isolates indicating the necessity for a current and comprehensive database. Of 64 isolates submitted to us as *Nocardia asteroides* or *N. asteroides* complex, only a single isolate was identified as *Nocardia asteroides*, suggesting that the previously most commonly reported *Nocardia* species is actually a rare cause of human infection.

Keywords: 16S ribosomal RNA, Nocardiaceae, Nocardia infections, Molecular diagnostic technique

Résumé

Les espèces *Nocardia* sont associées à des agents pathogènes opportunistes responsables d'une variété d'infections chez l'humain. Des descriptions récentes de nouvelles espèces provenant de sources environnementales et d'infections humaines compliquent la tenue d'une base de données détaillée d'un panel d'examen biochimiques et le séquençage des gènes ARNr 16s a été proposé comme moyen d'identification alternatif. Nous avons obtenu des séquençages de gènes ARNr 16s pour 49 espèces valides et six espèces reconnues de *Nocardia* ainsi que 23 groupes taxonomiques provenant de la base de données GenBank et nous avons utilisé ces séquences pour identifier 100 isolats cliniques et 88 isolats de référence *Nocardia*. Notre base de données a identifié 98% des isolats cliniques de *Nocardia*, *N. farcinica* (24 isolats) étant l'espèce la plus fréquemment identifiée, suivie de *N. cyriacigeorgica* (23 isolats) et *N. nova* (22 isolats) représentant 69% de toutes les espèces *Nocardia* soumises pour identification. L'espèce *Nocardia*, décrite valablement depuis l'année 2000, y compris six isolats de *N. beijingensis*, soit le premier rapport de cette espèce provenant de matériel clinique humain, comprenait 42% de tous les isolats, indiquant la nécessité d'une base de données actuelle et détaillée. Des 64 isolats qui nous ont été présentés comme étant des complexes *Nocardia asteroides* ou *N. asteroides*, un seul isolat a été identifié comme étant *Nocardia asteroides*, suggérant que l'espèce *Nocardia* la plus courante rapportée précédemment est en fait une rare cause d'infection chez l'humain.

“*Nocardia asteroides*”

ATCC 19247T *N. asteroides*

- Former “Miscellaneous” (Type VII?)
- Only rare strains molecularly identical to *N. asteroides* found in clinical samples to date
- Susceptible to Ampicillin, Amikacin, Ceftriaxone, Imipenem
- Resistant to Amoxicillin Clavulanic Acid, Clarithromycin, Ciprofloxacin



“*Nocardia asteroides* Type II”

- Former *N. asteroides* Type II (currently unnamed), related to *N. brevicatena*, *N. paucivorans*, *N. carnea*, *N. sienata*, *N. testacea* complex
- 16S rRNA gene sequences 99.6% similar (*N. brevicatena*/*N. paucivorans*)
- Distinct species by DNA-DNA hybridization
- Same as Type I but Kanamycin MICs $\leq 1 \mu\text{g/mL}$; often resistant to Gentamicin
- Rare human pathogens

Yassin, et. al. *Internat. J. Sys. Evol. Micro* 50:2000.

N. nova complex

- First described by Tsukamura, 1982. Submitted ATCC 33726T, ATCC 33727
- Biochemicals
 - ❖ (+) 14 d Arylsulfatase
 - ❖ Relatively asaccharolytic
- Only group suscept. to Ery_S/Clari_R
- Unusual inducible β -lactamase (Ampicillin , AmoxClav)
- Phenotypic characteristics alone do not reliably separate different species within *N. nova* complex

Wallace, et. al. *J. Clin. Micro.* 29:1991.

Conville and Witebsky, *Clin. Micro. News.* 26:2004

N. nova complex

- Formerly Type III *N. asteroides*
- ATCC 33726T = *N. nova*
- ATCC 33727 = *N. veterana*
- Currently includes *N. nova*, *N. veterana*, *N. africana*, *N. kruczakiae*, *N. elegans*, multiple unnamed taxa.
- *N. africana*, *N. elegans*, *N. veterana* can't be differentiated by 500 bp 16S rRNA gene sequencing.

Yano, et. al. *Internat. J. Syst. Bact.* 40:1990.

Conville, et. al. *J. Clin. Micro.*, 42:2004.

***N. transvalensis* complex**

- Composed of multiple species/taxa
 - Formerly Type IV *N. asteroides*
 - *N. transvalensis* ATCC 6865T
 - New unnamed taxa
- All generally R to all aminoglycosides
- *N. transvalensis* sensu stricto
- *N. wallacei* (formerly Type IV *N. asteroides*)
- *N. blacklockiae*

Patel, et. al, JCM, 2004


Conville, et. al., JCM 2008

N. cyriacigeorgica complex?

- 2000 Yassin described from bronchial secretions
- Type strain DSM 44484T
- 16S rRNA gene sequence identical to formerly *N. asteroides* Type VI, reference strain ATCC 14759T
- Difficult to ID by phenotypic methods alone
- Resistant to Ampicillin, Amox-Clav, Clari, Cipro
- Common pattern in USA, probably represents species known as *N. asteroides* previously

Yassin, et al. *Int. J. Syst. Evol. Micro.* 51:1419-1423. 2001.

***N. otitidiscaviarum* complex ?**

- Formerly “*N. caviae*”
- Former ID by hydrolysis (Xan, Hypoxan)
- Genetic heterogeneity of species/”Complex”
- Phenotypic characteristics alone usually not sufficient to ID
- Antimicrobial Susceptibility:
R to -lactams (Ampicillin, Amox/Clav),
Imipenem, Cephalosporins
S to Amik, Cip, Sulfa, Kana


Patel, et. al., *J. Clin. Micro.* 42:2530-2540, 2004.

***N. beijingensis* complex?**

Sequences of isolates of this group are similar, but not identical to the type strains of the species. May ultimately be split into several different species.

Yassin, et.al. *Internat. J. Syst. Evol. Micro.*50:2000.

Nocardia abscessus

- Yassin, 2000 (Formerly Type I Drug Susceptibility Pattern, ATCC 23824)
Type strain DSM 44432T
- Unique β -lactam susceptibility pattern
 - ❖ S to Amp, Amox Clav, Cefotax, Ceftriax
 - ❖ IPM –hi MICs (8-32 g/ml)
 - ❖ Also usually S to SMX, GM, Kana, Amik
R to Cip, Ery, Clari

Yassin, et.al. *Internat. J. Syst. Evol. Micro.*50:2000.

N. farcinica

- Morphologically, genetically distinct
- ATCC 3318T
- ATCC 3308, Preceptrol Strain (Prev. *N. asteroides*)
- Distinctive drug pattern
- ❖ R to all AMG except Amikacin
- ❖ R to β lactams including Broad Spectrum Cephs
- Circular Chromosome Genomic Sequence >6 Million Base Pairs and 5674 putative proteins

Wallace, et al. *J. Clin. Micro.* 28:1990.

Ishikawa, et. al. *Proc. Natl. Acad. Sci.* 101:2004.

N. brasiliensis

- Primary cutaneous infections
- Previous ID by hydrolysis (casein, tyrosine)
- Typically Cipro, Clari resistant; Most S to Amox/Clav, Mino
- 1996 study showed some isolates previously ID as *N. brasiliensis* were different
 - ❖ + Hydrolysis of Adenine
 - ❖ - Nitrate Reduction
 - ❖ Tetracycline (minocycline) resistance
 - ❖ Most often associated with extra-cutaneous infection
 - ❖ Different Iso-electric patterns (R to Imipenem, Ceph, Cefoxitin, Benzyl penicillin)
 - ❖ Different HPLC pattern

N. pseudobrasiliensis

- Primary extracutaneous infection
- Different PRA pattern from *N. brasiliensis*
- By DNA-DNA hybridization proved to be distinct species
- Typically Cipro, Clari susceptible, most resistant to Amox/Clav and Mino

N. brasiliensis pseudobrasiliensis **Antimicrobial Susceptibility Pattern**

<u><i>N. brasiliensis</i></u>		<u><i>N. pseudobrasiliensis</i></u>
Cipro	R	S
Clari	R	S
Kana	R	R
Mino	S/I	R
AmoxClav	S	R
SMX	S	S

Wallace, et al. *J. Clin. Micro.* 33:1528-1533, 1995.

Wallace, et al., *J. Infect. Dis.* 156:959-966, 1987

Identification of *Nocardia* sp. by Other Genes


- *secA* 1 “housekeeping gene” that codes for essential protein, *secA* 1
- *secA* 1 is a key component of protein secretion across cytoplasmic membrane
- Range of similarity among type strains
85.0 – 98.7%
- Short region (~470 bp) clearly discriminates all pathogenic species studied to date

Identification of *Nocardia* sp. by Other Genes (cont'd)

- *gyr B* encodes β subunit of DNA gyrase, a type II DNA topoisomerase
- Interspecies similarities range from 82.4 – 99.9% [3X greater than 16S rRNA gene (94.4 – 100%)]
- Phylogenetic relationships similar to those with 16S rRNA gene
- 12 primers amplify ~ 1200 bp

Takeda et al., *J. Med. Microbiol.* 2010; 59:165.

Other Genes

- *sod* (Superoxide dismutase)
- *rpo*  (RNA polymerase beta subunit)
- 16S – 23S ribosomal RNA intergenic spacer region

Entries in GenBank, no description in literature

Pyrosequencing

- Based on presence of variable gene regions unique for a particular species
- 44 isolates from 7 species and 1 complex
- *N. abscessus* 3/3; *N. farcinica* 5/5, *N. brasiliensis* 7/7; *N. otitiscaviarum* 0/1; *N. cyriacigeorgica* 8/10; *N. transvalensis* complex 3 / 4; *N. pseudobrasiliensis* 1 / 2; *N. nova* 2/12
- Targets 2 areas, the “Variable region” and a second region that discriminates between *N. farcinica* and *N. cyriacigeorgica*

Tuohy, et al. Abstract 2007 ASM General Meeting

PCR *hsp 65*

4 restriction enzymes: *BstE* II, *Msp* I,
BsaH I, *Hinf* I

N. farcinica

N. brasiliensis

N. pseudobrasiliensis

N. asteroides Type II

N. transvalensis complex

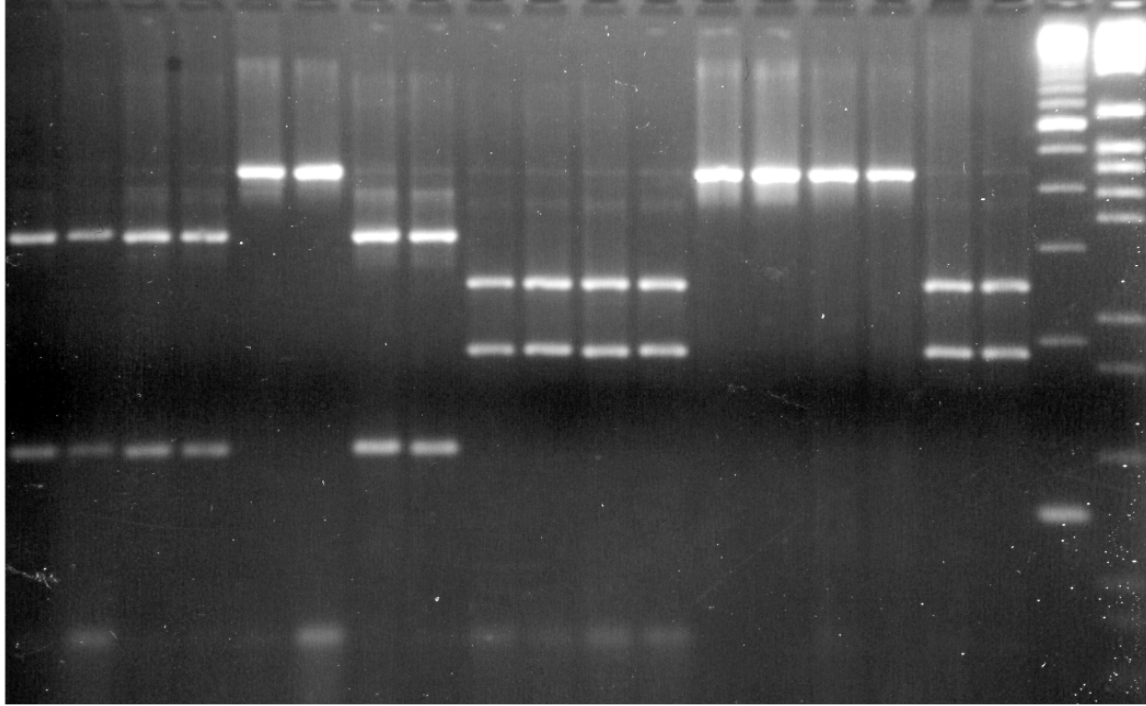
N. nova complex

Steingrube, et al., J. Clin. Microbiol, 1995; 33:3096-3101.

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PRA patterns (Hinf I)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



N. brasiliensis
N. otitidiscaviarum
N. asteroides Type VI
N. abscessus
N. asteroides Type IV
N. pseudobrasiliensis
N. nova
N. farcinica
N. transvalensis complex
pGem ladder
100 bp ladder

PCR *hsp* 65

(Rodriguez-Nava et al)

***Msp* I, *Bst*E II, *Hinf* I**

Group I: *N. asteroides* Type IV; *N. vacinii*

Group II: *N. fluminea*, *M. salmonicida*

Group III: *N. carnea*, *N. flavorosea*, *N. uniformis*,
N. asteroides Type II

Group IV: *N. abscessus*, *N. araoensis*, *N. asiatica*,
N. asteroides VI (ATCC 14759), *N. asteroides*
(ATCC 19247T), *N. brevicatena*, *N.*
cyriacigeorgica, *N. higoensis*, *N. neocaledoniensis*,
N. paucivorans, *N. puris*, *N. vinacea*

Rodriguez-Nava et al., J. Clin. Microbiol. 2006: 44:535-546.

PCR *hsp 65* (cont'd)

Msp I, BstE II, Hinf I

Group V: *N. brasiliensis, N. inohanensis, N. niigatensis, N. otitidiscaviarum, N. shimofusensis, N. yamanashensis*

Group VI: *N. africana, N. cummidelens, N. nova, N. pneumoniae, N. soli, N. veterana*

hsp65 and 16S rRNA Database for comparison

Rodriguez-Nava et al., J. Clin. Microbiol. 2006: 44:535-546.

MALDI – TOF MS

Matrix-Assisted Laser Desorption-Time-of-Flight Mass Spectrometry

- Proteomic based ID method
- Differentiate *Nocardia* by analysis of protein profiles from disrupted cells
- Detects hi and low Mol. Wt. proteins (2000-20,000 Daltons)
- 29/43 type strains have unique patterns

Stevenson, et al., Abstract ASM General Meeting 2009



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SUMMARY

- *Nocardia* species described from environmental sources are not uncommon from clinical samples
- **Phenotypic methods** are not sufficient for the ID of *Nocardia*.
- **Molecular methods** of identification are necessary to ID most species of *Nocardia*.
- Accurate ID of *Nocardia* sp. may require **sequencing of genes other than the 16S rRNA gene.**

SUMMARY

(cont'd)

In North America:

- *N. asteroides* is a rare human pathogen (<1% incidence)
- *N. cyriacigeorgica*, *N. farcinica*, and *N. nova* (sensu stricto) are the 3 most commonly isolated human pathogens of *Nocardia*.
- Undescribed species are not uncommon in clinical samples

SUMMARY

(cont'd)

- **Full sequencing** of the 16S rRNA, *hsp65*, *sec A 1* or *gyr B* genes can identify all species of *Nocardia* if they are sufficiently similar to a type strain.
- Always research unusual species
PubMed:
<http://www.ncbi.nlm.nih.gov/sites/entrez?>
- LSPN List of Prokaryotic Names with Standing in Nomenclature
<http://www.bacterio.cict.fr/index.html>

SUMMARY

(cont'd)

- Sequencing for ID requires
 - * Good quality sequence data
 - * Valid targets
 - Type strains
 - Distinct groups
 - * Comprehensive databases
 - All known species/taxa
 - Multiple species for each species/group

SUMMARY

(cont'd)

- Sequencing for ID requires (cont'd)

- * Databank pedigree

- Quality of sequence

- Length of sequence (partial vs. full)

- Public databases (NCBI, EMBL, etc.)

- Private databases (ABI, colleagues)

SUMMARY

(cont'd)

- Has the species been described in the literature? Is it a valid species?
Has it been implicated as a human pathogen?
- If your laboratory is unable to ID an isolate to species or complex level, consider sending the isolate to a reference laboratory, especially if the isolate is clinically significant.



Antimicrobial Susceptibility Testing (AST)

Antimicrobial Susceptibility Testing (AST)

CLSI (M24A, 2003)

Broth Microdilution

2-fold dilutions in CAMHB 1^o agents

72 hr incubation

Amikacin

Amox-Clavulanic Acid

Ceftriaxone

Ciprofloxacin

Clarithromycin

Imipenem

Linezolid

Minocycline

SMX/TMP-SMX

Tobramycin

Antimicrobial Susceptibility Testing (AST)

(cont'd)

- Inoculum density / Use of sterile pestles
- Indeterminate reads
- Agar disk diffusion / Broth microdilution discrepancies
- In-house validation necessary
- Proficiency testing

Nocardiae Broth Microdilution Breakpoints

Primary Agents

Resistance Breakpoints

($\mu\text{g/mL}$)

Amikacin	≥ 16
Minocycline	≥ 8
Sulfamethox	≥ 64
Amox Clav Acid	$\geq 32/16$
Ceftriaxone	≥ 64
Ciprofloxacin	≥ 4

Nocardiae Broth Microdilution Breakpoints (cont'd)

<u>Primary Agents</u>	<u>Resistance Breakpoints</u> ($\mu\text{g/mL}$)
Clarithromycin	≥ 8
Imipenem	≥ 16
TMP/SMX	$\geq 4/76$
Tobramycin	≥ 16
Linezolid	No "R"

Antimicrobial Susceptibility Testing (AST)

(cont'd)

<u>Secondary Agents</u>	<u>Resistance Breakpoints</u> ($\mu\text{g/mL}$)
Cefepime	≥ 32
Cefotaxime	≥ 64
Doxycycline	≥ 8
Gentamicin	≥ 16
Moxifloxacin*	

*No current CLSI breakpoints established.

Antimicrobial Susceptibility Testing (AST)

Linezolid (Oxazolidinone)

1st antimicrobial (other than sulfa) active
against all clinically significant species of
Nocardia

$$\text{MIC}_{90} \leq 4 \mu\text{g/mL}$$

Other Susceptibility Methods

Agar dilution

Agar disk diffusion

E-Tests (Gradient MICs)

**As with broth microdilution, interpretations
require skill and experience.**

**Currently accepted patterns may not be
valid as more species tested**

❖ Generally AST of *Nocardia* necessary



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