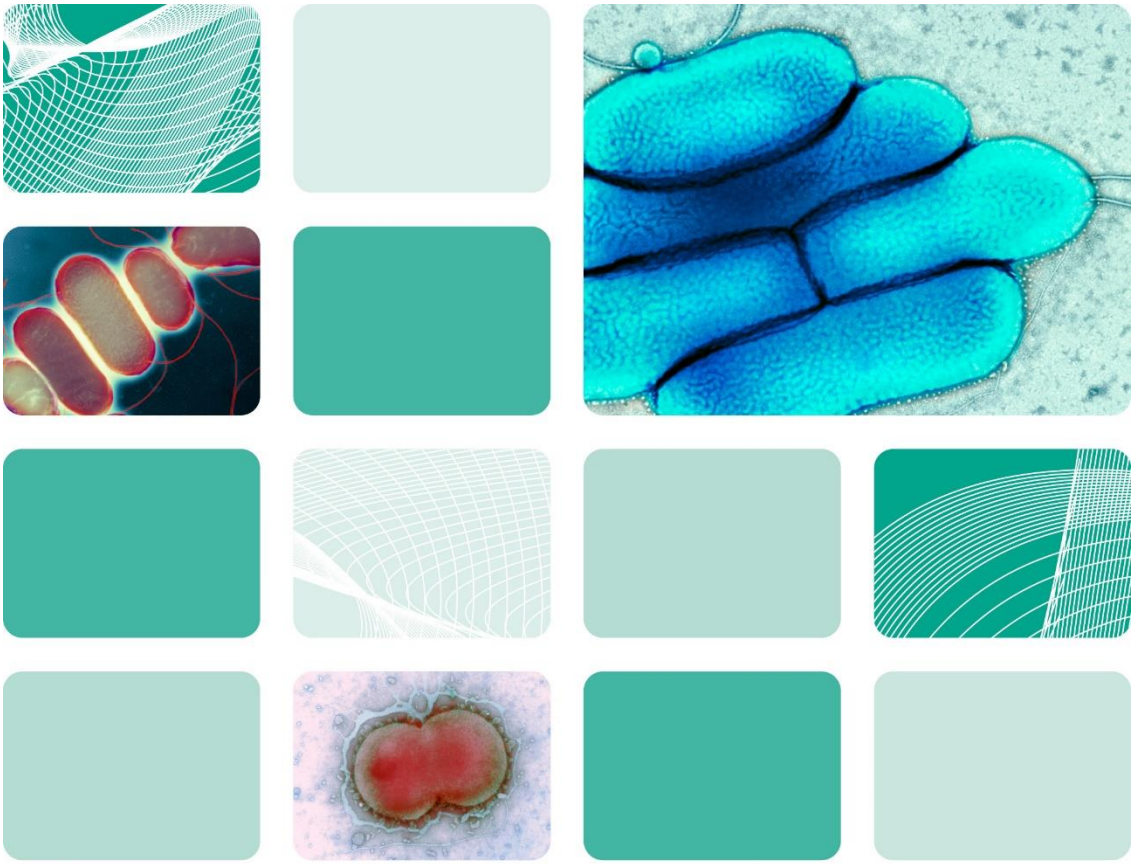
UK Health Security Agency

UK Standards for Microbiology Investigations

Identification of Corynebacterium species



National Institute for Health and Care Excellence (NICE) has renewed accreditation of the process used by the **UK Health Security Agency** to produce **UK Standards for Microbiology Investigations (UK SMIs)**. The renewed accreditation is valid until **30 June 2026** and applies to guidance produced using the processes described in '**UK Standards for Microbiology Investigations Development Process' (2021)**. The original accreditation term began on 1 July 2011.

Issued by the Standards Unit, Specialised Microbiology and Laboratories, UKHSA

Acknowledgments

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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.

UK SMIs are produced in association with:

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Contents

Amendment table

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

Any alterations to this document should be controlled in accordance with the local document control process.

Amendment number/date	8/16.05.2023
Issue number discarded	4.1
Insert issue number	5
Anticipated next review date*	16.05.2026
Section(s) involved	**Amendment**
Whole document	Updated to a new template with new layout
Target organisms	Main potentially toxigenic target organisms summarised.
Identification	Table of culture media and biochemical tests split and placed under relevant headings for ease.
Further Identification and characterisation	MLST and AFLP removed
Reporting and Flowchart section	Updated.

*Reviews can be extended up to 5 years where appropriate

1 General information

View general information related to UK SMIs.

2 Scientific information

View scientific information related to UK SMIs.

3 Scope of document

This UK SMI describes the identification to species level of the 3 potentially toxigenic *Corynebacterium* species which can cause respiratory or cutaneous diphtheria: *Corynebacterium diphtheriae, Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis.* These species are isolated from throat, nose, skin, ulcers and other sites in suspected cases of classical diphtheria or cutaneous diphtheria. The importance of prompt referral for confirmation of identification and toxigenicity testing for patient management and public health actions is emphasised. Diphtheria was once one of the most feared childhood diseases in the UK, but both cases and deaths dramatically reduced following introduction of mass immunisation in the 1940s.

There have been significant changes in diphtheria epidemiology over time in the UK. Until early 1990, toxigenic infections were more commonly caused by *C*. *diphtheriae* than *C. ulcerans*, whereas between 1990 and 2008, *C. ulcerans* was the predominant cause of UK toxigenic infection. From 2009 to 2017 there was an increase in *C. diphtheriae* cases and from 2018 onwards, the majority of cases have been *C. ulcerans*.

More recently, an increase in imported cases of toxigenic *C. diphtheriae* has been identified (1). Cases have also been reported in Switzerland and in eight EU/EEA countries: Austria, Belgium, France, Germany, Italy, the Netherlands, Norway and Spain (ECDC, 2022).

The document also describes the identification of non-toxigenic species, *Corynebacterium jeikeium, Corynebacterium striatum* and other clinically significant species.

Identification of *Arcanobacterium haemolyticum* is covered in ID 3: Identification of *Listeria* species and other non-sporing Gram Positive Rods (except *Corynebacterium*).

Identification of *C. urealyticum* is not covered in this document; however, isolation of this may be a significant finding if associated with urinary tract infection.

This UK SMI includes both biochemical tests and automated methods for the identification of microorganisms. Some biochemical tests may not be done routinely in the laboratory except in cases where confirmation by an alternative technique is required or automated methods are not available.

UK SMIs should be used in conjunction with other relevant UK SMIs.

4 Introduction

4.1 Taxonomy and characteristics

There are 134 validly published species in the *Corynebacterium* genus at the time of writing , approximately half of which (45%) have been isolated from humans (2). Some

species previously assigned to the genus *Corynebacterium* with genetic and chemotaxonomic features inconsistent with those currently attributed to the genus have since been reassigned to other genera. Conversely, relevant taxa assigned to other genera and those with *Corynebacterium*-like features, have been added to the genus (3). Some species are part of the normal human flora, but may opportunistically cause disease, and some are transmitted to humans by zoonotic contact.

The potentially toxigenic *Corynebacterium* species comprise *C. diphtheriae, C. pseudotuberculosis* and *C. ulcerans.* Toxigenic strains are lysogenic for a family of corynebacteriophages that carry the structural gene for diphtheria toxin, *tox* (4). These species can cause diphtheria - a potentially fatal disease. *C. diphtheriae* consists of 4 biovars: gravis, mitis, intermedius and belfanti (5). The biovar belfanti was reported to be clearly separated phylogenetically from *C. diphtheriae* biovar mitis and gravis and a new species, *Corynebacterium belfantii* sp. nov. has been proposed (6).

Corynebacterium species are Gram positive non-motile rods, often with clubbed ends, occurring singly or in pairs. Some cells may stain unevenly giving a beaded appearance and their size is between 2 to 6µm in length and 0.5µm in diameter. They are arranged together in a characteristic way, which has been described as ‘V’ or ‘L’ shaped ‘palisades’. Metachromatic granules are usually present representing stored phosphate regions. The species are aerobic or facultatively anaerobic and exhibit a fermentative metabolism (carbohydrates to lactic acid) under certain conditions. They are fastidious organisms, growing slowly even on enriched medium (7).

All species are catalase positive and most are oxidase negative with the exception of *C. bovis, C. aurimucosum, C. doosanense*, and *C. maris* (5). *C. diphtheriae, C. ulcerans* and *C. pseudotuberculosis* are facultatively anaerobic, non-sporing, non-capsulated and non-acid fast. These organisms are non-motile.

C. ulcerans and *C. pseudotuberculosis* are both urease positive which may be used to distinguish them presumptively from *C. diphtheriae* (4).

Non-toxigenic strains of *C. diphtheriae*, *C. ulcerans*, *C. pseudotuberculosis* as well as other *Corynebacterium* species such as *C. jeikeium* and *C. striatum* may also cause disease including pulmonary infection and endocarditis (8). Both *C. jeikeium* and *C. striatum* are non-haemolytic, urease negative and catalase positive (9).

Recommendations for the laboratory investigation of potentially toxigenic *Corynebacterium* species; *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* can be found in the WHO laboratory manual for the diagnosis of diphtheria and other related infections (10). Note that selective medium should be used in parallel with a non-selective media such as blood agar.

Agar containing blood and potassium tellurite, such as Hoyle’s medium, serves as a semi-selective and differential medium. On blood agar, they form small greyish colonies with a granular appearance, mostly translucent, but with opaque centres, convex, with continuous borders. Their optimum growth temperature is 37°C (7).

Corynebacterium diphtheriae

C. diphtheriae is transmitted by respiratory droplets through person to person, with an incubation of 2 to 5 days. An individual person is infectious when virulent bacteria are present in respiratory secretions, usually for 2 weeks without antibiotics (11).

Diphtheria is a potentially life threatening, but vaccine preventable infection. In England there were 3 reports of toxigenic *C. diphtheriae* isolation during 2021, and 1 non-toxigenic toxin-bearing strain (11).

C. diphtheriae grows as grey or black colonies on blood tellurite agar in 16 to 18 hours and produces characteristic colonies after 48 hours. Colony morphology of isolates will vary in size and appearance but generally appear 1 to 3 mm at 24 hours on blood agar (except for biovar intermedius). Modified Tinsdale agar is another selective and differential medium that contains tellurite, L-cystine and sodium thiosulphate. Colonies on modified Tinsdale agar are 1 to 2 mm, black or charcoal grey and have a brown-black halo visible in the agar due to cysteinase activity.

Corynebacterium ulcerans

On Tinsdale medium colonies appear brown with halos with the production of cystinase and do not produce pyrazinamidase. Colonies may be slightly β-haemolytic on blood agar.

Corynebacterium pseudotuberculosis

C. pseudotuberculosis colonies may be slightly β-haemolytic on blood agar.

4.2 Principles of identification

Isolates from primary culture are identified by colonial appearance, Gram stain, and 4 preliminary tests (this includes nitrate, urease, catalase and pyrazinamidase tests) which permit the presumptive identification of the potentially toxigenic *Corynebacterium* species within 4 hours. Additional identification may be made using a commercial identification kit in conjunction with toxin testing. It is advisable that suspected toxigenic cultures are sent promptly to a diphtheria reference laboratory for confirmation of identification and toxigenicity testing.

Use of Albert's stain is not recommended in this UK SMI, as metachromatic granules are not specific to *C. diphtheriae* or any of the potentially toxigenic *Corynebacterium* species.

The interpretation of the clinical significance of *Corynebacterium* species isolated from microbiological samples can be problematic. *Corynebacterium* isolated as a predominant organism from a specimen from a normally sterile site, wound, abscess or purulent sputum, from more than 1 blood culture set or present at greater than or equal to 10^4 cfu/mL in a pure culture from urine should be considered for identification to species level (7). The clinical significance is strengthened when isolating *Corynebacterium* species from multiple samples or when they are seen in a Gram stained smear as the predominant organism or associated with a significant leucocyte response (12).

Identification to species level is recommended especially if the organism is isolated from normally sterile body sites, from adequately collected clinical material if the *Corynebacterium* species is the predominant organism, and if recovered from urine specimens.

5 Technical information and limitations

Corynebacterium pseudotuberculosis

C. pseudotuberculosis can give a variable nitrate test result. This is because it consists of 2 biovars: biovar *equi* (from horses or cattle) that reduces nitrate and the biovar *ovis* (from sheep or goats) that fails to do so (9).

Agar media

The classic colonial morphology has been reported to develop better on media containing sheep blood rather than horse in some *Corynebacterium* species. For example, the degree of haemolysis in *Arcanobacterium haemolyticum*, formerly known as *C. haemolyticum* is far greater on sheep blood agar plate than most other *Corynebacterium* species (13).

6 Safety considerations

This section covers specific safety considerations (14-36) related to this UK SMI, and should be read in conjunction with the general safety considerations on GOV.UK.

C. diphtheriae, C. ulcerans and C. pseudotuberculosis are Hazard Group 2 organisms, and in some cases the nature of the work may dictate full Containment Level 3 conditions. All laboratories should handle specimens as if potentially high risk.

All suspected isolates of potentially toxigenic *Corynebacterium* species should always be handled in a microbiological safety cabinet. For the urease test, a urea slope is considered safer than a liquid medium.

C. diphtheriae and *C. ulcerans* cause severe and sometimes fatal diseases. Laboratory acquired infections have been reported (37,38). The organism infects primarily by the respiratory route. Vaccination against diphtheria is available; guidance is given in Diphtheria: the green book, chapter 15 (39). Individuals who may be exposed to diphtheria in the course of their work, in microbiology laboratories and clinical infectious disease units, are at risk and must be protected (40,41).

Diphtheria antitoxin for the treatment of clinical cases is distributed by UKHSA Immunisation Department and should be given without waiting for bacteriological confirmation (36).

Refer to current guidance on the safe handling of all Hazard Group 2 organisms documented in this UK SMI.

Laboratory procedures that may give rise to infectious aerosols must be conducted in a microbiological safety cabinet (25).

The above guidance should be supplemented with local COSHH and risk assessments and read in conjunction with the general safety considerations on GOV.UK.

Compliance with postal and transport regulations is essential.

7 Target organisms

The main potentially toxigenic target organisms are *Corynebacterium diphtheriae, Corynebacterium ulcerans and Corynebacterium pseudotuberculosis* (7). Other, non-toxigenic, *Corynebacterium* species have been known to cause human infection (9).

8 Identification

8.1 Microscopic appearance

Gram stain TP 39 - Staining procedures

Gram positive rods, pleomorphic, slightly curved with tapered or clubbed ends.

Cells may occur singly or in pairs, often in a 'V' or 'L' formation.

Cells usually stain weakly and unevenly giving a beaded appearance.

8.2 Primary isolation media

Blood agar skin swabs incubated in 5 to 10% CO_2 at 35 to 37°C for 40 to 48 hours and throat swabs incubated anaerobically at 35 to 37°C for 16 to 24 hours. β-haemolytic streptococci may also be present, particularly in throat swabs.

Blood tellurite agar incubated in air at 35 to 37°C for 16 to 48 hours.

8.3 Colonial appearance

Appearance varies among species on blood agar plates. For more information, refer to the table below.

Strain	Culture media	
	Blood tellurite agar	**Blood agar**
C. diphtheriae biotype biovar *gravis* (42)	Dull, grey or black, opaque colonies, 1.5 to 2.0mm in diameter, matte surface, friable - tending to break into small segments when touched with a straight wire	Non-haemolytic
C. diphtheriae biotype biovar *mitis* (42)	Grey or black, opaque colonies, 1.5 to 2.0mm in diameter, entire edge and glossy smooth surface; size variation is common	colonies exhibit a small zone of β-haemolysis
C. diphtheriae biotype biovar *intermedius* (42)	small, grey or black, shiny surface, discrete, translucent colonies, 0.5 to 1.0mm in diameter	colonies exhibit a small zone of β-haemolysis
C. diphtheriae biotype biovar *belfanti* (42)	grey or black, opaque colonies, 1.5 to 2.0mm in diameter, entire edge and glossy smooth surface; size variation is common	colonies exhibit a small zone of β-haemolysis
C. ulcerans (42)	grey or black, very dry opaque colonies	colonies exhibit a small zone of β-haemolysis
C. pseudo-tuberculosis (7,9,43)	grey or black, very dry opaque colonies	colonies exhibit a small zone of β-haemolysis

C. striatum (7,43)	grey or black, colonies	Non-haemolytic white moist smooth colonies greater than 2mm after 24 hours
C. jeikeium	grey or black, colonies	Non-haemolytic Grey or white low convex colonies

8.4 Test procedures

8.4.1 Biochemical tests

Rapid 4 hour tests should be performed for urease, pyrazinamidase, catalase and nitrate reduction.

Catalase test TP 8 - Catalase test

All potentially toxigenic corynebacteria are catalase positive. For non-toxigenic *Corynebacterium* species the catalase test results are varied.

Pyrazinamidase test

All potentially toxigenic *Corynebacterium* species (*C. diphtheriae, C. ulcerans* and *C. pseudotuberculosis*) are pyrazinamidase negative while other *Corynebacterium* species are positive.

Urease test TP 36 - Urease test

The urease test is used to determine the ability of an organism to split urea through the production of the enzyme urease. *C. ulcerans* and *C. pseudotuberculosis* are urease positive.

Nitrate reduction test - see table below

Strain	Biochemical tests†			
	Nitrate	Urease*	Catalase	Pyrazinamidase
C. diphtheriae biotype biovar *gravis* (42)	Positive	Negative	Positive	Negative
C. diphtheriae biotype biovar *mitis* (42)	Positive	Negative	Positive	Negative
C. diphtheriae biotype biovar *intermedius* (42)	Positive	Negative	Positive	Negative
C. diphtheriae biotype biovar *belfanti* (42)	Negative	Negative	Positive	Negative
C. ulcerans (42)	Negative	Positive	Positive	Negative

C. pseudo-tuberculosis (7,9,43)	Positive or Negative	Positive	Positive	Negative
C. striatum (7,43)	Positive or Negative	Negative	Positive	Positive
C. jeikeium	Negative	Negative	Positive	Positive

† Refer to TP 36 - Urease Test

*If results of these “4 hour” tests indicate *Corynebacterium* species, immediately inform the Infection Specialist any isolates of presumptive *C. diphtheriae*, *C. ulcerans*, *C. pseudotuberculosis* should be referred promptly to the diphtheria National Reference Laboratory. *C. xerosis* can be used as a positive control for this test.

If these preliminary tests do not indicate *Corynebacterium* species then consider further identification tests if clinically indicated.

Results for the nitrate test can be variable for *C. pseudotuberculosis*. This is because it consists of 2 biovars: biovar *equi* (from horses or cattle) that reduce nitrate and the biovar *ovis* (from sheep or goats) that fail to do so.

Use a commercial identification kit and refer isolate to the reference laboratory if clinically indicated.

Note: Fresh culture of control organism is advisable.

These test results are consistent with taxonomy from widely published systems.

It is important that a preliminary identification of possible colonies of *C. diphtheriae* or other potentially toxigenic *Corynebacterium* species is made as rapidly as possible with the use of “4 hour” tests. The preliminary tests provide an indication of the likely presence or absence of *C. diphtheriae, C. ulcerans* or *C. pseudotuberculosis*. The results should be considered together with the clinical details.

All suspected isolates of *C. diphtheriae* or other potentially toxigenic *Corynebacterium* species should be sub-cultured to a blood agar plate for purity and to a blood or chocolate agar slope (preferably) or Loeffler’s media (to expedite referral to a reference laboratory) at the time that the tests are set up.

8.4.2 Commercial identification systems

Laboratories should follow manufacturer’s instructions and rapid tests and kits should be validated and be shown to be fit for purpose prior to use.

8.4.3 Matrix-assisted laser desorption ionisation–time of flight mass spectrometry (MALDI-TOF)

MALDI-TOF MS has been used successfully to identify potentially toxigenic *Corynebacterium* species at the species level in clinical isolates within 15 minutes (44,45). This technology can be used as a rapid screening method helping to decide whether suspicious colonies should be referred for toxigenicity testing. It can also

discriminate *C. aurimucosum* from the closely related *C. minutissimum* - previously considered difficult to differentiate (46).

Refer to UK SMI TP 40: Matrix-assisted laser desorption ionisation - time of flight mass spectrometry (MALDI-TOF MS) test procedure for more information.

8.4.4 Nucleic acid amplification tests (NAATs)

Initially, conventional PCR assays designed to detect the diphtheria toxin gene (*tox*), particularly against the region responsible for the biologically active (Fragment A), were described (40). Subsequently, real-time PCR protocols facilitating more rapid detection of the tox gene and confirmation of identification and were developed (47-51).

Confirmation of identification and presence of the toxin gene is rapid and can be completed within 4 hours of receipt of the strain. However, the presence of the toxin gene as demonstrated by qPCR may not always predict toxin expression, thus, a phenotypic test for toxigenicity must always be performed to confirm expression of diphtheria toxin (52).

8.5 Further identification and characterisation

8.5.1 Typing methods

A variety of rapid typing methods have been developed for isolates from clinical samples; these include molecular techniques such as sequence analysis, 7-allele multi-locus sequence typing (MLST) (53) core genome MLST using 1,305 genes (54) and whole genome sequencing.

8.5.2 Whole genome sequencing (WGS)

Whole genome sequencing is the principle of sequencing the entire genome of an organism and can be achieved through the use of various available sequencing technologies. Several *Corynebacterium* species have had complete genomes sequenced (3). Genome sequences are available online for *C. glutamicum*, *C. efficiens*, *C. diphtheriae, C. jeikeium*, *C. pseudotuberculosis* and *C. ulcerans*.

Sequencing can provide valuable information complementing routine microbiological and epidemiological investigations. This can include identification of unknown clusters, antimicrobial resistance and can support results obtained by NAATs (including PCR) (55).

8.5.3 16S rRNA gene (rDNA) sequence analysis

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 use of molecular genetic methods such as 16S rRNA gene (rDNA) sequence analysis has facilitated a much tighter circumscription of the genus *Corynebacterium*, and the availability of comparative 16S rRNA gene sequence data with improved phenotypic data has resulted in much improved and more reliable species identification; however, *rpoB* gene sequences are used as they are more polymorphic than the 16S rDNA and can ensure reliable phylogenetic studies (46,56).

8.6 Storage and referral

Refer presumptive *C. diphtheriae, C. ulcerans* or *C. pseudotuberculosis* isolate on a Loeffler or blood agar or chocolate agar slope immediately to a reference laboratory.

Isolates should also be saved and stored locally.

8.7 Antimicrobial susceptibility testing

Report susceptibilities as clinically indicated. Prudent use of antimicrobials according to local and national protocols is recommended.

Refer to EUCAST guidelines (57).

All *C. diphtheriae* isolates should undergo antimicrobial susceptibility testing.

9 Reporting

9.1 Infection Specialist

Inform the Infection Specialist of presumptive and confirmed *C. diphtheriae, C. ulcerans* or *C. pseudotuberculosis* species. The infection specialist should also be informed of relevant information in the request, for example:

- membranous or pseudomembranous pharyngitis or tonsillitis
- contact with a confirmed case within the last 10 days
- travel abroad to a high risk area within the last 10 days
- contact with someone who has been to a high risk area within the last 10 days
- contact with any animals (including household pets, visiting a farm or petting zoo) within the last 10 days
- recent consumption of any type of unpasteurised milk or dairy products
- the patient works in a clinical microbiology laboratory, or similar occupation, where *Corynebacterium* species may be handled

For presumptive and confirmed non-toxigenic *Corynebacterium* species, the infection specialist should be informed when the request bears relevant information for example:

- cases of suspected endocarditis associated with appropriate specimen
- infection of indwelling medical devices (prosthetic valves, pacemakers, peritoneal and vascular catheters, CSF shunts)
- history of substance abuse, alcoholism, immunodeficiency or other serious underlying disorder such as cancer, or patients receiving treatment for cancer, inducing neutropenia or mucositis

Follow local protocols for reporting to the clinician.

9.2 Preliminary identification

Presumptive identification may be made if appropriate characteristics are demonstrated. This may include growth characteristics, colonial appearance, Gram stain of the culture, “4 hour” test results and results of rapid methods.

9.3 Confirmation of identification

For confirmation and identification please see Specialist and reference microbiology: laboratory tests and services page on GOV.UK for reference laboratory user manuals and request forms.

9.4 Health Protection Team (HPT)

Refer to local agreements in devolved administrations.

9.5 UK Health Security Agency

Refer to current guidelines on Second Generation Surveillance System (SGSS) reporting (30).

As diphtheria is a notifiable disease in the UK all suspected cases should be notified immediately to the local UK Health Security Agency laboratory for public health management of cases, contacts and outbreaks.

9.6 Infection prevention and control team

Inform the infection prevention and control team of presumptive and confirmed isolates of potentially toxigenic strains of *Corynebacterium* species according to local protocols.

10 Referral to reference laboratories

Potentially toxigenic strains from the species *C. diphtheriae*, *C. ulcerans* and *C*. *pseudotuberculosis* should be referred to a diphtheria reference laboratory for toxigenicity testing as soon as possible (11).

For information on the tests offered, turnaround times, transport procedure and the other requirements of the reference laboratory see user manuals and request forms

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

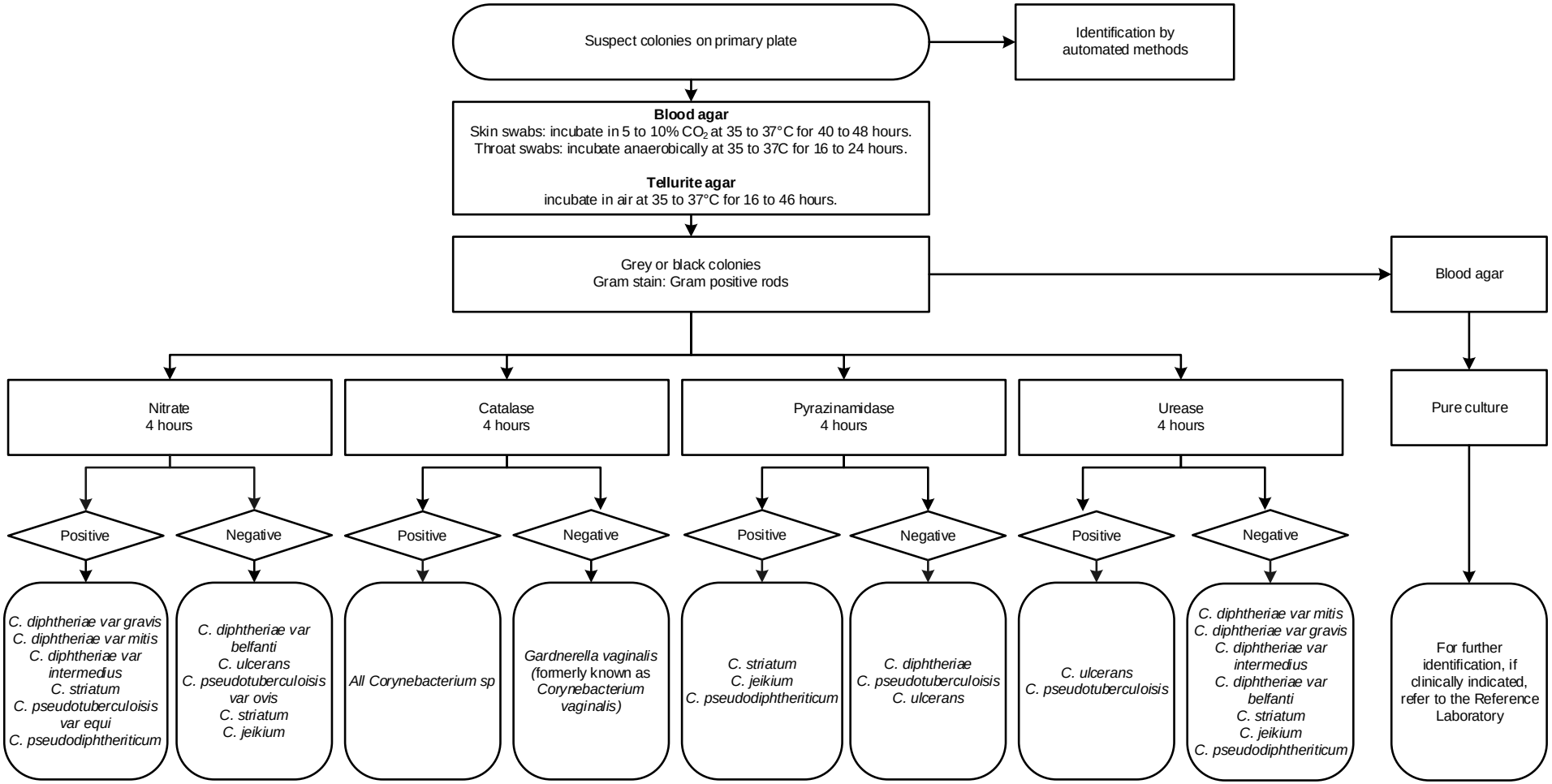
England

Wales

Scotland

Northern Ireland

Algorithm: Identification of *Corynebacterium* species



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An explanation of the reference assessment used is available in the scientific information section on the UK SMI website.

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