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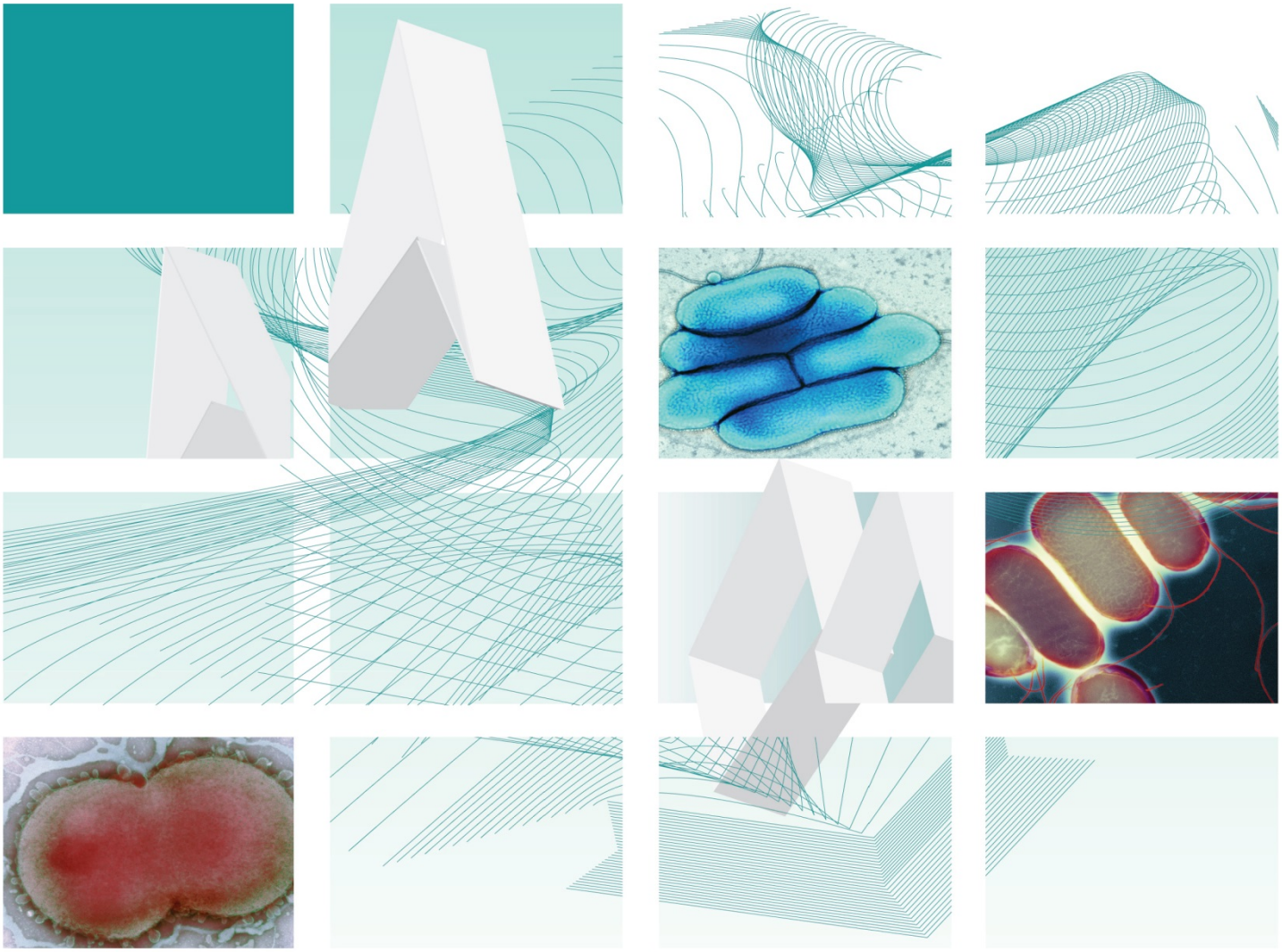
UK Standards for Microbiology Investigations

Identification of aerobic actinomycetes



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Acknowledgments

UK Standards for Microbiology Investigations (SMIs) are developed under the auspices of Public Health England (PHE) working in partnership with the National Health Service (NHS), Public Health Wales and with the professional organisations whose logos are displayed below and listed on the website https://www.gov.uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories. SMIs are developed, reviewed and revised by various working groups which are overseen by a steering committee (see https://www.gov.uk/government/groups/standards-for-microbiology-investigations-steering-committee).

The contributions of many individuals in clinical, specialist and reference laboratories who have provided information and comments during the development of this document are acknowledged. We are grateful to the medical editors for editing the medical content.

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UK Standards for Microbiology Investigations are produced in association with:

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Contents

Amendment table

Each SMI method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@phe.gov.uk.

New or revised documents should be controlled within the laboratory in accordance with the local quality management system.

Amendment no/date.	6/28.10.16
Issue no. discarded.	2.1
Insert issue no.	2.2
Section(s) involved	**Amendment**
3.2 Primary isolation media.	The note in Section 3.2 has been clarified.

Amendment no/date.	5/21.03.16
Issue no. discarded.	2
Insert issue no.	2.1
Section(s) involved	**Amendment**
Whole document.	Spelling errors corrected. Minor formatting amendments.

Amendment no/date.	4/15.01.15
Issue no. discarded.	1.3
Insert issue no.	2
Section(s) involved	**Amendment**
Whole document.	Hyperlinks updated to gov.uk.
Page 2.	Updated logos added.
Whole document.	Document presented in a new format. Reorganisation of some text. Edited for clarity. Test procedures updated. Updated contact detail of Reference Laboratory.
Introduction.	The taxonomy of the pathogenic genera within the

	aerobic Actinomycetes has been updated. More information has been added to the Characteristics section. The medically important species have been grouped and their characteristics described. Use of up-to-date references. Information on the section on Principles of Identification has been moved from the technical limitations/information section.
Technical information/limitations.	Some information has been removed from this section and put into the appropriate section. Information on commercial identification systems has been updated.
Target organisms.	The section on the Target organisms has been updated and presented clearly. References have been updated.
Identification.	Amendments and updates have been done on 3.1, 3.2, 3.3 and 3.4 have been updated to reflect standards in practice. The table in 3.3 has also been amended and updated. Subsection 3.5 has been updated to include the Rapid Molecular Methods.
Identification flowchart.	Information has been provided as to how to identify these organisms being that there is constantly considerable morphological diversity among genera.
Reporting.	Subsections 5.1, 5.3, 5.5 and 5.6 have been updated to reflect reporting practice.
Referral.	The address of the reference laboratory has been updated.
References.	Some references updated.

UK SMI[#]: scope and purpose

Users of SMIs

Primarily, SMIs are intended as a general resource for practising professionals operating in the field of laboratory medicine and infection specialties in the UK. SMIs also provide clinicians with information about the available test repertoire and the standard of laboratory services they should expect for the investigation of infection in their patients, as well as providing information that aids the electronic ordering of appropriate tests. The documents also provide commissioners of healthcare services with the appropriateness and standard of microbiology investigations they should be seeking as part of the clinical and public health care package for their population.

Background to SMIs

SMIs comprise a collection of recommended algorithms and procedures covering all stages of the investigative process in microbiology from the pre-analytical (clinical syndrome) stage to the analytical (laboratory testing) and post analytical (result interpretation and reporting) stages. Syndromic algorithms are supported by more detailed documents containing advice on the investigation of specific diseases and infections. Guidance notes cover the clinical background, differential diagnosis, and appropriate investigation of particular clinical conditions. Quality guidance notes describe laboratory processes which underpin quality, for example assay validation.

Standardisation of the diagnostic process through the application of SMIs helps to assure the equivalence of investigation strategies in different laboratories across the UK and is essential for public health surveillance, research and development activities.

Equal partnership working

SMIs are developed in equal partnership with PHE, NHS, Royal College of Pathologists and professional societies. The list of participating societies may be found at https://www.gov.uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories. Inclusion of a logo in an SMI indicates participation of the society in equal partnership and support for the objectives and process of preparing SMIs. Nominees of professional societies are members of the Steering Committee and working groups which develop SMIs. The views of nominees cannot be rigorously representative of the members of their nominating organisations nor the corporate views of their organisations. Nominees act as a conduit for two way reporting and dialogue. Representative views are sought through the consultation process. SMIs are developed, reviewed and updated through a wide consultation process.

Quality assurance

NICE has accredited the process used by the SMI working groups to produce SMIs. The accreditation is applicable to all guidance produced since October 2009. The process for the development of SMIs is certified to ISO 9001:2008. SMIs represent a good standard of practice to which all clinical and public health microbiology laboratories in the UK are expected to work. SMIs are NICE accredited and represent

[#] Microbiology is used as a generic term to include the two GMC-recognised specialties of Medical Microbiology (which includes Bacteriology, Mycology and Parasitology) and Medical Virology.

neither minimum standards of practice nor the highest level of complex laboratory investigation possible. In using SMIs, laboratories should take account of local requirements and undertake additional investigations where appropriate. SMIs help laboratories to meet accreditation requirements by promoting high quality practices which are auditable. SMIs also provide a reference point for method development. The performance of SMIs depends on competent staff and appropriate quality reagents and equipment. Laboratories should ensure that all commercial and in-house tests have been validated and shown to be fit for purpose. Laboratories should participate in external quality assessment schemes and undertake relevant internal quality control procedures.

Patient and public involvement

The SMI working groups are committed to patient and public involvement in the development of SMIs. By involving the public, health professionals, scientists and voluntary organisations the resulting SMI will be robust and meet the needs of the user. An opportunity is given to members of the public to contribute to consultations through our open access website.

Information governance and equality

PHE is a Caldicott compliant organisation. It seeks to take every possible precaution to prevent unauthorised disclosure of patient details and to ensure that patient-related records are kept under secure conditions. The development of SMIs is subject to PHE Equality objectives https://www.gov.uk/government/organisations/public-health-england/about/equality-and-diversity.

The SMI working groups are committed to achieving the equality objectives by effective consultation with members of the public, partners, stakeholders and specialist interest groups.

Legal statement

While every care has been taken in the preparation of SMIs, PHE and any supporting organisation, shall, to the greatest extent possible under any applicable law, exclude liability for all losses, costs, claims, damages or expenses arising out of or connected with the use of an SMI or any information contained therein. If alterations are made to an SMI, it must be made clear where and by whom such changes have been made.

The evidence base and microbial taxonomy for the SMI is as complete as possible at the time of issue. Any omissions and new material will be considered at the next review. These standards can only be superseded by revisions of the standard, legislative action, or by NICE accredited guidance.

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Scope of document

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

This SMI should be used in conjunction with other SMIs.

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.

Characteristics[1]

Most actinomycetes are typically Gram positive, filamentous, partially acid-fast, branched bacteria that have many microbiological characteristics in common with members of the genera *Mycobacterium* and *Corynebacterium*. The major groups of the order *Actinomycetales,* are actinoplanetes, maduromycetes, nocardioform actinomycetes, and streptomycetes[2].

Although the aerobic actinomycetes are infrequently encountered in clinical practice, they are important potential causes of serious human and animal infections.

The pathogenic genera within the aerobic actinomycetes are *Nocardia*, *Actinomadura*, *Streptomyces*, *Rhodococcus*, *Gordonia*, *Tsukamurella* and *Tropheryma whipplei*.

Nocardia species[2-5]

The genus *Nocardia* currently contains 100 species that have been characterised by phenotypic and molecular methods, and over 30 species are associated with humans. A few of these species have also been recently assigned to other genera. It comprises several species that are known to be unusual causes of a wide spectrum of clinical diseases in both humans and animals. While the majority of nocardial infections have been attributed to *Nocardia asteroides*, other pathogenic *Nocardia* species that have been described include *Nocardia brasiliensis, Nocardia otitidiscaviarum*, and *Nocardia transvalensis*. In a recent taxonomic revision of the *N. asteroides* taxon, two new species- *N. farcinica* and *Nocardia nova* were separated from it.

Nocardia species produce rudimentary to extensively branched vegetative hyphae, 0.5 - 1.2µm 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. They are usually partially acid-fast due to the presence of intermediate-length mycolic acids in their cell wall. Growth is aerobic, producing chalky, matt or velvety colonies. Macroscopically, visible aerial hyphae may be lacking, sparse, or very abundant. 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 superficially smooth and moist or granular, irregular, wrinkled or heaped with a velvety surface due to aerial filamentation or, more commonly, a chalky appearance. Soluble brown or yellow pigments may be produced.

Nocardia are catalase positive and grow on Sabouraud 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* species.

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 nocardial 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 with N-acetyl-L-cysteine or sodium hydroxide) may produce false negative results on some *Nocardia* positive sputum specimens. There are currently no serodiagnostic tests available to identify patients with active nocardiosis more quickly.

Important biochemical tests that differentiate the three major pathogenic *Nocardia* species, *N. asteroides, N. brasiliensis*, and *N. otitidiscaviarum*, include the decomposition of casein, xanthine, tyrosine, and hypoxanthine. However, this identification method does not differentiate the *N. asteroides* complex from the non-pathogenic *Nocardia* species, *Nocardia camea, Nocardia amarae,* and *Nocardia brevicatena* or from species of the related genera *Mycobacterium, Rhodococcus, Gordonia*, and *Tsukamurella*. In the past, the use of these few biochemical tests and morphology alone resulted in the genus *Nocardia* being characterized by extreme heterogeneity. In particular, the consistency and composition of the growth medium can affect the growth and stability of both aerial and substrate hyphae. An inconsistent morphologic feature of the genus *Nocardia* includes well-developed conidia in *N. brevicatena* and less well-formed spores in some *N. asteroides* strains.

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* species and problems in identification and taxonomic classification.

N. farcinica is commonly misidentified as *N. asteroides*, or *Rhodococcus* or *Gordonia* species.

Streptomyces species[1,4,6]

Streptomyces is the largest genus of Actinobacteria and the type genus of the family *Streptomycetaceae*. Currently 600 species and 38 subspecies of *Streptomyces* bacteria have been described. *Streptomyces* species are Gram positive and produce vegetative hyphae 0.5 - 2.0μm 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 not acid-alcohol fast. The cell wall lacks mycolic acids but contains major amounts of L- diaminopimelic acid (L-DAP). Growth is obligately aerobic and the optimum growth temperature is 25°C – 35°C, although some species grow at temperatures within the psychrophilic and thermophilic range. 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. They are also positive for catalase test as well as reducing nitrates to nitrites, degrading aesculin, casein, gelatin, starch and L. tyrosine.

Streptomyces species are most widely known for their ability to synthesize antibiotics. Over 50 different antibiotics have been isolated from *Streptomyces* species, providing most of the world's antibiotics.

These species are widely distributed and abundant in soil. A few are pathogenic for humans. *S. somaliensis* and *S. sudanensis* are associated with infections such as mycetoma. *Aspergillus nidulans* and *Curvularia lunata* are also associated with mycetoma in the Sudan.

The differentiation of the genus *Streptomyces* remains difficult because its physical measurements do not seem practicable and there seems to be nothing known of the chemistry of the pigments responsible for the colours of the aerial mycelium.

Rhodococcus species[1,4,7,8]

There are currently 50 species of *Rhodococcus* and 11 have been re-assigned to other genera. *Rhodococcus* species usually stain Gram positive. Cells form as cocci or short rods which grow in length, and may form an extensively branched vegetative mycelium which may fragment. Microscopic aerial hyphae and spores are not usually produced. They are also non-motile. They are usually partially acid-fast due to the mycolic acid in their cell walls. All rhodococci from clinical specimens are weakly acid-fast. 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 *Rhodococcus equi.* Incubation at 30°C also increases recovery. Growth occurs aerobically.

Although biochemical tests help to distinguish *Rhodococcus* from other organisms, differentiation from other aerobic actinomycetes can be difficult. Colonial and cell morphology cannot be used to distinguish among *Rhodococcus, Gordonia* and *Tsukamurella* species. *Rhodococcus* species typically react positively in catalase, nitrate reduction, and urea hydrolysis tests and negatively with oxidase, gelatin hydrolysis, and carbohydrate reduction. Their inability to ferment carbohydrate is important in distinguishing them from Corynebacteria.

Of the species that have remained in the revised genus, *R. equi* appears to have the most clinical significance as a potential cause of infections in animals and humans. It has been identified as the cause of potentially life-threatening infections in severely immunocompromised patients, in particular, patients with HIV infections and has been associated with pulmonary and cutaneous infections

The microscopic morphology of *R. equi* in cultures is cyclic, varying from bacillary to coccoid, depending upon incubation time and growth conditions. All of the rhodococci from clinical specimens are generally weakly acid- fast when stained by either the modified Kinyoun or the Ziehl-Neelsen method. The colony morphology of *R. equi* is diverse and consists of three major varieties. The classic colony type is pale pink and slimy in 2 to 4 days on brain heart infusion agar or heart infusion agar containing 5% rabbit blood when incubated aerobically at 35°C. The second most frequent colony type is coral and non-slimy when grown on the same media under similar incubation conditions. The third and least common colony type is pale yellow, non-slimy, more opaque than the classic slimy type of colony, and identical to that of the *R. equi* type strain.

Oerskovia species[1,4,9]

There are currently 5 species. *Oerskovia* species produce extensively branching vegetative hyphae approximately 0.5μm 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. Most strains may be pigmented yellow. Glucose is metabolised both oxidatively and fermentatively.

Most *Oerskovia* infections are associated with an indwelling prosthetic device and are resolved following the removal of the devices.

Actinomadura species[1,4,10]

There are currently 75 species and 2 subspecies of this genus but 37 species are with validly published names in nomenclature, 20 species have been re-classified to other genera. Although the species status of some strains remains uncertain, and further comparative studies are needed.

Actinomadura madurae and *Actinomadura pelletieri* are the only two species of clinical importance in this genus.

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.

A. madurae can be distinguished reliably from *A. pelletieri* on the basis of biochemical tests. They both hydrolyse casein and may hydrolyse hypoxanthine and tryrosine but only *A. madurae* hydrolyses aesculin. *A. pelletieri* are asaccharolytic in contrast to *A. madurae*. The former produce acid only from glucose and trehalose whereas,

A. madurae produce acid from adonitol, arabinose, cellobiose, mannitol, trehalsoe, xylose, glycerol, mannose, mannitol and rhamnose.

Tsukamurella species[1,11,12]

There are currently 11 species. *Tsukamurella* species are straight to slightly curved rods 0.5 - 0.8 x 1.0 – 5.0µm. 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 - 2.0mm 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.*

All *Tsukamurella* species are resistant to lysozyme*,* positive for catalase, tween 80 hydrolysis, urease, pyrazinamidase, iron uptake, and tolerance to 5% sodium chloride and negative for nitrate reduction and arylsulftase except *Tsukamurella wratislaviensis.*

Tsukamurella species cause disease mainly in immunocompromised individuals. Infections with these microorganisms have been associated with chronic lung diseases, immune suppression (leukaemia, tumours and HIV/AIDS infection) and post-operative wound infections.

Gordonia species[4,13-15]

The genus *Gordonia* belongs phylogenetically to the suborder *Corynebacterineae,* the mycolic acid group within the order *Actinomycetales*, and its classification has changed drastically in recent years, with several species being reclassified and many novel species being described. At present, the genus *Gordonia* comprises 36 validly published species, and 9 species are known to cause infections in humans.

Cells are short rods or cocci which resemble thin beaded coccobacilli. They stain Gram positive or Gram-variable and are usually partially acid-fast. They do not generate spores. The colony morphology of *Gordonia* species varies from slimy, smooth, and glossy to irregular and rough; it may even differ within one species depending on the medium used for growth. Colonies on blood agar are dry, wrinkled, raised and beige, brownish, pink, or orange and red after 3 to 7 days incubation. On further incubation, colonies may become salmon coloured, particularly on chocolate agar. Growth is aerobic. Colonial and cell morphology cannot distinguish among *Rhodococcus, Gordonia* and *Tsukamurella.* They also have an oxidative carbohydrate metabolism.

Gordonia species can be separated from the *Nocardia* species by its inability to produce aerial hyphae and inability to grow in the presence of lysozyme.

Gordonial infections are rather rare in comparison to reports on infections caused by other pathogenic bacteria belonging to related genera such as *Rhodococcus* and *Nocardia.*

Tropheryma species[16,17]

This genus is phylogenetically placed with the Gram positive Actinobacteria and *Tropheryma whippeli* is the only species that is validly published. This causes Whipples disease, a systemic infection with symptoms of fever, weight loss, diarrhoea, polyadenopathy and polyarthritis. They are occasionally responsible for cardiac manifestations.

They are non-motile short rods, 0.25- 0.3µm in diameter and 0.8-1.7µm long, sometimes longer when cell division is impaired. On Gram stain, bacterial structures are all poorly stained and always appear as Gram negative. It does not grow on axenic media or in culture media with lysed eukaryotic cells. The presence of intact cells appears to be necessary for bacterial growth. It grows well in or associated with HEL and MRC-5 cells in minimal essential medium with 10% foetal calf serum and 2 mM l-glutamine when incubated at 37°C in a 5% CO_2 atmosphere. They are equally well preserved by rapid freezing and storage at - 80°C.

Morphologically similar organisms

Amycolata species (now *Pseudonocardia* species)[1,18]

There were only four validly published species but these are now classified in an emended genus *Pseudonocardia* because the sequences of species belonging to the genera *Amycolata* and *Pseudonocardia* were always recovered as a mixed group in phylogenetic trees and this was strongly supported by previously published lipid, ribosomal protein and ultrastructure data.

Amycolata species produce branching vegetative hyphae 0.5 - 2.0µm in diameter which tends 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. They are mesophilic but some strains are facultatively autotrophic. These species do not grow in the presence of lysozyme and they all produce acid from galactose, glucose, mannitol, maltose, arabinose, xylose, trehalose and fructose but not from lactose, raffinose, rhamnose and starch.

Amycolatopsis species[1,19]

There are currently 63 species and 4 subspecies. Amycolatopsis species produce branching substrate hyphae about 0.5 - 2.0µm 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. Cells stain Gram positive and are non-acid fast.

They are mesophilic but some strains are facultatively autotrophic. They are non-motile, catalase positive and do not grow in the presence of lysozyme.

Dermatophilus congolensis[1,4]

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. Growth is aerobic and facultatively anaerobic.

Depending on the age of the isolate and the type of medium used for culturing, completely coccal elements, many with flagellae or irregularly arranged cells in packets; germinating spores; or branched segmented or nonsegmented filaments can be seen. Motility is usually evident in isolates from fresh cultures. If cocci only are seen and *D. congolensis* is suspected, younger cultures should be examined for hyphae. At 24hr, on brain heart infusion agar containing horse blood, tiny (0.5- to 1.0-

mm), round beta-haemolytic colonies can be seen. This beta- haemolysis is also more prominent on areas of the medium in which colonies are crowded. The appearance of these colonies may vary, but they are usually grey-white and adherent and pit the medium. In 2 to 5 days, they develop an orange pigment. There is no growth on Sabouraud dextrose agar.

Isolation of *D. congolensis* may be difficult because they are relatively slow-growing and are readily overgrown by other bacteria. Clinical material, preferably the underside of freshly removed scabs, should be streaked on a blood plate and incubated aerobically or with added carbon dioxide at 35°C – 37°C. Special isolation techniques are required for contaminated specimens. Alternatively, Haalstra's method may be used[20]. The method depends on the release from the scab of the motile cocci of *D. congolensis* and their chemotropic attraction towards the carbon dioxide rich atmosphere of the candle jar.

They are positive for catalase, urea and casein (which could take up to 7 days) hydrolysis. They are also negative for nitrate reduction, tyrosine, hypoxanthine and xanthine hydrolysis. The metabolism is non-fermentative but acid is produced from some carbohydrates. The optimum growth temperature is 37°C.

The colonies can be differentiated from *Nocardia* sp and *Streptomyces* sp, neither of which produces filaments that break up into multiple rows of motile cocci.

Nocardiopsis species

Nocardiopsis species produce a well-developed substrate mycelium. The colour of the aerial and substrate mycelium varies – orange, brown, blue, white, yellow, cream, grey and colourless. 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.

Rothia species[21,22]

Rothia species are Gram positive cocci with a variable microscopic morphology. Their cells occur singly, in pairs, in clusters or in chains. They are weakly catalase positive and weakly proteolytic. *Rothia* species are positive for nitrate and nitrite reduction, liquefaction of gelatin and fermentation of sugars with the production of acid; while negative for motility, urease and indole. Colonies on agar surface may appear branched which rapidly fragment into bacillary or coccoid forms, resembling *Actinomyces* or *Nocardia* species. They exhibit good growth under aerobic or microaerophilic conditions, but poor or no growth anaerobically.

Rothia species are susceptible to penicillin but because rare isolates may be resistant, susceptibility testing should be performed.

There are currently 7 species of *Rothia* and 2 have been known to cause infections in humans - *Rothia dentocariosa* and *Rothia mucilaginosa.*

Rothia dentocariosa[23-25]

R. dentocariosa is an irregular Gram positive non-spore forming bacterium and cells occur singly, in pairs, in clusters or in chains. Colonial pleomorphism can also be observed. Microscopically, the morphology varies from coccoid to diphtheroid (with clavate ends) to filamentous. In broth cultures, cells may be coccoid, which distinguishes them from *Actinomyces* species and appears in filamentous forms on plates, but mixtures may appear in any culture. They may show rudimentary branching

and loss of the Gram positive appearance in ageing cultures. *R. dentocariosa* grows faster under aerobic than under anaerobic conditions, and does not need CO_2 or lipids for growth. It grows well on simple media (except Sabouraud dextrose agar) and colonies may be creamy, dry, crumbly or mucoid, non-haemolytic and may adhere to the agar surface. They are non-motile, catalase positive and ferment carbohydrates with the end-products being lactic and acetic acid.

Catalase negative strains of *R. dentocariosa* have been reported and this will be more difficult to recognise with traditional tests, since they may mimic the rare *Bifidobacterium* strains that are able to grow aerobically, as well as *Actinomyces* and *Arcanobacterium sp*, *Propionibacterium propionicum* and catalase negative *Listeria* strains.

R. dentocariosa is distinct from *Dermabacter* species in that it is nitrate and pyrazinamidase positive.

***Rothia mucilaginosa*[26-28]**

(was previously known as *Stomatococcus mucilaginosus, Micrococcus mucilaginosus* or *Staphylococcus salivarius*).

This Gram positive coccus is found in clusters. Cells display variable catalase reactions ranging from negative to weakly positive to strongly positive, oxidase negative, and exhibit facultatively anaerobic metabolism. They are able to use glucose fermentatively. Optimum growth temperature is 30-37°C. Their white to greyish non-haemolytic colonies may be mucoid, rubbery, or sticky in consistency and adherent to agar due to the mucilagenous capsular material produced. The inability to grow in the presence of 5% NaCl distinguishes *R. mucilagino*sa from members of the genera *Staphylococcus* and *Micrococcus*.

It is isolated primarily from mouth and respiratory tract of humans, and is capable of growth and producing diseases like endocarditis and meningitis in mammals.

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.

Technical information/limitations

Method for demonstrating the micromorphology of cultures

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.

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 - 1.0mm) round colonies may be seen on brain heart infusion agar containing blood which is incubated for 24 hours.

Rhodococci can be easily distinguished from most *Corynebacterium s*pecies which, except for *Corynebacterium aquaticum, Corynebacterium minutissimum* and the Centers for Disease Control and Prevention (CDC) group B-1, have a fermentative metabolism.

Commercial identification systems

Commercial identification systems do not provide reliable identification of *Rhodococcus* species and clinically important isolates should be referred to the Reference Laboratory[8].

1 Safety considerations[29-45]

Hazard Group 2 organisms.

Refer to current guidance on the safe handling of all Hazard group 2 organisms documented in this UK Standard for Microbiology Investigations.

The above guidance should be supplemented with local COSHH and risk assessments.

Compliance with postal and transport regulations is essential.

2 Target organisms

***Norcardia* species which have been associated with infection[2,3] - *N. asteroides, N. brasiliensis*,** *N. abscessus, N. africana, N. anaemia, N. aobensis, N. araoensis, N. arthritidis, N. asiatica, N. beijingensis, N. blacklockiae, N. brevicatena, N. carnea, N. concave, N. cyriacigeorgica, N. elegans, N.exalbida, N. farcinica, N. higoensis, N. inohanensis, N. kruczakiae, N. Mexicana, N. mikamii, N. niigatensis, N. ninae, N. niwae, N. nova, N. otitidiscaviarum, N. paucivorans, N. pseudobrasiliensis, N. pneumoniae, N. puris, N. sienata, N. terpenica, N. testacea, N. thailandica, N. transvalensis, N. vermiculata, N. veteran, N. vinacea, N. wallacei, N. yamanashiensis*

Other species that are associated in infections in humans are[4,8,12,18,46-48] - *Actinomadura madurae, Actinomadura pelletieri, Streptomyces somaliensis, Tsukamurella paurometabola,* *Rhodococcus equi, Rhodococcus erythropolis, Rhodococcus rhodochrous, Streptomyces sudanensis, Tsukamurella strandjordii, Tsukamurella inchonensis, Tsukamurella pulmonis, Tsukamurella tyrosinosolvens, Gordonia terrae, Gordonia sputi, Gordonia bronchialis, Gordonia polyisoprenivorans, Gordonia rubripertincta, Gordonia otitidis, Gordonia effuse, Gordonia araii, Gordonia aichiensis, Nocardiopsis dassonvillei, Dermatophilus congolensis, Pseudonocardia autotrophica (*formerly *Amycolata autotrophica), Amycolatopsis orientalis*

3 Identification

3.1 Microscopic appearance

(TP 39 – Staining procedures)

Gram stain

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

Norcardia species – In direct Gram smears, organisms appear as very long branching, thin and finely beaded Gram positive rods. When prepared from cultures, smears may show streptococcus-like chains or small branching filaments.

Rhodococcus, Gordonia, Tsukamurella - diphtheroid-like with minimal branching or coccobacilary.

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 stain

If the stain is positive the isolate is probably a partially acid fast aerobic actinomycete. *Nocardia, Rhodococcus, Gordonia* and *Tsukamurella* species are usually negative with this stain.

Acid-fast stain (Modified Kinyoun Method)

Nocardia species are variably acid-fast.

Rhodococcus and *Gordonia* species are usually partially acid-fast.

Most strains of *Tsukamurella* species are acid-fast by the Kinyoun method.

Actinomadura species are non-acid-fast.

3.2 Primary isolation media

Chocolate agar incubated in 5 - 10% CO_2 at 35°C - 37°C for 16 - 48hr.

Blood agar incubated in 5 - 10% CO_2 at 35°C - 37°C for 16 - 48hr.

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

Note: If selective agar plates are used, they should be incubated for 2 to 3 weeks. Most *Streptomyces* species grow best at 25-35°C.

3.3 Colonial appearance

Genus	**Characteristics of growth on fastidious anaerobe agar after incubation at 35 - 37°C for 40 – 48hr**
Nocardia species	Wrinkled often dry, crumbly, chalky-white appearance to orange or tan pigment
Streptomyces species	Waxy heaped colonies with variable morphology
Oerskovia species	Yellow pigmented, extensive branching that grows on the surface and in to the agar
Gordonia, Rhodococcus, and *Tsukamurella* species	Non-haemolytic, round, often mucoid with salmon-pink/red colonies developing within 4 to 7 days
Dermatophilus congolensis	Round adherent grey-white colonies, that later develop orange pigments; often beta-haemolytic. Colonies may be adherent to the agar.
Actinomadura species	White to pink colour. Colonies are usually mucoid, wrinkled and have a molar

	tooth appearance
Rothia species	Small, smooth to rough colonies and dry in appearance
Nocardiopsis species	Coarsely wrinkled and folded with well- developed aerial mycelium

3.4 Test procedures

3.4.1 Differentiation of branching Gram positive rods

Smears (in duplicate) from both colonies and clinical material should be stained with Gram 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).

3.4.2 Commercial identification Systems[12]

The commercial identification systems when used, gave reproducible results on repeated testing for sugar assimilation.

This was most helpful in distinguishing *T. paurometabola* and *T. pulmonis* from the other *Tsukamurella* species. Testing with the commercial identification system in combination with standard biochemicals for mycobacteria, temperature responses, and degradation agars allowed us to identify all Tsukamurellae to the species level.

3.4.3 Matrix-assisted laser desorption/ionisation - time of flight (MALDI-TOF) mass spectrometry

Matrix-assisted laser desorption ionisation - time of flight mass spectrometry (MALDI-TOF MS), which can be used to analyse the protein composition of a bacterial cell, has emerged as a new technology for species identification. This has been shown to be a rapid and powerful tool because of its reproducibility, speed and sensitivity of analysis. The advantage of MALDI-TOF as compared with other identification methods is that the results of the analysis are available within a few hours rather than several days. The speed and the simplicity of sample preparation and result acquisition associated with minimal consumable costs make this method well suited for routine and high-throughput use[49].

The use of this technique for the identification of fastidious, slow-growing organisms such as *Nocardia* species, which are notoriously difficult to identify by conventional tests, in the routine laboratory has been very beneficial and of major interest[50].

MALDI-TOF has also been used successfully to reclassify *Streptomyces* species and in identification of *Oerskovia* species as well morphologically similar organisms like the *Rothia species*[51,52].

The one factor limiting the use of MALDI-TOF MS remains the limited availability of reference data sets for microorganisms that are infrequently isolated from clinical specimens, and it has been shown previously that the absence or the availability of only a small number of isolates of a given species in the reference database may

account for most of the cases in which no identification can be obtained by the MALDI-TOF MS method[53].

Further expansion of the database with a larger number of isolates including the less commonly described *Nocardia* species is also clearly warranted.

3.4.4 Nucleic acid amplification tests (NAATs) and PCR-RFLP molecular analysis (PRA)

PCR is usually considered to be a good method as it is simple, sensitive and specific. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.

This has been used for the characterisation of isolates belonging to the genus *Nocardia* or for the identification of *Nocardia* species from those belonging to other genera of actinomycetes[54]. PCR tests, using primers targeted at species-specific sequences in the 16S rRNA gene, were successfully developed for *Rhodococcus globerulus, Rhodococcus erythropolis, Rhodococcus opacus* and *Rhodococcus ruber*[55]. This has helped facilitate rapid diagnosis and prompt the initiation of the appropriate chemotherapy as well as used for epidemiological studies.

PRA techniques involve PCR amplification of portions of the *hsp65* gene or the 16*S* rRNA gene and subsequent digestions with specific restriction endonucleases for each gene. PRA techniques take advantage of the variations in gene sequences of species within a genus and of the presence or absence of restriction endonuclease recognition sites within variable regions of the gene. This has been used successfully to *Nocardia* isolates from those belonging to the genus *Mycobacterium* as well as allowed differentiation of most of the species of *Nocardia* commonly isolated from clinical specimens[56].

3.5 Further identification

Rapid molecular methods

Molecular methods have had an enormous impact on the taxonomy of aerobic actinomycetes. Analysis of gene sequences has increased understanding of the phylogenetic relationships of aerobic actinomycetes and related organisms and has resulted in the recognition of numerous new species. Molecular techniques have made identification of many species more rapid and precise than is possible with phenotypic techniques.

A variety of rapid typing methods have been developed for isolates from clinical samples; these include molecular techniques such as Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Analysis (MLSA), and 16S rRNA gene sequencing. All of these approaches enable subtyping of unrelated strains, but do so with different accuracy, discriminatory power, and reproducibility.

However, some of these methods remain accessible to reference laboratories only and are difficult to implement for routine bacterial identification in a clinical laboratory.

16S rRNA gene sequencing

A genotypic identification method, 16S rRNA gene sequencing is used for phylogenetic studies and has subsequently been found to be capable of re-classifying

bacteria into completely new species, or even genera. It has also been used to describe new species that have never been successfully cultured.

The availability of gene sequencing has revolutionized the taxonomy of the aerobic actinomycetes and has become an invaluable tool for the identification of clinical isolates. With the rapidly changing *Nocardia* taxonomy, the species associated with some sequences may not have the correct nomenclature based on today's standards and there has been significant criticism of the GenBank database because sequences submitted to the database are not checked for accuracy or for appropriate species assignment and it is inadequate[2]. There is a high degree of sequence divergence which exists within many species and many taxa within the *Nocardia* species are unnamed[57].

16SrRNA sequencing may also not be a definitive method for distinguishing between *G. sputi* and *G. aichiensis*. However, it aids in the identification of isolates with indeterminate phenotypic or PRA results[57].

This has also been used to identify a new bacterium, *Tsukamurella strandjordii* as well as *Tropheryma whipplei*[12,17].

MultiLocus sequence analysis (MLSA)

MLSA has been used as a method to examine prokaryote taxonomy because of its ease of use, accuracy, and discriminating power.

Nocardia species identification is difficult due to a complex and rapidly changing taxonomy, the failure of 16S rRNA and cellular fatty acid analysis to discriminate many species, and the unreliability of biochemical testing. However, *Nocardia* species identification can be achieved through multilocus sequence analysis (MLSA) of gyrase B of the β subunit of DNA topoisomerase (*gyrB*), 16S rRNA (*16S*), subunit A of SecA preprotein translocase (*secA1*), the 65-kDa heat shock protein (*hsp65*), and RNA polymerase (*rpoB*) and this would be more feasible for routine use in a clinical reference microbiology laboratory[58].

The identification and classification of species within the genus *Streptomyces* is difficult but this technique has helped in the taxonomy and has been extremely successful in the elucidation of interspecies relationships within the *Streptomyces griseus* rRNA gene clade[59,60].

Pulsed field gel electrophoresis (PFGE)

PFGE detects genetic variation between strains using rare-cutting restriction endonucleases, followed by separation of the resulting large genomic fragments on an agarose gel. PFGE is known to be highly discriminatory and a frequently used technique for outbreak investigations and has gained broad application in characterizing epidemiologically related isolates. However, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies. However, due to its time-consuming nature (30hr or longer to perform) and its requirement for special equipment, PFGE is not used widely outside the reference laboratories[61,62].

This has been used to differentiate epidemiologically related isolates of *Nocardia farcinica* in nocardial endemics or epidemics[63]. This information has been helpful for understanding the spread of disease in both hospitals and communities.

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 aerobic actinomycetes flowchart

Due to the considerable morphological diversity seen among genera and also among strains of the same taxon, refer to the current journal articles for identification.

5 Reporting

5.1 Presumptive identification

Presumptive identification may be made if appropriate growth characteristics, colonial appearance, Gram 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 bears relevant information.

5.4 CCDC

Refer to local Memorandum of Understanding.

5.5 Public Health England[64]

Refer to current guidelines on CIDSC and COSURV reporting.

5.6 Infection prevention and control team

N/A

6 Referrals

6.1 Reference laboratory

Contact appropriate devolved national reference laboratory for information on the tests available, turnaround times, transport procedure and any other requirements for sample submission:

Molecular Identification Service Unit (MISU)
Microbiology Services
Public Health England
61 Colindale Avenue
London
NW9 5EQ

Contact PHE's main switchboard: Tel. +44 (0) 20 8200 4400

England and Wales

https://www.gov.uk/specialist-and-reference-microbiology-laboratory-tests-and-services

Scotland

http://www.hps.scot.nhs.uk/reflab/index.aspx

Northern Ireland

http://www.belfasttrust.hscni.net/Laboratory-MortuaryServices.htm

7 Notification to PHE[64,65], or equivalent in the devolved administrations[66-69]

The Health Protection (Notification) regulations 2010 require diagnostic laboratories to notify Public Health England (PHE) when they identify the causative agents that are listed in Schedule 2 of the Regulations. Notifications must be provided in writing, on paper or electronically, within seven days. Urgent cases should be notified orally and as soon as possible, recommended within 24 hours. These should be followed up by written notification within seven days.

For the purposes of the Notification Regulations, the recipient of laboratory notifications is the local PHE Health Protection Team. If a case has already been notified by a registered medical practitioner, the diagnostic laboratory is still required to notify the case if they identify any evidence of an infection caused by a notifiable causative agent.

Notification under the Health Protection (Notification) Regulations 2010 does not replace voluntary reporting to PHE. The vast majority of NHS laboratories voluntarily report a wide range of laboratory diagnoses of causative agents to PHE and many PHE Health protection Teams have agreements with local laboratories for urgent reporting of some infections. This should continue.

Note: The Health Protection Legislation Guidance (2010) includes reporting of Human Immunodeficiency Virus (HIV) & Sexually Transmitted Infections (STIs), Healthcare Associated Infections (HCAIs) and Creutzfeldt–Jakob disease (CJD) under ‘Notification Duties of Registered Medical Practitioners’: it is not noted under ‘Notification Duties of Diagnostic Laboratories’.

https://www.gov.uk/government/organisations/public-health-england/about/our-governance#health-protection-regulations-2010

Other arrangements exist in Scotland[66,67], Wales[68] and Northern Ireland[69].

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