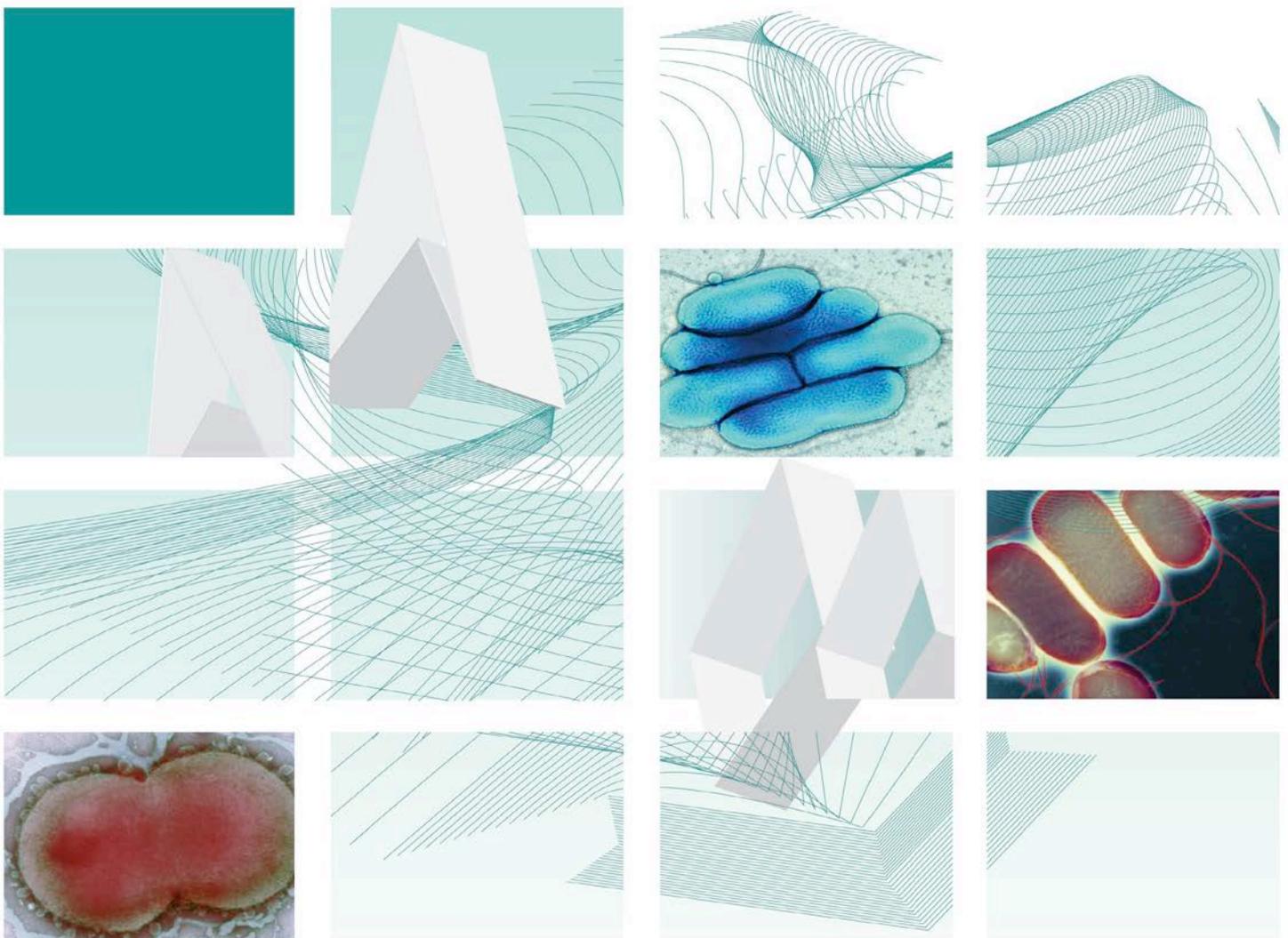




UK Standards for Microbiology Investigations

Identification of *Staphylococcus* species, *Micrococcus* species and *Rothia* species



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.

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Contents

ACKNOWLEDGMENTS	2
AMENDMENT TABLE	4
UK STANDARDS FOR MICROBIOLOGY INVESTIGATIONS: SCOPE AND PURPOSE.....	6
SCOPE OF DOCUMENT	9
INTRODUCTION	9
TECHNICAL INFORMATION/LIMITATIONS.....	15
1 SAFETY CONSIDERATIONS	17
2 TARGET ORGANISMS.....	17
3 IDENTIFICATION.....	18
4 IDENTIFICATION OF <i>STAPHYLOCOCCUS</i> SPECIES, <i>MICROCOCCUS</i> SPECIES AND <i>ROTHIA</i> SPECIES.....	23
5 REPORTING	24
6 REFERRALS.....	25
7 NOTIFICATION TO PHE OR EQUIVALENT IN THE DEVOLVED ADMINISTRATIONS	25
REFERENCES	27



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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.	5/12.11.14
Issue no. discarded.	2.3
Insert Issue no.	3
Section(s) involved	Amendment
Whole document.	Hyperlinks updated to gov.uk.
Page 2.	Updated logos added.
Whole document.	Document presented in a new format.
Scope of document.	The scope has been updated to include webpage links for B 29 and ID 4 documents.
Introduction.	The taxonomy of <i>Staphylococcus</i> , <i>Micrococcus</i> and <i>Rothia</i> species has been updated. More information has been added to the Characteristics section. The medically important species are mentioned and their characteristics described. Use of up-to-date references. Section on Principles of Identification has been updated to reflect rapid methods used for identification.
Technical Information/Limitations.	Addition of information regarding agar media, coagulase test and common issues with <i>S. aureus</i> has been described and referenced.
Target Organisms.	The section on the Target organisms has been updated and presented clearly. References have been updated.
Identification.	Minor amendments have been made to 3.1 and 3.2. 3.3 and 3.4 have been updated to reflect standards in practice. Subsection 3.5 has been updated to include the Rapid Molecular Methods.

Identification of *Staphylococcus* species, *Micrococcus* species and *Rothia* species

Identification Flowchart.	Modification of flowchart for identification of species has been made for easy guidance.
Reporting.	Subsection 5.1 - 5.4 has been updated to reflect reporting practice.
Referral.	The address of the reference laboratory has been updated.
References.	Some references updated.

UK Standards for Microbiology Investigations[#]: Scope and Purpose

Users of SMIs

- SMIs are primarily intended as a general resource for practising professionals operating in the field of laboratory medicine and infection specialties in the UK.
- SMIs 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.
- SMIs 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.

[#]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.

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 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.

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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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Suggested Citation for this Document

Public Health England. (2014). Identification of *Staphylococcus* species, *Micrococcus* species and *Rothia* species . UK Standards for Microbiology Investigations. ID 7 Issue 3. <https://www.gov.uk/uk-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories>

Scope of Document

This SMI describes the identification of *Staphylococcus* species, *Micrococcus* species and *Rothia* species. Details on MRSA screening can be found in [B 29 - Investigation of Specimens for Screening MRSA](#).

For the identification of catalase negative Gram positive cocci, see [ID 4 - Identification of *Streptococcus* species, *Enterococcus* species and Morphologically Similar Organisms](#).

This SMI should be used in conjunction with other SMIs.

Introduction

Taxonomy

Taxonomically, the genus *Staphylococcus* is in the bacterial family Staphylococcaceae, which includes five lesser known genera, *Gemella*, *Jeotgalicoccus*, *Macrococcus*, *Nosocomiicoccus* and *Salinicoccus*. There are currently 47 recognised species of staphylococci and 21 subspecies most of which are found only in lower mammals¹. The staphylococci most frequently associated with human infection are *S. aureus*, *S. epidermidis* and *S. saprophyticus*. Other *Staphylococcus* species may also be associated with human infection².

The genus *Micrococcus* belongs to the bacterial family Micrococcaceae which currently contains 16 species. These have been isolated from human skin, animal and dairy products as well as environment (water, dust and soil)³. Some of these species have been re-classified to other genera. Former members of the genus *Micrococcus*, now assigned to other genera, include *Arthrobacter agilis*, *Nesterenkonia halobia*, *Kocuria kristinae*, *K. rosea*, *K. varians*, *Kytococcus sedentarius*, and *Dermacoccus nishinomiyaensis*. The *Micrococcus* species that are associated with infections are *Micrococcus luteus* and *Micrococcus lylae*.

The genus *Rothia* belonged to the bacterial family Actinomycetaceae as described by Georg and Brown in 1967 but more recent molecular studies placed the genus in the family Micrococcaceae, suborder *Micrococcineae*, order *Actinomycetales*, subclass *Actinobacteridae* and class *Actinobacteria*. It is therefore in the same family as the genera *Micrococcus*, *Arthrobacter*, *Kocuria*, *Nesterenkonia*, *Renibacterium* and *Stomatococcus*, all of which show characteristic signature nucleotides in their 16S rDNA sequences⁴. There are currently 6 species, *Rothia dentocariosa* and *Rothia mucilaginoso* are the only two which have been known to cause infections in humans⁵.

Characteristics

Staphylococcus species are Gram positive, non-motile, non-sporing cocci of varying size occurring singly, in pairs and in irregular clusters. Colonies are opaque and may be white or cream and occasionally yellow or orange. The optimum growth temperature is 30°C-37°C. They are facultative anaerobes and have a fermentative metabolism. *Staphylococcus* species are usually catalase positive and are also oxidase negative with the exception of the *S. sciuri* group (*S. sciuri*, *S. lentus* and *S. vitulinus*), *S. fleuretti* and the *Macrococcus* group to which *S. caseolyticus* has been assigned^{2,6,7}. This is also a distinguishing factor from the genus streptococci, which are catalase negative, and have a different cell wall composition to staphylococci.

Nitrate is often reduced to nitrite. Some species are susceptible to lysis by lysostaphin but not lysozyme and are able to grow in 6.5% sodium chloride. Some species produce extracellular toxins. Staphylococci may be identified by the production of deoxyribonuclease (DNase) and/or a heat-stable DNase (thermostable nuclease)⁸.

Coagulase positive staphylococci

Staphylococcus aureus

S. aureus are cocci that form irregular grape-like clusters. They are non-motile, non-sporing and catalase positive. They grow rapidly and abundantly under aerobic conditions. On blood agar, they appear as glistening, smooth, entire, raised, translucent colonies that often have a golden pigment. The colonies are 2-3mm in diameter after 24hr incubation and most strains show β -haemolysis surrounding the colonies.

There are currently 2 subspecies of *S. aureus*; these are *S. aureus* subspecies *aureus* and *S. aureus* subspecies *anaerobius*.

S. aureus* subspecies *aureus is commonly isolated from human clinical specimens. All strains are able to grow on thioglycolate medium within 24hr. Most strains produce a wide zone of strong haemolysis within 24 to 36hr. They show positivity for D-glucose, D-fructose, D-mannose, D-maltose, D-lactose, D-trehalose, D-mannitol, sucrose, N-acetyl-glucosamine, D-celiobiose, and D-turanose; meanwhile, no acid production was demonstrated by utilization of D-ribose, xylitol, xylose, D-melibiose, raffinose, L-arabinose, and α -methyl-D-glucoside. They are also positive for catalase, coagulase, and benzidine reactions and are capable of nitrate reduction and acetylmethylcarbinol (acetoin) production. Results for DNase, clumping factor, urease, arginine dihydrolase, pyrrolidonyl arylamidase, leucine arylamidase, β -N-acetylglucosaminidase, α -chymotrypsin, α -glucosidase, β -glucosidase, alkaline phosphatase, esterase C-4 and C-8, lipase (C-14), phosphatase acid, and naphthol- AS-BI-phosphohydrolase are positive.

There is no production of oxidase, α -galactosidase, β -glucuronidase, β -galactosidase, valine arylamidase, cystine arylamidase, arginine arylamidase, trypsin, ornithine decarboxylase, α -mannosidase, and α -fucosidase. All strains are resistant to novobiocin⁹.

S. aureus* subspecies *anaerobius is rarely isolated from clinical specimens. They are 0.8 to 1.0 μ m in diameter and occur singly, in pairs, and predominantly in irregular clusters. On the primary isolation medium, growth is obtained only in media that are supplemented with blood, serum, or egg yolk and incubated microaerobically or anaerobically. Colonies on blood agar after 2 days of incubation are very small (1 to 3mm in diameter), low convex, circular, entire, smooth, glistening, and opaque. Pigment is not produced. Luxuriant growth is obtained on Dorset egg medium, with colony diameters of 4 to 6mm. The strains produce unevenly disseminated growth on brain heart infusion agar after 3 days of microaerophilic incubation. They grow as dwarf colonies, among which a few colonies of normal size are observed¹⁰.

It grows poorly aerobically and growth may be CO₂ dependent. It is slide coagulase negative and thermonuclease negative and may be catalase negative. Strains may be identified by better growth anaerobically and they may give a positive coagulase test result. However, because growth may be poor, the coagulase result may be negative and suspected isolates should be referred to the Reference Laboratory.

Staphylococcus aureus may be associated with severe infection and it is important to distinguish it from the opportunistic coagulase negative staphylococci. In routine laboratory practice, the production of coagulase is frequently used as the sole criterion to distinguish *S. aureus* from other staphylococci. It is also important to note that coagulase negative strains of *S. aureus* have been reported¹¹.

Other coagulase positive staphylococcal species such as *S. hyicus*, *S. schleiferi* subspecies *coagulans*, *S. pseudintermedius* or *S. intermedius* may be coagulase positive but have been found only occasionally to be associated with human infection or carriage^{12,13}. The production of coagulase and thermostable nuclease by these staphylococci may lead to their misidentification as *S. aureus*. *Staphylococcus delphini* is coagulase positive and thermostable nuclease positive (rarely isolated from humans).

Carbon dioxide dependent strains of *S. aureus* can be recovered from clinical material¹⁴. The significance of these strains in the laboratory is that they pose a significant technical problem when performing antibiotic susceptibility testing as they fail to grow in air. Therefore susceptibility testing should be performed in a CO₂ enriched atmosphere. These strains although referred to as dwarf strains in the past should not be confused with the slow-growing small colony variants (SCV's) of *S. aureus* which have decreased metabolism and a defective electron transport system and are auxotrophic for substrates such as haemin, menadione, thiamine or thymidine¹⁵. Such strains are meticillin resistant and have an intrinsic resistance to aminoglycoside antibiotics such as gentamicin and are most frequently identified in patients with chronic or persistent infections¹⁶.

Multi resistance to antibiotics has most often been associated with meticillin resistant strains¹⁶.

Staphylococcus aureus produces virulence factors such as protein A, capsular polysaccharides and α toxin. Some strains of *S. aureus* produce toxic shock syndrome 1 toxin (TSST-1), Panton-Valentine Leucocidin or other toxins.

Coagulase negative staphylococci (CoNS)¹⁷

Coagulase negative staphylococci (CoNS) are normal commensals of the skin, anterior nares, and ear canals of humans. They have long been considered as non-pathogenic, and were rarely reported to cause severe infections. However, as a result of the combination of increased use of intravascular devices and an increase in the number of hospitalized immunocompromised patients, CoNS have emerged as a major cause of nosocomial bloodstream infections.

They are opportunistic pathogens which lack many of the virulence factors associated with *S. aureus*. There are more than 30 species of CoNS. The taxonomy of these coagulase negative staphylococci (CoNS) fall into clusters based on 16s rRNA sequences¹⁸.

S. epidermidis and *S. saprophyticus* are the species most often associated with infection but *Staphylococcus capitis*, *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Staphylococcus sciuri*, *Staphylococcus schleiferi* subspecies *schleiferi*, *Staphylococcus simulans*, *Staphylococcus saccharolyticus* (previously known as *Peptococcus saccharolyticus*) and *Staphylococcus warneri* have also been implicated^{19,20}. Many of these species are also thermostable nuclease negative. *S. lugdunensis* is coagulase negative but some strains may be positive for the slide coagulase test or clumping factor²¹.

Multi resistance to antibiotics also occurs in some strains of *S. epidermidis* which are thermostable nuclease negative. *S. saprophyticus*, *S. cohnii* and *S. sciuri* groups are generally novobiocin resistant as is *S. hominis* subsp. *novobiosepticus*²².

Staphylococcus pasteurii can be phenotypically distinguished from all of the other novobiocin-susceptible staphylococci except *S. warneri*, from which it can only be differentiated by genotyping²³.

Staphylococcus epidermidis

S. epidermidis are approximately 0.5 to 1.5µm in diameter and arranged in grape-like clusters. They are facultative anaerobes that can grow by aerobic respiration or by fermentation. Some strains may not ferment.

It forms greyish-white, raised, circular, smooth, glistening, and translucent to slightly opaque, cohesive colonies approximately 1–2mm in diameter after overnight incubation, and is non-haemolytic on blood agar. They grow well at NaCl concentrations up to 7.5%, poorly at 10% and fail to grow at 15%. They are positive for catalase, urease and exhibit a weak positive reaction for the nitrate reduction test. They are negative for coagulase, oxidase and gelatin hydrolysis tests. They utilize glucose, fructose, sucrose, and lactose to form acid products aerobically. In the presence of lactose, they will also produce gas.

They are either susceptible or slightly resistant to lysostaphin and are resistant to lysozyme.

S. epidermidis is sensitive to novobiocin, and this test distinguishes it from *Staphylococcus saprophyticus*, which is coagulase negative, as well, but novobiocin resistant²⁴.

Staphylococcus saprophyticus

They are positive for catalase and urease tests while they are negative for motility, coagulase, nitrate reduction and oxidase tests. They utilize fructose, maltose, sucrose and trehalose to form acid products. They grow well on 10% NaCl agar, but only 11-89% strains tolerate 15% NaCl. Colonies appear as raised to slightly convex, circular, usually entire, 4.0 to 9.0mm in diameter, smooth, glistening, and usually opaque. Colony pigment is variable; however, most strains are not pigmented or might have a slight yellow tint which increases in intensity with age.

Two subspecies for *S. saprophyticus* exist: *S. saprophyticus* subsp. *bovis* and *S. saprophyticus* subsp. *saprophyticus*, the latter is more commonly found in human UTIs. *S. saprophyticus* subsp. *saprophyticus* is distinguished by its being nitrate reductase and pyrrolidonyl arylamidase negative while *S. saprophyticus* subsp. *bovis* is nitrate reductase and pyrrolidonyl arylamidase positive²⁵.

S. saprophyticus is resistant to the antibiotic novobiocin, a characteristic that is used in laboratory identification to distinguish it from *S. epidermidis*, which is also coagulase negative but novobiocin sensitive²².

***Micrococcus* species**

Micrococcus species are strictly aerobic Gram positive cocci arranged in tetrads or irregular clusters, not in chains and cells range from 0.5 to 3µm in diameter. They are seldom motile and are non-spore forming. They are also catalase positive and often oxidase positive, although weakly. Micrococci may be distinguished from staphylococci by a modified oxidase test^{26,27}. Their colonies are usually pigmented in shades of yellow or red and grow on simple media. The optimum growth temperature is 25-37°C. They

have a respiratory metabolism, often producing little or no acid from carbohydrates and are usually halotolerant, growing in 5% NaCl. They contain cytochromes and are resistant to lysostaphin⁸.

They are generally considered harmless saprophytes that inhabit or contaminate the skin, mucosa, and also the oropharynx; however they can be opportunistic pathogens in certain immunocompromised patients²².

There are currently 9 species of *Micrococcus* and 2 have been known to cause infections in humans - *Micrococcus lylae* and *Micrococcus luteus*³.

Micrococcus lylae

They are mostly arranged in tetrads. They are positive for catalase and oxidase and negative for urease. They grow in circular, entire, convex and usually not pigmented or cream white colonies having diameters of approximately 4mm after 2-3 days on plate at 37°C. They assimilate D- Maltose, D- Trehalose, Maltitol, acetate, citrate, d-Glucose, sucrose, d-fructose, d, fumarate, dl-3-hydroxybutyrate, dl-lactate, pyruvate, l-aspartate, l-histidine, l-leucine, 3-hydroxybenzoate and 4-hydroxybenzoate and also hydrolyse l-proline pNA, Tween 20 and Tween 80²⁸.

M. lylae can be distinguished from the closely related species *M. luteus* by lysozyme susceptibility, genetic compatibility, and the type of cell-wall peptidoglycan. There are also some differences between these species in the parameters of pigmentation, nitrogen requirements, nitrate reduction and acid from maltose and sucrose.

It has been isolated from human skin.

Micrococcus luteus

They are mostly arranged in tetrads. They are positive for catalase and oxidase. They grow in circular, entire, convex and creamy yellow pigmented colonies having diameters of approximately 4mm after 2-3 days at 37°C. Several uncommon strains produce raised colonies with translucent, depressed centres. Colony pigmentation varies considerably but are usually different shades of yellow or cream-white²⁹.

Growth or weak growth is observed at 45°C, at pH 10 and in the presence of 10% NaCl; no growth is observed in the presence of 15% NaCl. D-Glucose, sucrose and D-mannose are assimilated while L-proline pNA, and Tween 20 are hydrolysed.

There are 3 biovars of *M. luteus* and they possess quite diverse chemotaxonomic features with respect to their menaquinone systems, cell-wall compositions and Fourier transform-infrared (FT-IR) spectroscopy (FT-IR) patterns, as well as biochemical properties. The recognition of three different biovars within the species *M. luteus* has the advantage that the three groups can be differentiated without nomenclatural changes having to be introduced²⁸.

It has been isolated from human skin.

***Rothia* species**

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* species³⁰. 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*⁵.

Rothia dentocariosa

R. dentocariosa 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 culture⁴. 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 CO₂ 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 acid³¹.

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* species, *Propionibacterium propionicum* and catalase negative *Listeria* strains⁴.

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

Rothia mucilaginosa (was previously known as *Stomatococcus mucilaginosus*, *Micrococcus mucilaginosus* or *Staphylococcus salivarius*^{32,33}).

This 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. mucilaginosa* from members of the genera *Staphylococcus* and *Micrococcus*³⁴.

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

Presumptive staphylococci need to be quickly differentiated into two groups:

- Probable *S. aureus* - a potential pathogen when isolated from most sites
- Other staphylococci - usually not significant in skin and superficial wound swab sites, but a possible pathogen in some circumstances

Staphylococcus aureus has traditionally been identified by tube coagulase tests that detect staphylocoagulase or "free coagulase". However, detection of surface proteins such as clumping factor (slide coagulase test) and/or protein A (commercial latex tests) may be used for rapid identification. Inclusion of latex particles sensitized with antibodies against specific capsular antigens has enabled commercial manufacturer's to improve the sensitivity of latex tests to detect atypical strains of *S. aureus* and MRSA that fail to express the major characteristics listed above³⁵. Positive results or suspected erroneous slide tests may be confirmed by a tube coagulase test.

Full molecular identification using for example, MALDI-TOF MS can be used to identify CoNS isolates to species level.

Typing and differentiation between strains of *S. aureus* can be achieved using a range of molecular techniques eg *spa* typing, Pulsed Field Gel Electrophoresis (PFGE), Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA), Multi-locus sequence typing (MLST), Microarrays, Next Generation Sequencing, etc³⁶.

Technical Information/Limitations

Agar Media

The use of conventional media such as blood agar has the advantage that they may also be useful for the simultaneous isolation of other pathogens such as streptococci. The disadvantage of such media is that confirmatory tests are necessary to differentiate *S. aureus* from other staphylococci³⁵. Performing such tests on all colonies resembling staphylococci can be time-consuming and labour intensive.

The use of chromogenic media, if sufficiently sensitive and specific, can potentially reduce the number of confirmatory tests and achieve isolation and presumptive identification in a single step. Another advantage is that they require fewer reagents for confirmation of suspect colonies of *S. aureus* and hence may be cost effective^{37,38}.

Chromogenic media for *S. aureus* may be supplemented with appropriate antimicrobials (eg oxacillin or ceftiofuran) for the detection of MRSA³⁹.

Note: Chromogenic media are affected by direct light and plates should be stored in the dark and not left in the light long before or after inoculation.

Coagulase test

S. aureus is differentiated from other staphylococci by the coagulase test. However it is now known that not all *S. aureus* are coagulase positive and not all coagulase positive staphylococci are *S. aureus*⁴⁰.

S. lugdunensis is coagulase negative but some strains may be slide coagulase or clumping factor positive.

For the tube coagulase test, citrate-utilizing organisms such as *Enterococcus faecalis*, *Pseudomonas species*, *Serratia marcescens*, and strains of *Streptococcus* will clot citrated plasma⁴¹.

S. hyicus, *S. intermedius*, *S. pseudintermedius* and *S. schleiferi* may be tube coagulase positive.

Common issues with *S. aureus*

Yeasts can be misidentified as coagulase negative staphylococci on the basis of colony morphology and a negative slide agglutination test. Speciation of staphylococci should be considered for isolates from sterile sites and blood cultures to avoid missing *S. aureus*, *S. lugdunensis* or yeasts.

Staphylococcus sciuri can give positive results with DNA and *Staph aureus* latex tests and can have the *mecA* gene and therefore grow on chromogenic MRSA medium with a blue green pigment. On blood agar, they appear as large yellow colonies resembling *S. aureus*. It is easily distinguished from other staphylococci as it is oxidase positive.

Other non *S. aureus* species such as *S. intermedius* could also be misidentified as MRSA/MSSA.

1 Safety Considerations⁴²⁻⁵⁸

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

Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet⁵⁰.

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

Compliance with postal and transport regulations is essential.

2 Target Organisms

***Staphylococcus* species reported to have caused human infections^{13,21,22,59-64}**

S. aureus* group - *Staphylococcus aureus* subsp *aureus*, *Staphylococcus aureus* subsp *anaerobius

S. epidermidis* group - *Staphylococcus epidermidis*, *Staphylococcus capitis* subsp *capitis*, *Staphylococcus capitis* subsp *urealyticus*, *Staphylococcus caprae*, *Staphylococcus saccharolyticus

S. saprophyticus* group - *Staphylococcus saprophyticus* subsp *saprophyticus*, *Staphylococcus cohnii* subsp *cohnii*, *Staphylococcus cohnii* subsp *urealyticus*, *Staphylococcus xylosus

S. hyicus-intermedius* group - *Staphylococcus hyicus*, *Staphylococcus intermedius*, *Staphylococcus pseudintermedius*, *Staphylococcus schleiferi* subsp *coagulans*, *Staphylococcus schleiferi* subsp *schleiferi

S. simulans* group - *Staphylococcus simulans

S. haemolyticus* group - *Staphylococcus hominis* subsp *hominis*, *Staphylococcus hominis* subsp *novobiosepticus*, *Staphylococcus haemolyticus

S. lugdunensis* group - *Staphylococcus lugdunensis

S. warneri* group - *Staphylococcus warneri*, *Staphylococcus pasteurii

S. auricularis* group - *Staphylococcus auricularis

S. carnosus* group - *Staphylococcus massiliensis*, *Staphylococcus pettenkoferi

***S. sciuri* group - *Staphylococcus sciuri* subsp *sciuri*, *Staphylococcus sciuri* subsp *rodentium*,**

Staphylococcus sciuri subsp *carnaticus*, *Staphylococcus lentus*, *Staphylococcus vitulinus*

Other species reported to have caused human infections- *Micrococcus luteus*, *Micrococcus lylae*, *Micrococcus mortus* (not officially recognised), *Rothia mucilaginoso*, *Rothia dentocariosa*

3 Identification

3.1 Microscopic Appearance

Gram stain ([TP 39 - Staining Procedures](#))

Gram positive cocci occurring singly, in pairs, tetrads or in irregular clusters.

3.2 Primary Isolation Media

Blood agar incubated in 5 - 10% CO₂ at 35°C - 37°C for 16 - 24hr.

These organisms may be isolated from other media including CLED, Staph/Strep selective and Mannitol Salt agar (MSA).

3.3 Colonial Appearance

Staphylococcus species usually grow as opaque, 1 – 5mm, smooth colonies; white, cream or yellow to orange on blood agar. Haemolysis may be detected. They appear as white or yellow-green, 1 - 2mm colonies on CLED agar.

Note: Small colony variant strains of *S. aureus* and strains resistant to vancomycin (VRSA) may require 72hr incubation to become visible.

S. lugdunensis gives a prominent β -haemolysis and a characteristic *Eikenella*-like odour after 2 days of incubation on Columbia agar with 5% sheep blood, which, combined with colony pleomorphism, helps in its initial recognition⁶⁵.

Note: Avoid smelling or wafting the bacteria on the plates towards you because of inhalation of spores and becoming contaminated. This is prohibited in the laboratory.

Micrococcus species produce yellow or red-pigmented colonies on blood agar.

Rothia species are round, convex, mucoidy and adhere to the agar. Colonial morphology varies with species.

3.4 Test Procedures

3.4.1 Biochemical tests

Catalase test (see [TP 8 - Catalase Test](#))

Staphylococcus, *Micrococcus* and *Rothia* species are catalase positive.

S. aureus subspecies *anaerobius* and *S. saccharolyticus* are catalase negative²².

Coagulase and other tests to detect *S. aureus* (see [TP 10 - Coagulase Test](#))

Protein A, clumping factor (slide coagulase or latex), thermostable nuclease or tube coagulase tests may be used. Positive results or suspected erroneous slide tests (listed above) may be confirmed by a tube coagulase test.

S. aureus, some strains of *S. hyicus*, *S. intermedius*, and *S. schleiferi* subspecies *coagulans* are coagulase positive and thermostable nuclease positive.

Other species of staphylococci are coagulase negative and thermostable nuclease negative or weak positive. *S. lugdunensis* is coagulase negative but some strains may be slide coagulase or clumping factor positive.

DNase test (see [TP 12 – Deoxyribonuclease Test](#))

Commercially available DNA containing agars are used to detect thermolabile nuclease activity. Addition of a weak acid (1N HCl) solution to an 18 – 24hr culture plate will demonstrate clearing around colonies of DNase positive species and if toluidine blue O solution is added, a bright rose-pink zone around colonies of DNase positive species can be seen.

Note: It is important that both positive and negative screening tests for *S. aureus* are verified using a second confirmatory test to detect false positive and false negative primary screens eg Protein A latex and DNase.

3.4.2 Commercial identification Systems

Several commercial identification systems are available for the speciation of Staphylococci. Results should be interpreted in conjunction with the key test results indicated above.

3.4.3 Matrix-Assisted Laser Desorption/Ionisation - Time of Flight (MALDI-TOF)

Matrix-assisted laser desorption ionization–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 use⁶⁶.

MALDI-TOF MS method has been found to be useful as an additional test for the description of new staphylococcal species and in the profiling of staphylococcal strains and it has also revealed different clonal lineages of *S. epidermidis* that were of either human or environmental origin⁶⁷. However, further studies are required to test this technology with a large collection of staphylococci of diverse origins.

3.4.4 Nucleic Acid Amplification Tests (NAATs)

PCR is usually considered to be a good method for bacterial detection as it is simple, sensitive and specific. However, it does have limitations. Although the 16S rRNA gene is generally targeted for the design of species-specific PCR primers for identification, designing primers is difficult when the sequences of the homologous genes have high similarity. In the case of staphylococci, therefore, ribotyping, internal transcribed spacer PCR and various other methods have been used. There are different PCRs for the different groups (coagulase positive and the coagulase negative staphylococcal species) and their target genes and depending on clinical details, the appropriate PCR will be performed^{68,69}. However, the development of a species-specific quantitative PCR methodology has proved difficult.

Multiplex PCR assay has also been used for detection of genes encoding surface protein adhesins, toxins or antibiotic resistance in Staphylococci and more recently, for species identification of coagulase positive Staphylococci by targeting the thermonuclease (*nuc*) gene locus⁷⁰⁻⁷². This has also been used to discriminate simultaneously between *mecA* and *mecA_{LGA251}* alongside the detection of Pantone–Valentine leucocidin (PVL) and *nuc* genes of Meticillin resistant *S. aureus* and it

provides a valuable tool for the rapid and accurate characterization of staphylococci which is essential in modern hospital practice⁷³. This approach would also be valuable for surveys.

3.5 Further Identification

Toxin studies

Occasionally *S. aureus* is recovered from cases of suspected toxin-mediated disease eg staphylococcal scalded skin syndrome, toxic shock syndrome, PVL toxin, necrotising pneumonia, bullous impetigo and food poisoning. The Staphylococcus Reference Laboratory welcomes such isolates being referred for toxin gene profiling and typing studies.

Rapid Methods

A variety of current rapid typing methods have been developed for isolates from clinical samples; these include molecular techniques such as Pulsed-Field Gel Electrophoresis (PFGE), 16S rRNA gene sequencing, PCR- restriction fragment length Polymorphism (PCR-RFLP), *spa* typing, Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) and Multi-locus sequence typing (MLST). All of these approaches enable subtyping of strains, but do so with different accuracy, discriminatory power, and reproducibility.

For further molecular investigation, Microarray analysis, single nucleotide polymorphism (SNPs) – these are looking to replace PFGE and MLVA) and even whole-genome sequencing (WGS) may be options, as new technology (eg Ion Torrent Sequencing) allows WGS results within a few days³⁶.

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

16S rRNA gene sequences has been useful in phylogenetic studies at the genus level, its use has been questioned in studies at the *Staphylococcus* species level¹⁸. This stems from the fact that closely related species may have identical 16S rRNA sequences or, alternatively, that divergent 16S rRNA sequences may exist within a single organism⁷⁴.

S. caprae and *S. capitis* cannot be distinguished by their 16S rRNA gene sequences. Similarly, some *Staphylococcus* taxa have the same 16S rRNA gene sequences in variable regions V1, V3, V7, and V9, with identical sequences occurring in, eg *S. vitulinus*, *S. saccharolyticus*, *S. capitis* subsp *urealyticus*, *S. caprae*, the two subspecies of *S. aureus*, and the two subspecies of *S. cohnii*⁷⁵.

PCR- restriction fragment length Polymorphism (PCR-RFLP)

Due to the limited number of stable features that can be used for species discrimination, many taxa remain difficult to distinguish from one another and are misidentified by phenotypic tests.

However, restriction fragment length polymorphism (RFLP) analysis of the *dnaJ* gene of PCR products has been reported for use for the identification of staphylococci. This has proved to be an adequate tool for the correct identification of almost all prevalent species and subspecies of *Staphylococcus*, irrespective of their phenotypic characterization. This method requires only PCR and one or two enzymes and thus is

technically less demanding than the majority of other molecular approaches. It is easier to use, less expensive and less equipment dependent than sequencing.

This method is also able to discriminate subspecies of the species *S. capitis*, *S. carnosus*, *S. cohnii*, and *S. hominis*⁷⁴.

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. However, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies. Due to its time-consuming nature (30hr or longer to perform) and its requirement for special equipment and the interpretation of its results often being subjective, PFGE is not used widely outside reference laboratories. These problems make the exchange of strain typing information difficult and complicate the creation of an *S. aureus* and MRSA typing database⁷⁶.

Presently, pulsed-field gel electrophoresis (PFGE) remains the most discriminatory technique for *S. aureus* typing, but it allows the constitution of shared databases only at the national level and is not appropriate for population studies⁷⁷.

More recently, PFGE has been used for epidemiological typing of Meticillin resistant *Staphylococcus aureus* (MRSA)⁷⁸.

Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA)

Multiple-Locus Variable number tandem repeat Analysis (MLVA) is a method used to perform molecular typing of particular microorganisms. It utilizes the naturally occurring variation in the number of tandem repeated DNA sequences found in many different loci in the genome of a variety of organisms. The molecular typing profiles are used to study transmission routes, to assess sources of infection and also to assess the impact of human intervention such as vaccination and use of antibiotics on the composition of bacterial populations.

This has been used successfully for the genotyping of *S. aureus*⁷⁷.

***spa* sequence typing**

spa sequencing appears to be a highly effective rapid typing tool for *S. aureus* that, despite some expense of specificity, has significant advantages in terms of speed, ease of use, ease of interpretation, and standardization among laboratories. It provides a suitable discrimination for outbreak investigation. Another technique that can be used in outbreaks is *SCCmec* typing depending on the local epidemiology although better *SCCmec* methods need to be developed³⁶. An additional advantage of *spa* typing is that adequate typing information is obtained from a single locus, as opposed to MLST, which requires the combination of allelic information from many genes.

It has been documented that *spa* repeat sequences by themselves define excellent resolving power among strains of *S. aureus*^{79,80}. *spa* typing correctly assigns staphylococcal strains to the appropriate phylogenetic groups and performs better than multi locus enzyme electrophoresis (MLEE) and PFGE, and it facilitates the detection of both macro- and micro-variation⁸¹.

However, *spa* does have some disadvantages. It is insufficiently discriminatory in regions where a particular clone/small number of clones are endemic; it is not recommended for smaller local hospital laboratories and is currently not yet used universally³⁶.

Multi-locus sequence typing (MLST)

MLST measures the DNA sequence variations in a set of housekeeping genes directly and characterizes strains by their unique allelic profiles. The principle of MLST is simple: the technique involves PCR amplification followed by DNA sequencing. Nucleotide differences between strains can be checked at a variable number of genes depending on the degree of discrimination desired.

This has been used to provide a reliable method of characterising MRSA clones as well as investigating the epidemiology and phylogeny of *S. lugdunensis*⁸². It has also been used for analysing the evolution of *S. epidermidis* as well.

Microarrays

DNA microarray technology can provide detailed, clinically relevant information on the isolate by detecting the presence or absence of a large number of virulence-associated genes simultaneously in a single assay; however, their clinical value has been limited by a complicated methodology that is unsuitable for routine use in diagnostic microbiology laboratories.

This has been used to successfully differentiate between isolates representative of a spectrum of *S. aureus* types, including meticillin susceptible, meticillin resistant, community-acquired, and vancomycin resistant *S. aureus*, and to simultaneously detect clinically relevant virulence determinants⁸³.

Whole Genome Sequencing (WGS)

This is also known as full genome sequencing, complete genome sequencing, or entire genome sequencing. It is a laboratory process that determines the complete DNA sequence of an organism's genome at a single time. There are several high-throughput techniques that are available and used to sequence an entire genome such as pyrosequencing, nanopore technology, Illumina sequencing, Ion Torrent sequencing, etc. This sequencing method holds great promise for rapid, accurate, and comprehensive identification of bacterial transmission pathways in hospital and community settings, with concomitant reductions in infections, morbidity, and costs⁸⁴.

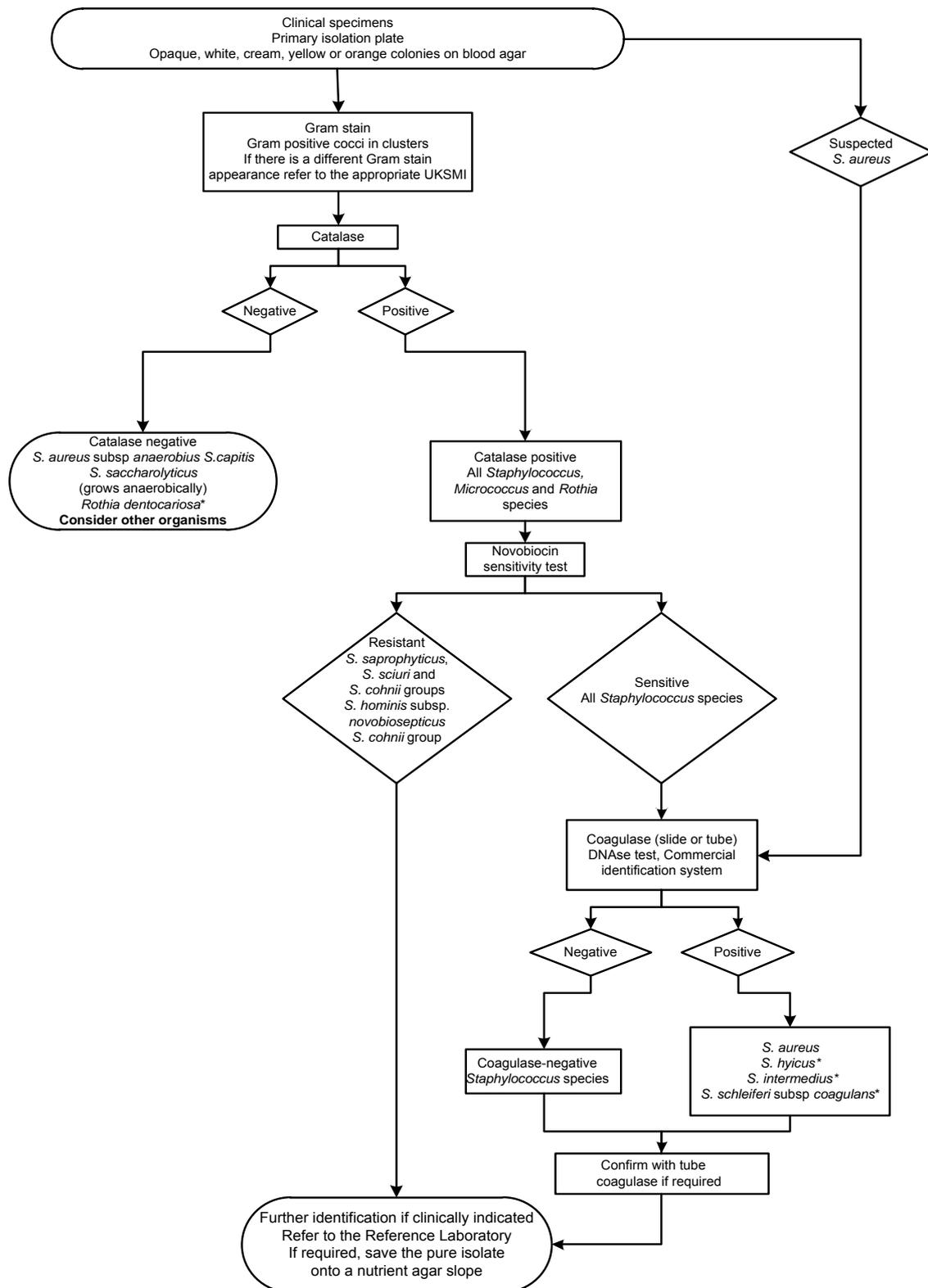
This has been useful in the detection of meticillin resistant *S. aureus* in an outbreak⁸⁵. It has also been used to highlight extensive differences in genome content between the closely related *Staphylococcus intermedius* group (*S. intermedius*, *S. pseudintermedius* and *S. delphini*) inhabiting distinct host niches as well as providing new avenues for research into pathogenesis and bacterial host-adaptation¹³.

3.6 Storage and Referral

If required, save pure isolate on a nutrient agar slope for referral to the Reference Laboratory.

Any strain of *S. aureus* suspected of demonstrating unusual resistance eg vancomycin, linezolid must be referred to the Staphylococcal Reference Service for further examination.

4 Identification of *Staphylococcus* Species, *Micrococcus* species and *Rothia* species



* Strains may be positive to certain tests

5 Reporting

5.1 Presumptive Identification

If appropriate growth characteristics, colonial appearance, Gram stain of the culture, catalase and slide coagulase or latex agglutination results are demonstrated.

5.2 Confirmation of Identification

Following confirmatory test results.

5.3 Medical Microbiologist

Inform the medical microbiologist of presumptive and confirmed *Staphylococcus aureus* when the request bears relevant information, eg:

- Toxin-mediated phenomena (eg Toxic Shock Syndrome, scalded skin syndrome, epidermal necrolysis, bullous impetigo, necrotising pneumonia, food poisoning)
- Suspected outbreaks or instances of cross-infection

The medical microbiologist should also be informed of presumptive and confirmed isolates of *Staphylococcus* species under the following circumstances:

- Osteomyelitis and septic arthritis
- Infections involving indwelling medical devices, eg prosthetic valves, pacemakers, CSF shunts, peritoneal or vascular catheters
- Endocarditis, haematogenous dissemination of infection, septicaemia
- Isolates from normally sterile sites
- Serious soft-tissue infections (cellulitis, erysipelas, necrotising myofasciitis, puerperal sepsis, surgical wound infection, pneumonia, peritonitis, meningitis, formation of abscesses or empyemas)

Follow local protocols for reporting to clinician.

5.4 CCDC

Refer to local Memorandum of Understanding.

5.5 Public Health England⁸⁶

Refer to current guidelines on CIDSC and COSURV reporting.

5.6 Infection Prevention and Control Team

Inform the infection prevention and control team of isolates of methicillin resistant *Staphylococcus aureus* and any *S. aureus* bacteremia (MSSA) in accordance with local protocols.

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:

Staphylococcus Reference Service
Antimicrobial Resistance and Healthcare Associated Infections Reference Unit
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^{86,87} or Equivalent in the Devolved Administrations⁸⁸⁻⁹¹

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 (HCAs) and Creutzfeldt–Jakob disease (CJD) under

Identification of *Staphylococcus* species, *Micrococcus* species and *Rothia* species

'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](#)^{88,89}, [Wales](#)⁹⁰ and [Northern Ireland](#)⁹¹.

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Identification of *Staphylococcus* species, *Micrococcus* species and *Rothia* species

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Identification of *Staphylococcus* species, *Micrococcus* species and *Rothia* species

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