

NATIONAL STANDARD METHOD

IDENTIFICATION OF AEROBIC ACTINOMYCETES

BSOP ID 10

Issued by Standards Unit, Department for Evaluations, Standards and Training
Centre for Infections



Scottish Microbiology Forum

Association of Medical Microbiologists
Association of Medical Microbiologists
Association of Medical Microbiologists

IDENTIFICATION OF AEROBIC ACTINOMYCETES

Issue no: 1 Issue date: 23.11.09 Issued by: Standards Unit, Department for Evaluations, Standards and Training Page: 1 of 15
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The reader is informed that all taxonomy in this document was correct at time of issue.

Please note the references are now formatted using Reference Manager software. If you alter or delete text without Reference Manager installed on your computer, the references will not be updated automatically.

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AMENDMENT PROCEDURE

Controlled document reference	BSOP ID 10
Controlled document title	Identification Of Aerobic Actinomycetes

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@hpa.org.uk.

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment

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IDENTIFICATION OF AEROBIC ACTINOMYCETES SPECIES

SCOPE OF DOCUMENT

This document describes the identification of branching Gram-positive bacilli isolated from clinical specimens. Colonies may be isolated on blood agar or egg containing media.

Anaerobic sporing organisms are described in [BSOPID 8 - Identification of *Clostridium* species](#)
Anaerobic cocci are described in [BSOP ID 14 – Identification of anaerobic cocci](#). Anaerobic Gram-negative rods are described in [BSOP ID 25 – Identification of anaerobic Gram negative rods](#).

INTRODUCTION

Taxonomy

The nomenclature of the group comprising the branching Gram-positive rods is complicated. Considerable morphological diversity is not only seen among genera but also among strains of the same taxon.

Characteristics²

Nocardia species

Nocardia species produce rudimentary to extensively branched vegetative hyphae, 0.5 - 1.2 µm in diameter which grow on the surface and penetrate agar media. The hyphae often fragment into rod-shaped or coccoid elements. Aerial hyphae are almost always produced. Short to long chains of conidia may be found on the aerial hyphae and occasionally on substrate hyphae. Cells stain Gram-positive to Gram-variable and are usually acid-fast. Growth is aerobic, producing chalky, matt or velvety colonies. Colonial morphology will vary according to the medium or incubation temperature used. The colonies may be brown, tan, pink, orange, red, purple, grey or white. Colonies on solid media may be smooth and moist or granular, irregular, wrinkled or heaped with a velvety surface due to aerial filamentation. Soluble brown or yellow pigments may be produced. *Nocardia* are catalase positive and grow on Sabouraud's glucose agar, blood agar, brain heart infusion agar and Lowenstein-Jensen medium. Added carbon dioxide (10%) promotes more rapid growth. On Sabouraud dextrose agar, colonies of *N. asteroides* complex vary from salmon pink to orange. *N. brasiliensis* colonies are usually orange-tan. *N. otitidiscavarum* colonies are pale tan whereas *N. transvalensis* may vary in colour from pale tan to violet. Colonies in pure culture can grow after only 48 hours incubation. In mixed cultures other rapidly growing bacteria may obscure small *Nocardia* species colonies which may take several weeks to develop. Modified Thayer-Martin medium or buffered charcoal-yeast extract agar may enhance recovery of *Nocardia* species³.

Microscopic examination of Gram-stained clinical specimens may give a rapid and specific diagnosis. Thin, delicate, weakly to strongly Gram-positive, irregularly stained or beaded branching filaments are characteristic of *Nocardia* species. Multiple clinical specimens should be submitted for culture. *Nocardia* species may not be detected unless pus from a discharging fistula or abscess is examined. Smears and cultures of specimens are often negative unless specimens are obtained by biopsy. Routine blood cultures are not usually positive. Many *Nocardia* species from clinical material are variably acid-fast on primary isolation. This is rapidly lost in subcultured colonies. Modified Kinyoun stain decolourised with a weak acid (1-2% sulphuric acid instead of acid-alcohol) should be used. A single *Nocardia* colony isolated from CSF or a normally sterile site such as soft tissue abscess, pleural space or joint fluid from a patient with an appropriate clinical presentation should never be ignored. These organisms are seldom laboratory contaminants and are not part of the body's normal flora. Sputum digestion procedures (eg N-acetyl-L-cysteine or sodium hydroxide) may produce negative *Nocardia* species cultures. There are currently no serodiagnostic tests available⁴.

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Since *Nocardia* species are ubiquitous in nature, the isolation of these microorganisms from specimens may not be significant clinically. The presence of *Nocardia* in sputum culture may not always indicate invasive infection but may reflect laboratory contamination or respiratory colonization. The clinical and microbiological difficulties include the non-specific presentation of the infection, a frequent requirement for invasive diagnostic biopsy procedures, difficulty in isolating the *Nocardia* and problems in identification and taxonomic classification. *N. farcinica* is commonly misidentified as *N. asteroides*, or *Rhodococcus* or *Gordona* species.

Streptomyces species

Streptomyces species produce vegetative hyphae 0.5 - 2.0 µm in diameter which form an extensively branched mycelium which rarely fragments. This matures to form chains of three to many non-motile spores. A few species produce spores on the substrate mycelium. Cells are Gram-positive but not acid-alcohol fast. Growth is obligately aerobic and the optimum growth temperature is 25°C – 35°C. Initially the colonies produced are relatively smooth surfaced but later they develop aerial mycelium which may appear floccose, granular, powdery or velvety. Colonies are discrete, lichenoid, leathery or butyrous. The vegetative and aerial mycelia may be pigmented and diffusible pigments may also be produced. Metabolism is oxidative and the catalase test is positive. Nitrates are reduced to nitrites and aesculin is degraded.

Rhodococcus species

Rhodococcus species produce cocci which may germinate into short rods, form filaments with side projections, branching or extensively branched hyphae. The next generation of cocci or short rods is produced by fragmentation of the rods, filaments or hyphae. Microscopic aerial hyphae and spores are not usually produced, and spores are not produced. Cells stain Gram-positive and are usually partially acid-fast. Growth occurs aerobically.

There are three main colony types of *R. equi*. The classic colony type is pale pink and slimy. The second is colonet and non-slimy, and the third is pale yellow, non-slimy, and more opaque. Colonies of other rhodococci may be rough, smooth or mucoid and pigmented cream, buff, yellow, coral, orange or red. Colourless variants may occur particularly of *R. equi*. *R. equi* has a variable microscopic morphology (bacillary to coccoid forms) and may be discarded as a contaminant⁵. The cyclic variation in morphology of *R. equi* and some non-*equi* rhodococci depends upon incubation time and growth conditions. All rhodococci from clinical specimens are weakly acid-fast. Colonial and cell morphology cannot be used distinguish among *Rhodococcus*, *Gordonia* and *Tsukamurella* species. Commercial identification systems do not provide reliable identification of *Rhodococcus* species and clinically important isolates should be referred to the Reference Laboratory⁶.

Oerskovia species

Oerskovia species produce extensively branching vegetative hyphae approximately 0.5 µm in diameter which grow on the surface and penetrate into agar. The hyphae break up into rod-shaped, motile, flagellate rods. Non-motile strains may also occur. An aerial mycelium is not formed. Cells stain Gram-positive, although part of the thallus may become Gram-negative with age and coryneforms may be seen. Growth is facultatively anaerobic and the catalase test is positive when grown aerobically and negative when grown anaerobically. The species may be pigmented yellow. Glucose is metabolised both oxidatively and fermentatively.

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Morphologically similar organisms

***Actinomadura* species**

Actinomadura species produce extensively branching vegetative hyphae which form a dense non-fragmenting substrate mycelium. The aerial mycelium may be absent or moderately developed to form short or occasionally long chains of arthrospores when mature. The spore chains are straight, hooked or irregular spirals. The aerial mycelium may be blue, brown, cream, grey, green, pink, red, white or yellow. The colonies have a leathery or cartilaginous appearance when the aerial mycelium is absent. Colonies are usually mucoid and have a molar tooth appearance after 2 days incubation at 35°C. Growth is aerobic and occurs within the temperature range 10°C – 60°C. Cells stain Gram-positive and are non-acid-fast.

***Amycolata* species**

Amycolata species produce branching vegetative hyphae 0.5 - 2.0 µm in diameter which tend to fragment into squarish elements. Aerial mycelium may be produced which may remain stable or differentiate into long chains of smooth-walled ellipsoidal to cylindrical spores. Chains of spores are also produced on vegetative hyphae.

Amycolata autotrophica is a rare human pathogen. It is Gram-positive with branched filaments which are not acid-fast by the Kinyoun method. Aerial hyphae are abundant and aesculin is hydrolysed.

***Amycolatopsis* species**

Amycolatopsis species produce branching substrate hyphae 0.5 - 2.0 µm in diameter which fragment into squarish elements. Aerial mycelium may be produced and the aerial hyphae may be sterile or differentiate into long chains of smooth-walled, squarish to ellipsoidal spore-like structures. Spores may be produced on vegetative hyphae.

Dermatophilus congolensis

Dermatophilus congolensis grows only on complex media and the aerial mycelium will grow only in atmospheres containing added carbon dioxide. The substrate mycelium consists of long tapering filaments which branch laterally at right angles. *D. congolensis* may be easily recognised microscopically. Septa are formed in transverse, horizontal and vertical longitudinal planes to produce up to eight parallel rows of motile spores. Cells stain Gram-positive but are not acid-fast.

Isolation of *D. congolensis* may be difficult. Clinical material, preferably the underside of scabs, should be streaked on a blood plate and incubated aerobically or with added carbon dioxide at 35°C – 37°C. Growth is aerobic, facultatively anaerobic and catalase positive. The metabolism is non-fermentative but acid is produced from some carbohydrates. The optimum growth temperature is 37°C.

***Gordonia* species**

Cells are short rods or cocci which resemble thin beaded coccobacilli. They stain Gram-positive or Gram-variable and are usually partially acid-fast. Colonies on blood agar are dry, wrinkled, raised and beige, brownish, pink, or orange and red after 3 to 7 days incubation. Growth is aerobic. Colonial and cell morphology cannot distinguish among *Rhodococcus*, *Gordonia* and *Tsukamurella*.

***Nocardiopsis* species**

Nocardiopsis species produce a well developed substrate mycelium. The hyphae are long and densely branched and may fragment into coccoid and bacillary forms. The aerial mycelium is also well developed and abundant and the aerial hyphae fragment completely into spores of various lengths. The growth temperature range is 10°C - 45°C.

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Rothia species

Rothia contains 5 species including *Rothia mucilaginosa* (previously named *Stomatococcus mucilaginosus*). In Gram's stains a mixture of cocci, rods and filaments are seen. It is catalase positive and optimum growth temperature is 35°C - 37°C. *R. dentocariosa* is a facultative anaerobe: it produces spider-like, filamentous colonies when grown anaerobically. Under aerobic conditions colonies are convex or convoluted and glisten. *Rothia mucilaginosa* is a coccus 0.9 – 1.3 mm in diameter, usually arranged in clusters.

Tsukamurella species

Tsukamurella species are straight to slightly curved rods 0.5 - 0.8 x 1.0 – 5.0 µm. Very short rods may also be present. Cells are Gram-positive and weakly to strongly acid-fast and occur singly, in pairs or in masses. They are non-motile, non-sporing and do not produce aerial hyphae. Growth is obligately aerobic producing white/creamy to orange small, convex colonies 0.5 - 2.0 mm in diameter with entire, sometimes rhizoidal, edges which are dry but easily emulsified. The preferred growth temperature is below 37°C. Colonial and cell morphology cannot distinguish among *Rhodococcus*, *Gordonia* and *Tsukamurella*.

Colonies of *Tsukamurella paurometabola*, the species associated with infection, grow on brain heart infusion agar containing blood and are 0.5 - 2.0 mm in diameter, circular with an entire and occasionally a rhizoid edge, dry, easily emulsified and white to creamy to orange. Rough colonies are produced after prolonged incubation for up to seven days. These colonies are cerebiform and do not produce aerial hyphae but resemble rapidly growing mycobacteria. Most strains of *T. paurometabola* are acid-fast by the Kinyoun method.

Mycetoma

The important agents of actinomycetoma in humans are:

Actinomadura madurae
Actinomadura pelletieri
Nocardia brasiliensis
Streptomyces somaliensis

Less commonly involved are, *N. asteroides*, *N. otitidiscaviarum*, *N. dassonvillei* and *N. transvalensis*. *Aspergillus nidulans* and *Curvularia lunata* are also associated with mycetoma in the Sudan.

Principles of identification

Reliable identification of clinically significant actinomadurae, nocardiae, actinomycetes and streptomycetes is possible only by detecting key chemical markers. Identification should be confirmed by a Reference Laboratory. The standard phenotypic identification tests will give only a presumptive identification.

Method for demonstrating the micromorphology of cultures (for information)

Slide culture should be made of undisturbed colonies grown on minimal medium, such as tap water medium or cornmeal medium without dextrose. The culture preparations are incubated at 25°C and examined periodically for 2 to 3 weeks. Examine the slide cultures under a microscope in order to recognise true branched substrate mycelium, aerial mycelium and sporulation. The substrate hyphae of *Nocardia* species appear as very fine, dichotomously branched filaments. Movement of the objective up and down through several planes will reveal aerial hyphae. The presence of aerial hyphae differentiates the genus *Nocardia* from other related genera (*Rhodococcus*, *Gordonia*, *Tsukamurella*, *Corynebacterium* and *Mycobacterium*). Only *Nocardia* species in this group of organisms have aerial hyphae. The rapidly growing mycobacteria, which phenotypically resemble the nocardiae, have simple, relatively short substrate hyphae that branch at acute angles. In contrast, the complex substrate hyphae of the nocardiae branch at right angles and usually have secondary branches. Rhodococci grow as coccobacilli arranged in a zigzag pattern.

A. pelletieri differs from *A. madurae* in that *A. madurae* hydrolyses aesculin and *A. pelletieri* does not.

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The microscopic morphology of *D. congolensis* in cultures is similar to that in clinical specimens. The typical appearance of branched filaments divided in their transverse and longitudinal planes is diagnostic. Wet mounts of colonies or smears of colonies or clinical material should be stained with methylene blue or by Giemsa's stain. A Gram-stained preparation is not helpful in visualising this organism because it is too dark and obscures crucial morphologic details. Completely coccal elements may be seen, many with flagellae or irregularly arranged cells in packets. Germinating spores and branched segmented or non-segmented filaments may be seen. Motility is usually seen in isolates from fresh cultures. If only cocci are seen and *D. congolensis* is suspected, prepare a younger culture to examine for hyphae. Very small (0.5 - 1.0 mm) round colonies may be seen on brain heart infusion agar containing blood which is incubated for 24 hours. The colonies are usually grey-white, adherent and pit the medium. After two to five days an orange pigment develops. β -haemolysis is frequently present and is more prominent on areas of the medium where the colonies are crowded. There is no growth on Sabouraud dextrose agar. *D. congolensis* is catalase positive and urea is hydrolysed in 24 hours. Nitrate is not reduced and acid but no gas is produced from glucose in 48 hours.

Rhodococci can be easily distinguished from most *Corynebacterium* species which, except for *Corynebacterium aquaticum*, *Corynebacterium minutissimum* and the CDC group B-1, have a fermentative metabolism.

TECHNICAL INFORMATION/LIMITATIONS

N/A

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1 SAFETY CONSIDERATIONS⁷⁻¹⁷

Hazard Group 2 organisms.

Refer to current guidance^{13,18,19} on the safe handling of all Hazard group 2 organisms documented in this National Standard Method.

The above guidance should be supplemented with local COSHH and risk assessments. Compliance with postal and transport regulations is essential.

2 TARGET ORGANISMS

Nocardia species which have been associated with infection:

N. asteroides sensu stricto

N. nova

N. farcinica

N. brasiliensis

N. otitidiscaviarum

N. transvalensis

N. brevicatena

N. carnea

N. pseudobrasiliensis

3 IDENTIFICATION

3.1 MICROSCOPIC APPEARANCE

[BSOPTP 39 – Staining Procedures](#)

Gram's stain

Gram-positive, may be Gram-variable depending on the age of the culture.

Nocardia species branching, fine, delicate filaments with fragmentation.

Rhodococcus, *Gordona*, *Tsukamurella* diphtheroid-like with minimal branching or coccobacillary.

Streptomyces species extensive branching with chains and spores; does not fragment easily.

Actinomadura species moderate, fine, intertwining branching with short chains of spores.

Dermatophilus species branched filaments divided into transverse and longitudinal planes; fine and tapered filaments.

Norcardiopsis species branching with internal spores.

Oerskovia species extensive branching; hyphae break up to motile, rod shaped elements.

Rothia species pleomorphic; predominately coccoid and bacillary (in broth) to branched filaments (solid media).

Modified ZN

If the stain is positive the isolate is probably a partially acid fast aerobic actinomycete.

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3.2. PRIMARY ISOLATION MEDIA

Chocolate agar incubated in 5 - 10% CO₂ at 35°C - 37°C for 16 - 48 h.

Blood agar incubated in 5 - 10% CO₂ at 35°C - 37°C for 16 - 48 h.

Fastidious anaerobe agar or equivalent, with or without neomycin (some anaerobic organisms may be inhibited by neomycin) 40 – 48 h incubation anaerobically at 35°C - 37°C.

Note: plates should be incubated for 2 to 3 weeks.

3.3 COLONIAL APPEARANCE

Genus	Characteristics of growth on fastidious anaerobe agar after incubation
<i>Nocardia</i> species	Wrinkled often dry, chalky-white appearance to orange tan pigment, crumbly
<i>Streptomyces</i> species	Waxy heaped colonies with variable morphology
<i>Oerskovia</i> species	Yellow pigmented, extensive branching that grows on the surface and in to the agar
<i>Gordonia</i> , <i>Rhodococcus</i> , and <i>Tsukamurella</i> species	Non-haemolytic, round, often mucoid with salmon-pink/red developing within 4 to 7 days
<i>Dermatophilus</i> <i>congolensis</i>	Round adherent grey-white colonies, that later develop orange pigments; often beta haemolytic
<i>Actinomadura</i> species	White to pink colour. Colonies are usually mucoid and have a molar tooth appearance
<i>Rothia</i> species	Small smooth to rough colonies dry appearance
<i>Nocardioopsis</i> species	Coarsely wrinkled and folded with well developed aerial mycelium

3.4 TEST PROCEDURES

Differentiation of branching Gram-positive rods

Smears (in duplicate) from both colonies and clinical material should be stained with Gram's stain and by the modified Kinyoun method. Isolates of *Streptomyces* species may show acid-fast coccoid forms and non-acid fast hyphae, but are considered non-acid fast. There must be a contrast between the carbol fuchsin and the counterstain. The demonstration of acid-fastness by isolates should be used only in conjunction with other tests as a supportive test and not as an absolute diagnostic test.

Nocardia species and *Streptomyces* species (β -galactosidase positive) may be differentiated from group IV mycobacteria (β -galactosidase negative) and rhodococci (β -galactosidase variable)¹⁵.

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3.5 FURTHER IDENTIFICATION

Commercial identification kit or molecular techniques.

3.6 STORAGE AND REFERRAL

If required, subculture to blood agar and save the isolate on blood agar slopes for referral to the Reference Laboratory.

4 IDENTIFICATION OF GRAM-POSITIVE BRANCHING RODS – FLOW CHART

N/A

5 REPORTING

5.1 PRESUMPTIVE IDENTIFICATION

Presumptive identification may be made if appropriate growth characteristics, colonial appearance, Gram's stain of the culture; and biochemical or molecular techniques.

5.2 CONFIRMATION OF IDENTIFICATION

Confirmation of identification can be made by the appropriate reference laboratory.

5.3 MEDICAL MICROBIOLOGIST

Inform the medical microbiologist when the request card bears relevant information.

5.4 CCDC

Refer to local Memorandum of Understanding.

5.5 CENTRE FOR INFECTIONS²⁰

Refer to current guidelines on Cfl and COSURV reporting.

5.6 INFECTION CONTROL STAFF

N/A

6 REFERRALS

6.1 REFERENCE LABORATORY

Confirmation of identification can be made by the appropriate reference laboratory.

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7 ACKNOWLEDGEMENTS AND CONTACTS

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