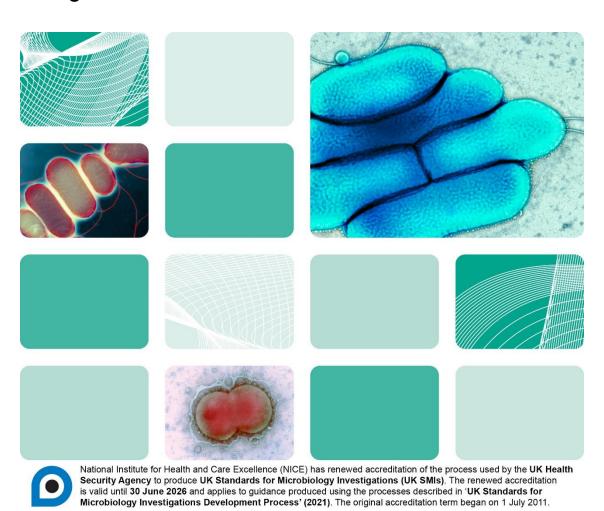


UK Standards for Microbiology Investigations

Identification of *Haemophilus* species and the HACEK group of organisms



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Acknowledgments

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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	8/17.09.24
Issue number discarded	4
Insert issue number	5
Anticipated next review date*	17.09.27
Section(s) involved	Amendment
	The document has been transferred into a new template and headings have been reorganised.
Whole document	The hyperlinks in the document have been updated to direct the reader to UK SMI webpages hosted on the Royal College of Pathologists website.
	The information and references in the document have been updated.
	The scope has been updated to reflect the focus of the document.
Scope of document	Additional links have been added to UK SMI TP 40 and other identification documents for further information.
Introduction	Descriptions of each species have been removed. Colonial morphology and microscopic appearance of relevant species have been summarised in table 1, section 8.2.
Technical information	Information has been moved to the relevant subheadings in section 8.
Safety considerations	References have been updated where available.
Identification	Subheadings have been restructured to reflect laboratory practices.

Identification of Haemophilus species and the HACEK group of organisms

	Table 1 summarises microscopic and colonial appearance of relevant species.
	Table of biochemical test results has been removed.
Reporting	Information and subheadings have been updated.
Referral to reference laboratory	Hyperlinks have been updated.
Algorithm	Overarching algorithm has been added to reflect current laboratory practices.
References	References updated where necessary.

^{*}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 Standards for Microbiology Investigations (UK SMI) document describes the identification of *Haemophilus* species and other members of the HACEK group of organisms (*Aggregatibacter, Cardiobacterium, Eikenella* and *Kingella* species). It includes culture, Gram stain and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). This document mentions molecular methods for alternative identification and confirmation. Some biochemical tests may not be performed routinely in the laboratory except in cases where confirmation by an alternative technique is required or automated methods are not available.

The test procedure for MALDI-TOF MS is covered in <u>UK SMI TP 40: Matrix-assisted</u> <u>laser desorption/ionisation - time of flight mass spectrometry (MALDI-TOF MS) test procedure.</u>

This document mentions the differentiation of *Kingella* species from pathogenic *Neisseria* and *Moraxella* species. The identification of these genera are covered in <u>UK SMI ID 6: Identification of Neisseria species</u> and <u>UK SMI ID 11: Identification of Moraxella species and morphologically similar organisms</u>.

The direct identification of microorganisms from samples is beyond the scope of this document. For information related to direct identification, please refer to the other <u>UK</u> SMI categories.

Antimicrobial Susceptibility Testing (AST) is also beyond the scope of this document. However, for effective antibiotic stewardship, laboratories should perform AST on all clinically significant isolates, particularly in cases of poor treatment response. For further information related to AST, please refer to the other UK SMI categories.

This document addresses laboratory processes for microorganism identification and is not intended for primary healthcare guidance. For relevant information please refer to the <u>UK SMI Syndromic documents</u>.

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

4 Introduction

4.1 Taxonomy and characteristics

Haemophilus species

The genus *Haemophilus* is part of the family Pasteurellaceae in the order Pasteurellales (1). There are currently 9 species of the genus *Haemophilus* associated with human infection (1,2). *Haemophilus aphrophilus* and *Haemophilus paraphrophilus* have been reclassified as a single species based on multilocus sequence analysis, *Aggregatibacter aphrophilus*, which includes V-factor dependent and V-factor independent isolates. *Haemophilus segnis* has been reclassified as *Aggregatibacter segnis* (3,4). *Haemophilus influenzae* is the type species.

There are six antigenically distinct capsular types of *H. influenzae*, designated 'a' to 'f' based on the polysaccharide composition of the capsular structure. Isolates that do not express a polysaccharide capsule are referred to as non-capsulated or non-typeable (5). Before the introduction of a vaccine against serotype b (Hib), the majority of infections were caused by serotype b strains, but incidence has significantly decreased following vaccination programme implementation (6). However, all types of *H. influenzae* (including non-typeable strains) can cause infections such as meningitis, bacteraemia, sepsis, otitis media and rhinosinusitis (7,8).

Other Haemophilus species associated with human infection are Haemophilus aegyptius, Haemophilus haemolyticus, Haemophilus parainfluenzae, Haemophilus pittmaniae, Haemophilus parahaemolyticus, Haemophilus paraphrohaemolyticus, Haemophilus sputorum and Haemophilus ducreyi (2).

Haemophilus species are fastidious, Gram negative coccobacilli or rods with marked pleomorphism. They are facultatively anaerobic, non-acid-fast, non-spore forming and non-motile (9). All species require either or both of two growth factors for growth: haemin (factor X) and/or nicotinamide adenine dinucleotide (factor V), which can be used to aid identification of species (2).

Other HACEK group of organisms

A systematic approach is used to differentiate the HACEK group of clinically encountered, morphologically similar, aerobic, and facultatively anaerobic Gram negative rods mainly associated with endocarditis and infections from normally sterile sites. These organisms are oropharyngeal/respiratory tract commensals (10,11).

Aggregatibacter species

Aggregatibacter species are members of the family Pasteurellaceae. The genus Aggregatibacter contains 4 species, Aggregatibacter actinomycetemcomitans, Aggregatibacter aphrophilus, Aggregatibacter segnis and Aggregatibacter kilianii. The type species is Aggregatibacter actinomycetemcomitans (1).

A. actinomycetemcomitans has been found in periodontitis, endocarditis, brain abscess and urinary tract infections (3,12).

Aggregatibacter species are Gram negative, non-motile, facultatively anaerobic, pleomorphic rods or coccobacilli (13). There is no dependence on X factor and the requirement for V factor is variable.

The species of the genus are intimately associated with humans. They are often recovered as part of the human oral flora, but are occasionally recovered from other body sites, including blood and brain, or as causes of infective endocarditis and abscesses (10).

Cardiobacterium species

The genus *Cardiobacterium* are members of the Cardiobacteriaceae family. The genus *Cardiobacterium* contains 2 species, *Cardiobacterium hominis* and *Cardiobacterium valvarum. C. hominis* is the type species (1,14). They are Gram negative, facultatively anaerobic, pleomorphic or straight rods and are arranged singly, in pairs, in short chains and in rosette clusters (15).

Eikenella species

The genus *Eikenella* is part of the Neisseriaceae family. Currently there are 5 species within the genus *Eikenella*. The type species is *Eikenella corrodens*, which is a coloniser of the oral mucous membranes, the upper respiratory tract and possibly the gastrointestinal tract. Other species include *Eikenella exigua*, *Eikenella glucosivorans*, *Eikenella halliae*, *Eikenella loninqua* (1). *Eikenella* species are Gram negative, facultatively anaerobic (except for *E. loninqua*) small rods with occasional filaments. They are non-motile; however, some species exhibit a "twitching" motility (16,17).

Kingella species

The genus *Kingella* is in the Neisseriaceae family and comprises of five species, *Kingella kingae, Kingella denitrificans, Kingella potus* and *Kingella oralis, Kingella negevensis,* with *K. kingae* being the type species (1). *Kingella indologenes* has been transferred to a new genus and classified as *Suttonella indologenes* (18). They are Gram negative, non-motile straight rods. They occur in pairs and sometimes short chains (19).

Kingella species may grow on Neisseria selective agar and therefore may be misidentified as pathogenic *Neisseria* species. The strain can be differentiated from *Moraxella* and *Neisseria* species by a catalase test. Most *Kingella* species are catalase negative; *Moraxella* and most *Neisseria* species (except *Neisseria elongata*) are catalase positive.

5 Technical information and limitations

With improvements to molecular taxonomy, some species previously included in the *Haemophilus* genus have been reclassified into the *Aggregatibacter* genus (3). Whilst no longer in the same genus, identification of these species can be difficult due to similarities in characteristics.

Clinicians are encouraged to stay informed about any further taxonomy changes and consider these when interpreting laboratory results. Changes in taxonomy should be considered when using commercial identification systems. Laboratories should ensure all databases, including MALDI-TOF MS, are regularly updated to reduce potential misidentification.

6 Safety considerations

The section covers specific safety considerations (20-40) related to this UK SMI, and should be read in conjunction with the general <u>safety considerations</u>.

All HACEK species are Hazard Group 2 organisms and processing of diagnostic samples should be carried out at Containment Level 2.

H. influenzae is a Hazard Group 2 organism, 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.

H. influenzae can cause serious invasive disease, especially in young children. Laboratory-acquired infections have been reported. The organism infects primarily by the respiratory route, autoinoculation or ingestion in laboratory workers (41).

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

For safety considerations for individual tests, please see <u>UK SMI Test Procedures</u> <u>documents</u>

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

Compliance with postal and transport regulations is essential.

7 Target organisms

Please refer to Table 1 for all HACEK species associated with human disease.

8 Identification

Identification of *Haemophilus* and other HACEK species requires a combination of methods. Colonies on blood or chocolate agar may be presumptively identified by colonial morphology, microscopy, requirement for X and V factors and MALDI-TOF MS. Biochemical tests can be used in laboratories when MALDI-TOF MS is unavailable. If confirmation or further identification is required, samples are transported to reference or specialised testing laboratories.

8.1 Culture methods

Culture can be used to provide presumptive identification of HACEK organisms. Initial assessments of colonial morphology can dictate future testing when investigating potential HACEK isolates. Following presumptive identification, further techniques, including MALDI-TOF MS or biochemical tests can be used to further identify the species.

8.1.1 Bacterial growth medium

Haemophilus species require enriched media to support growth. They require X and/or V factor. This can be added to medium unless chocolate blood agar is used (42). For the growth of *H. ducreyi* and *H. aegyptius*, growth medium should be further supplemented with growth factors, which are commercially available as a supplement (42).

All HACEK species are facultative anaerobes and grow best with 5-10% CO₂ present. HACEK species are slow growing and therefore most colonies can take between 24 and 48 hours, however *E. exhigua* and *C. valarum* can take up to 72 hours to become visible (17,43).

Primary isolation media

For *Haemophilus* species, incubation for 24-48 hours with enriched 5% chocolatised sheep blood agar at 35-7°C with 5-10% CO₂ is preferred (44). Blood agar can be used instead of chocolate agar, providing free V and X factor are supplemented.

Other HACEK species can be incubated for 24-48 hours on either chocolate blood agar or blood agar at 35-37°C with 5-10% CO₂ present (45).

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Selective media

Haemophilus selective agar is commercially available and contains horse blood and antibiotics (kanamycin and vancomycin). If not already present, bacitracin can be added to media, or bacitracin discs applied to the surface of inoculated agar to inhibit *Neisseria* species. Cultures should be incubated at 35-37°C with 5-10% CO₂ for 24-48 hours (42).

Selective media for *A. actinomycetemcomitans* is commercially available. Samples should be incubated at 35-37°C for 18-24 hours under anaerobic conditions (45).

8.1.2 Colonial appearance

Colonial appearance varies significantly with species, however generally:

- *Haemophilus* species produce colonies that are flat, convex and grey-white on blood agar (9).
- Aggregatibacter species produce colonies that are greyish-white/yellow, granular and rough (13)
- Cardiobacterium species produce smooth, convex and opaque colonies (15)
- Eikenella species produce colonies that may corrode the agar (16)
- Kingella species produce either spreading/corroding colonies or smooth, convex colonies (19)

For detailed descriptions of each species refer to section 8.2, Table 1. Refer to section 8.4.1, Table 2 for a summary of haemolysis of *Haemophilus* species on blood agar.

8.2 Microscopic appearance

8.2.1 Gram stain

Please refer to UK SMI TP 39 - Staining procedures.

All HACEK species are Gram negative; however, some species may stain weakly.

- Haemophilus species are small-medium sized pleomorphic rods; however, spheres and coccobacilli can be seen (9)
- Aggregatibacter species tend to be rod-shaped, but coccobacilli can also be observed (13)
- Cardiobacterium species are straight rods with rounded ends and occasional long filaments (15)
- Eikenella species are usually straight, unbranched rods with rounded edges (16)

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• *Kingella* species are straight rods with rounded/square ends. *K. kingae* does not Gram stain well (19)

For information on the microscopic appearance of individual species refer to table 1 below.

Table 1: Microscopic and Colonial appearance of HACEK species (9,13,17,42,43,46-52)

Please note that the information in this table provides general characteristics of colony appearance and can vary among different strains and culture conditions.

Species	Appearance	Additional Comments
H. influenzae	Small, regular rods that can be mixed with coccobacilli.	Indole producing strains have an amine-like odour.
	Colonies are smooth, low, convex, greyish, translucent. Encapsulated strains can appear mucoid. Nonencapsulated stains produce small, buff colonies.	
H. aegyptius	Slow-growing colonies. Colonies produced are smooth, low, convex and translucent.	Grow to 0.5mm diameter in 48 hours
H. ducreyi	Slender rods. Colonies are small, flat, smooth and grey. Larger colonies are sometimes seen mixed with smaller colonies.	Grows poorly. Can take 3-5 days to become visible. Can be surrounded by small zone of β-haemolysis.
H. pittmaniae	Small pleomorphic rods with occasional filamentous forms. Colonies are convex and grey-white.	None
H. parainfluenzae	Small pleomorphic rods interspersed with filamentous forms. Colonies are off-white to yellow coloured. Colony appearance can vary. They can be flat and smooth or granular or wrinkled.	Some strains show β haemolysis. Colony appearance may change with age.
H. haemolyticus	Small, regular rods or spheres with occasional filamentous forms. Colonies are translucent, smooth, and convex.	Produce a clear zone of β- haemolysis.
H. parahaemolyticus	Small, regular rods with occasional filamentous forms. Smooth colonies similar to <i>H. parainfluenzae</i> .	Produce zone β-haemolysis.
H. paraphrohaemolyticus	Small rods. Colonies similar to <i>H.</i> haemolyticus.	None
H. sputorum	Small regular rods, occasionally spheres.Colonies are convex, whiteish and opaque	Produce β-haemolysis on horse/sheep agar but some strains are non-haemolytic.
A. actinomycetemcomitans	Small pleomorphic rods. Rough, tenacious colonies with an internal, opaque pattern.	Colonies can be sticky if slime is produced.

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Species	Appearance	Additional Comments
A. aphrophilus	Short regular rods with occasional filamentous forms. Colonies are convex, opaque, granular, and yellowish.	None
A. segnis	Small pleomorphic rods, sometimes with irregular filamentous forms. Colonies are slow growing. They are smooth, granular, convex, greyishwhite, and opaque.	None
A. kilianii	Short regular rods with occasional filamentous forms. Supplemented with CO2 colonies are granular, yellowish, and opaque. Without CO2 colonies are small with larger colonies interspersed.	None
C. hominis	Small colonies produced unless in a humid atmosphere. Colonies are circular, smooth, moist and opaque.	Colonies can cause some α- haemolysis.
E. corrodens	Colonies are small with a moist clear centre surrounded by flat spreading growth. Pitting of the medium can occur.	Non-haemolytic. Older cultures can turn yellow.
K. denitrificans	Colonies are small and translucent. They may show pitting of the medium.	None
K. kingae	Colonies produce small depressions. They have a central pailla and spreading growth with granular zones surrounding. Colonies can also be small, delicate, translucent/opaque.	Colonies can cause β- haemolysis.
K. oralis	Colonies are round with irregular borders. They are flat to umbonate with a granular periphery.	None
K. potus	Colonies are circular, convex, and smooth. They are often yellow pigmented.	Non-haemolytic.
K. negevensis	Colonies are round and smooth. They can be pale yellow in colour.	Colonies are β-haemolytic.

8.3 Matrix-Assisted Laser Desorption/Ionisation - Time of Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS is used as the primary method for the identification of HACEK species in diagnostic laboratories. Therefore, it is important that this method is appropriately validated, manufacturer instructions carefully followed, available database updates are installed and reviewed, and the use of an extraction step that can contribute to a more reliable species identification is considered.

MALDI-TOF MS is used for the identification of several *Haemophilus* species, including *H.influenzae*, *H. parainfluenzae*, and *H. parahaemolyticus* (53,54). Databases that are used only for research may include other *Haemophilus* species including *H. haemolyticus*, however it should be noted that MALDI-TOF MS can incorrectly identify *H. haemolyticus* isolates as *H. influenzae* or *H. parainfluenzae* (55). In the case of suspected misidentification, results should be interpreted carefully, and further biochemical tests or molecular methods are recommended.

This technique accurately identifies members of the other HACEK genera, despite their fastidious nature (54,56). MALDI-TOF MS is effective for identification of *Aggregatibacter* species, *C. hominis, E. corrodens* and *K. kingae* (57). Some species, including *A. killianii, K. negevensis* and any new species resulting from taxonomy changes may not be included in the analyser databases. Laboratories are encouraged to check the MALDI-TOF MS databases used if these organisms are suspected. Biochemical testing is recommended for species not represented in the MALDI-TOF MS databases.

8.4 Further identification

8.4.1 Biochemical tests and commercial identification systems

Biochemical tests are no longer routine in laboratories but are used in cases when MALDI-TOF MS is unavailable or when MALDI-TOF MS results are inconclusive. Discrepancies in test results should be referred to the appropriate reference or specialist laboratories for further testing. Refer to manufacturer's guidance or other relevant sources for biochemical properties of individual HACEK species (42,45). Algorithms 2 and 3 contain examples of biochemical tests that may be used to differentiate between HACEK organisms.

Several commercial identification systems that use biochemical or enzymatic substrates are available for identification of *Haemophilus* species. The manufacturer's instructions should be followed precisely when using these kits. In many cases, the commercial identification system may not reflect recent changes in taxonomy.

X and V factor Test

Please refer to <u>UK SMI TP 38 – X and V Factor Test</u>

Haemophilus species have a requirement for V factor, which can be helpful in species identification. X and V factor test can provide initial information on the species. Porphyrin tests can identify X factor dependent species. Negative porphyrin tests suggest X factor dependence. Please refer to UK SMI TP 29 – Porphyrin synthesis (ALA) test.

For X & V factor requirements of the relevant *Haemophilus* species see table 2 below.

The X and V factor testing should be performed using a minimal nutrient agar, such as basic nutrient agar, but for which the X and V discs have been validated as any trace X or V factors could influence the results. Manufacturers' instructions should be followed when performing this test.

Table 2: Summary of Haemolysis and X/V factor requirements (42)

Organism	X factor	V factor	Haemolysis
H. influenzae ^a	+	+	-
H. parainfluenzae	-	+	-
H. haemolyticus ^b	+	+	+
H. parahaemolyticus	-	+	+
H. paraphrohaemolyticus	-	+	+
H. aegyptius	+	+	-
H. pittmaniae	-	+	+
H. sputorum	-	+	+
H. ducreyi	+	-	-

^a H. aegyptius is indistinguishable from H. influenzae biotype III in normal laboratory tests.

8.4.2 Serotyping *H. influenzae* with commercial type-specific antisera and PCR

If *H. influenzae* is detected, serotyping should be performed using slide agglutination or PCR testing. The presence of capsule polysaccharide can be detected by slide agglutination using commercial antisera. If positive, the individual serotype (a to f) can also be determined using antisera. Slide agglutination can sometimes generate ambiguous results and so the capsule type can be confirmed using multiple PCRs targeting regions within the capsule gene operon (58,59).

Some multi-species meningitis latex agglutination detection kits include antiserum against *H. influenzae* serotype b alone because of its historical dominance in causing meningitis and its relevance in detecting vaccine failures. However, it should be noted that not all latex agglutination detection kits are suitable for use on bacterial suspensions of *H. influenzae* (according to the manufacturer's instructions).

^b Traditionally described as β-haemolytic on horse blood agar, but non-haemolytic strains exist (2)

^{+;} factor is required for growth

^{-;} factor is not required for growth

8.4.3 Molecular Methods

Molecular techniques have made identification of many species more rapid and precise than is possible with phenotypic techniques. However, some of these methods are difficult to implement for routine bacterial identification in a clinical laboratory and may be better sourced from a reference laboratory.

Other tests such as nucleic acid amplification tests have been developed to identify *H.* influenzae and *H. parainfluenzae* in clinical specimens and some have been incorporated into commercial multi-pathogen detection systems (60). A commercial multiplex PCR assay has been developed that permits the simultaneous amplification of DNA targets from *H. ducreyi*, *Treponema pallidum*, and Herpes Simplex Virus types 1 and 2 directly from genital ulcer specimens (61).

A genotypic identification method, 16S rRNA gene sequencing has been used for better discrimination of closely related species such as *C. hominis* and *C. valvarum* (43,62). It has equally been used for identifying *Haemophilus* and *Aggregatibacter* species (2).

Next Generation Sequencing

Metagenomic NGS has been used to confirm identification of *A. segnis* and *H. influenzae* (63,64). Whilst currently limited to reference laboratories, NGS could become more accessible to diagnostic laboratories, however its performance compared to current gold standard methods still needs to be established.

9 Storage

For short term storage of *H. influenzae* and other HACEK organisms, isolates should be preserved on a chocolate agar slope at room temperature (45,65).

For long term storage of HACEK species, isolates should be frozen at -20°C to -80°C in a cryoprotective solution such as glycerol (45,66).

If required, save pure isolates on a chocolate agar slope for referral to the reference laboratory.

10 Reporting

10.1 Infection Specialist

Inform the medical microbiologist of all positive cultures from normally sterile sites.

Invasive *H. Influenzae* should be reported for surveillance purposes.

Certain clinical conditions must be notified to the laboratory associated infection specialist. Typically, these will include:

- Facial cellulitis
- Septic arthritis
- Osteomyelitis
- Epiglottitis, pneumonia, mastoiditis or empyema thoracis

Follow local protocols for reporting to clinician.

10.2 Presumptive identification

If appropriate growth characteristics, colonial appearance and Gram stain of the culture are demonstrated.

10.3 Confirmation of identification

Following identification, serotyping of *H. influenzae* can be obtained from the reference or specialist laboratory.

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

10.4 Health Protection Team (HPT)

Refer to local agreements in devolved administrations.

10.5 UK Health Security Agency

Refer to current guidelines on Second Generation Surveillance System (SGSS) reporting (67).

10.6 Infection prevention and control team

N/A

11 Referral to reference or specialist laboratories

If isolates are being transported to laboratories for further testing, ensure specimen is placed in a sealed container within appropriate packaging, following all relevant transport regulations. If required, save pure isolate on a chocolate agar slope for referral to the reference laboratory.

Isolates of invasive *H. influenzae* from normally sterile sites should be sent to the Vaccine Preventable Bacteria Section, Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU), UK Health Security Agency (UKHSA) for confirmation and typing.

For information on the tests offered, turnaround times, transport procedure and the other requirements of the reference or specialist laboratory <u>see user manuals and request forms</u>

Organisms with unusual/unexpected resistance, associated with a laboratory/clinical problem or an anomaly that requires investigation should be sent to the appropriate reference laboratory.

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

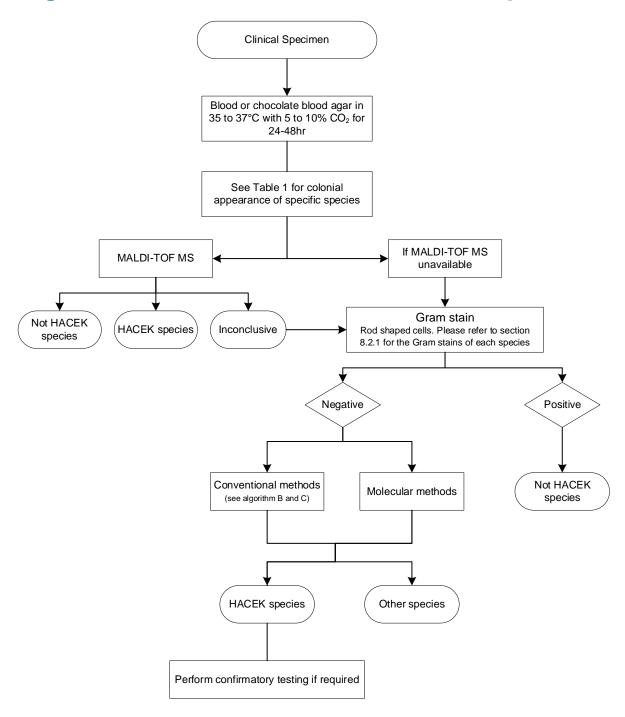
England

Wales

Scotland

Northern Ireland

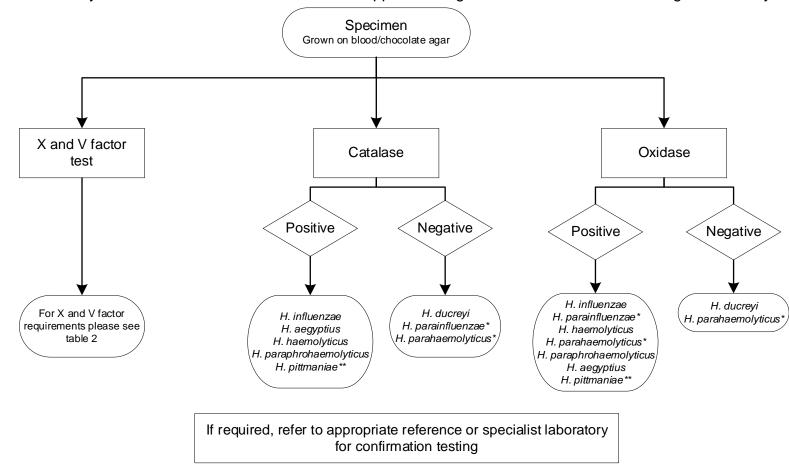
Algorithm 1: Identification of HACEK Species



This flowchart is for guidance only.

Algorithm 2: Identification of Haemophilus species

This flowchart provides a summary some common biochemical tests to supplement algorithm 1. This flowchart is for guidance only.



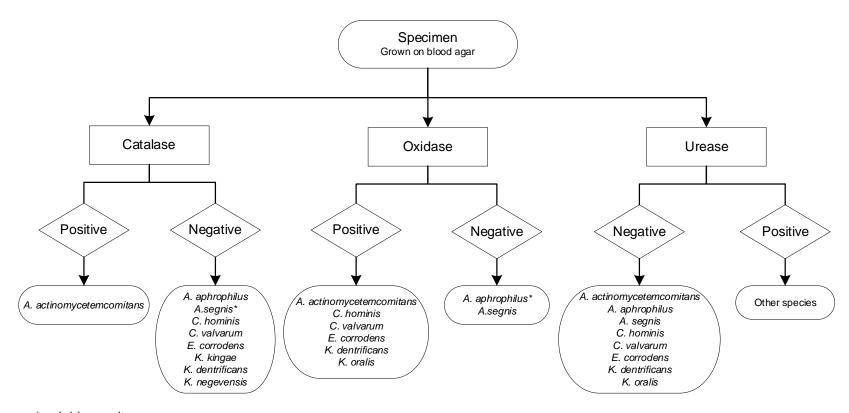
** weakly positive

* 11- 89% strains positive

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Algorithm 3: Identification of other HACEK organisms

This flowchart provides a summary of some common biochemical tests to supplement algorithm 1. This flowchart is for guidance only.



*variable results

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UK Standards for Microbiology Investigations | Issued by the Standards Unit, UK Health Security Agency

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An explanation of the reference assessment used is available in the <u>scientific</u> information section.

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