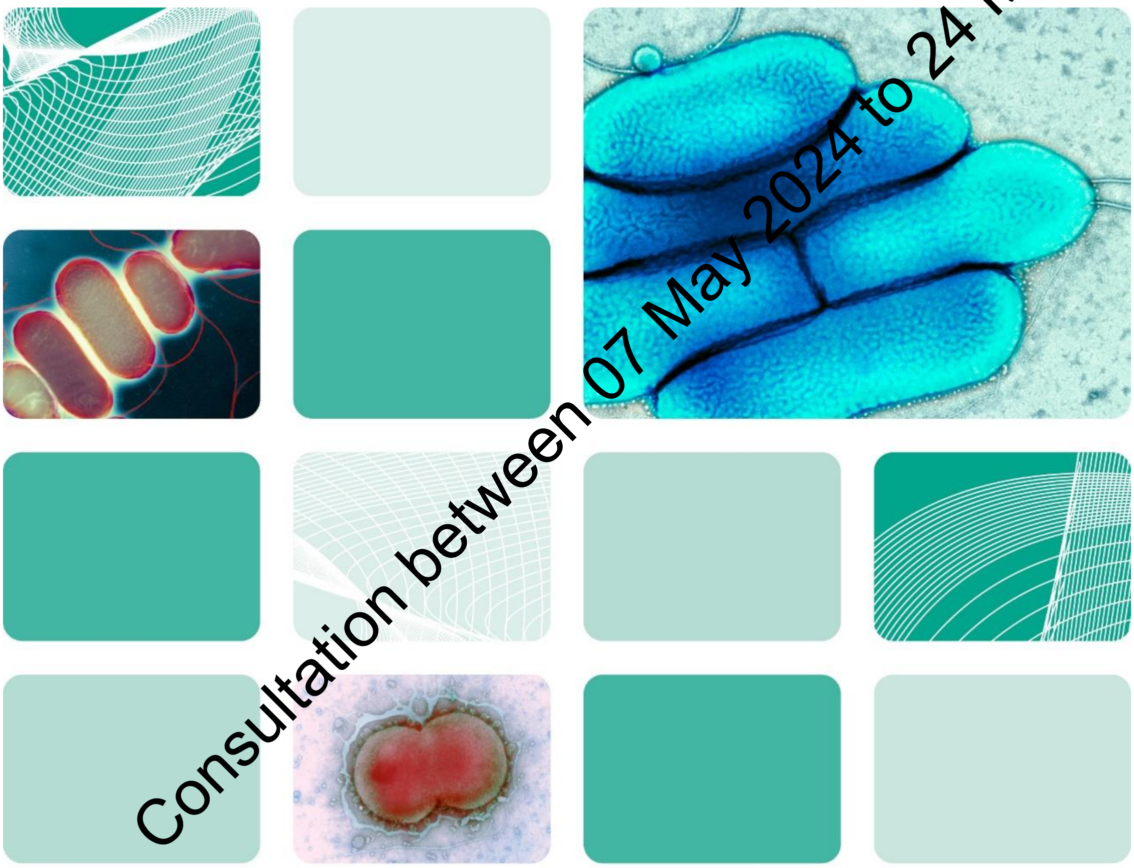
UK Standards for Microbiology Investigations

Identification of Haemophilus species and the HACEK group of organisms



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UK SMIs are produced in association with:

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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	x/dd.mm.yy
Issue number discarded	
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Anticipated next review date*	dd.mm.yy
Section(s) involved	**Amendment**

*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) for the identification of microorganisms from culture. Some biochemical tests may not be done routinely in laboratory except in cases where confirmation by an alternative technique is required or automated methods are not available.

The test procedure for matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is covered in UK SMI TP 40: Matrix-assisted laser desorption/ionisation - time of flight mass spectrometry (MALDI-TOF MS) test procedure. It also includes molecular methods for alternative identification and confirmation

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

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 UK 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 UK SMI Syndromic documents.

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 Pasteurellacae in the order Pasterurellales (1). There are currently 8 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* and *Haemophilus ducreyi* (9).

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 (2,9). 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 in identification of species (9,10).

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 (11,12).

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 endocarditis, brain abscess and urinary tract infections (3).

Aggregatibacter species are Gram-negative, non-motile, facultatively anaerobic, pleomorphic rods or coccobacilli. 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 part of the human oral flora and are occasionally recovered from other body sites, including blood and brain, and as causes of infective endocarditis and abscesses.

Cardiobacterium species

The genus Cardiobacterium are members of the Cardiobacteriacaea family. The genus Cardiobacterium contains 2 species, *Cardiobacterium hominis* and *Cardiobacterium valvarum. C. hominis* is the type species (1,13). They are Gram negative, facultatively anaerobic, pleomorphic or straight rods and are arranged singly, in pairs, in short chains and in rosette clusters (14).

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 (15,16).

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* (17). They are Gram negative, non-motile straight rods with rounded or square ends. They occur in pairs and sometimes short chains (18).

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, 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 ensure they are aware of any further taxonomy changes and take this into account when interpreting laboratory results. All databases including MALDI-TOF MS, should be updated accordingly. Changes in taxonomy should be considered when using commercial identification systems.

6 Safety considerations

The section covers specific safety considerations (19-40) related to this UK SMI, and should be read in conjunction with the general safety considerations on the RCPath website.

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. Invasive disease is usually caused by encapsulated strains of the organism. Laboratory acquired infections have been reported (41). The organism infects primarily by the respiratory route (inhalation), autoinoculation or ingestion in laboratory workers (42).

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

For safety considerations for individual tests, please see UK SMI Test Procedures documents

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 either X and/or V factor. This can be added to medium unless chocolate blood agar is used (9). 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 (9).

All HACEK species are facultative anaerobes and grow best with 5-10% CO_2 present. The optimum growth temperature is between 35 and 37°C (9,43). HACEK species are slow growing and therefore most colonies can take between 24 and 48 hours, however *E. exigua* and *C. valarum* can take up to 72 hours to become visible (16,44).

Primary isolation media

For Haemophilus species, incubation for 24-48 hours with enriched 5% chocolatised sheep blood agar at 35 to 37°C with 5 to 10% CO_2 is preferred (45). 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% CO_2 present (43).

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 inhibit Neisseria species. Cultures should be incubated at 35-37°C with 5-10% CO_2 for 24-48 hours (9).

Selective media for *A. actinomycetemcomitans* is commercially available. Samples should be incubated at 35-37°C for 18-24 hours under anaerobic conditions (43).

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 (10)
- Aggregatibacter species produce colonies that are greyish-white/yellow, granular and rough (46)
- Cardiobacterium species produce smooth, convex and opaque colonies (14)
- Eikenella species produce colonies that may corrode the agar (15)
- Kingella species produce either spreading/corroding colonies or smooth, convex colonies (18)

For detailed descriptions of each species refer to section 8.2, Table 1.

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 (10)
- Aggregatibacter species tend to be rod-shaped, but coccobacilli can also be observed (46)
- Cardiobacterium species are straight rods with rounded ends and occasional long filaments (14)
- Eikenella species are usually straight, unbranched rods with rounded edges (15)
- Kingella species are straight rods with rounded/square ends. *K. kingae* does not Gram stain well (18)

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

Table 1: Microscopic and Colonial appearance of HACEK species (9,10,16,44,46-53)

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. Colonies are smooth, low, convex, greyish, translucent. Encapsulated strains can appear mucoid. Non-encapsulated stains produce small, buff colonies.	In 24 hours colonies grow to 1-2mm in diameter. Indole producing strains have an amine-like odour.
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.	Grow to 1-2mm diameter in 24 hours.
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.	Grow to 1-2mm diameter in 24 hours. 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.	Colonies grow to 0.5-1.5mm diameter after 24 hours. Produce a clear zone of β-haemolysis.
H. parahaemolyticus	Small, regular rods with occasional filamentous forms. Smooth colonies similar to *H. parainfluenzae*.	Produce zone β-haemolysis.
H. paraphrohaemolyticus	Small rods. Colonies similar to *H. haemolyticus.*	None
A. actinomycetemcomitans	Small pleomorphic rods. Rough, tenacious colonies with an internal, opaque pattern.	Colonies grow to diameter of 1-2mm after 48 hours. Colonies can be sticky if slime is produced.
A. aphrophilus	Short regular rods with occasional filamentous forms. Colonies are convex, opaque, granular, and yellowish.	None

Species	Appearance	Additional Comments
A. segnis	Small pleomorphic rods, sometimes with irregular filamentous forms. Colonies are slow growing. They are smooth, granular, convex, greyish-white, 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 installed and reviewed, and the use of an extraction step that can contribute to a more reliable species identification should be considered.

MALDI-TOF MS is used for the identification of several *Haemophilus* species, including *H.influenzae*, *H. parainfluenzae, H. parahaemolyticus* (54,55). 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* (56). 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 (55,57). MALDI-TOF MS is effective for identification of Aggregatibacter species, *C. hominis, E. corrodens* and *K. kingae* (58). 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 the manufacturer's guidance or the relevant chapters in the *Manual of Clinical Microbiology* book (9,43) for biochemical properties of individual HACEK species. Algorithms B and C 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 UK SMI TP 38 – X and V Factor Test

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

The use of chocolate agar is preferable for species that require X and V factor for growth rather than blood agar or blood containing medium because of risk of carryover of X factor. The X and V factor testing could also be done using a basic nutrient agar, but for which the X and V discs have been validated in case it had trace factors that could influence the results. Manufacturers' instructions should be followed when performing this test.

Please note that sometimes the X and V factor tests can give false V dependent results if incubated in CO_2 (59).

Table 2: Summary of X and V test results (9)

Organism	X factor	V factor	β-haemolysis
H. influenzae[a]	+	+	
H. parainfluenzae	-	+	-
H. haemolyticus[b]	+	+	+
H. parahaemolyticus	-	+	+
H. paraphrohaemolyticus	-	+	+
H. aegyptius	+	+	-
H. pittmaniae	-	+	+
H. ducreyi	+	-	-

[a] *H. aegyptius* is indistinguishable from *H. influenzae* biotype III in normal laboratory tests.

[b] Traditionally described as β-haemolytic on horse blood agar, but non-haemolytic strains exist (60)

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 directed at targets within the capsule gene operon (61,62).

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).

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 NAATs have been developed to identify *H.* influenzae and *H. parainfluenzae* in clinical specimens and some have been incorporated into commercial multi-pathogen detection systems (63). NAATs have been used to identify *H. ducreyi* in clinical specimens. A commercial multiplex PCR assay has been developed that permits the simultaneous amplification of DNA targets from *H. ducreyi*, *Trepanemal pallidum*, and Herpes Simplex Virus types 1 and 2 directly from genital ulcer specimens (64).

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* (44,65). It has equally been used for identifying Haemophilus and Aggregatibacter species (60).

Next Generation Sequencing

Whilst currently limited to reference laboratories, Next Generation Sequencing (NGS) could become more common in clinical laboratories. Metagenomic NGS has been used to confirm identification of *A. segnis* and *H. influenzae* (66,67). NGS provides a quick and accurate means of identifying pathogens that could be potentially beneficial for routine diagnostics in the future (68).

9 Storage

For short term storage of HACEK species, isolates should be kept viable on chocolate blood agar supplemented with 5-7% CO_2 at 35-37°C (9).

For long term storage of HACEK species, isolates should be frozen at -80°C in a cryoprotective solution such as glycerol or trypticase soy broth (43,69).

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 Routine identification

Initially appropriate growth characteristics, colonial appearance and Gram stain of the culture are indicative of a fastidious organism. Identification is made using MALDI-TOF MS or where not available using biochemical methods and appropriate X and V factors.

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 Specialist and reference microbiology: laboratory tests and services page on GOV.UK 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 (35).

10.6 Infection prevention and control team

N/A

11 Referral to reference or specialist laboratories

If isolates are being transported to further laboratories for testing, ensure specimen is placed in a sealed containing 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 *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 see user manuals and request forms

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:

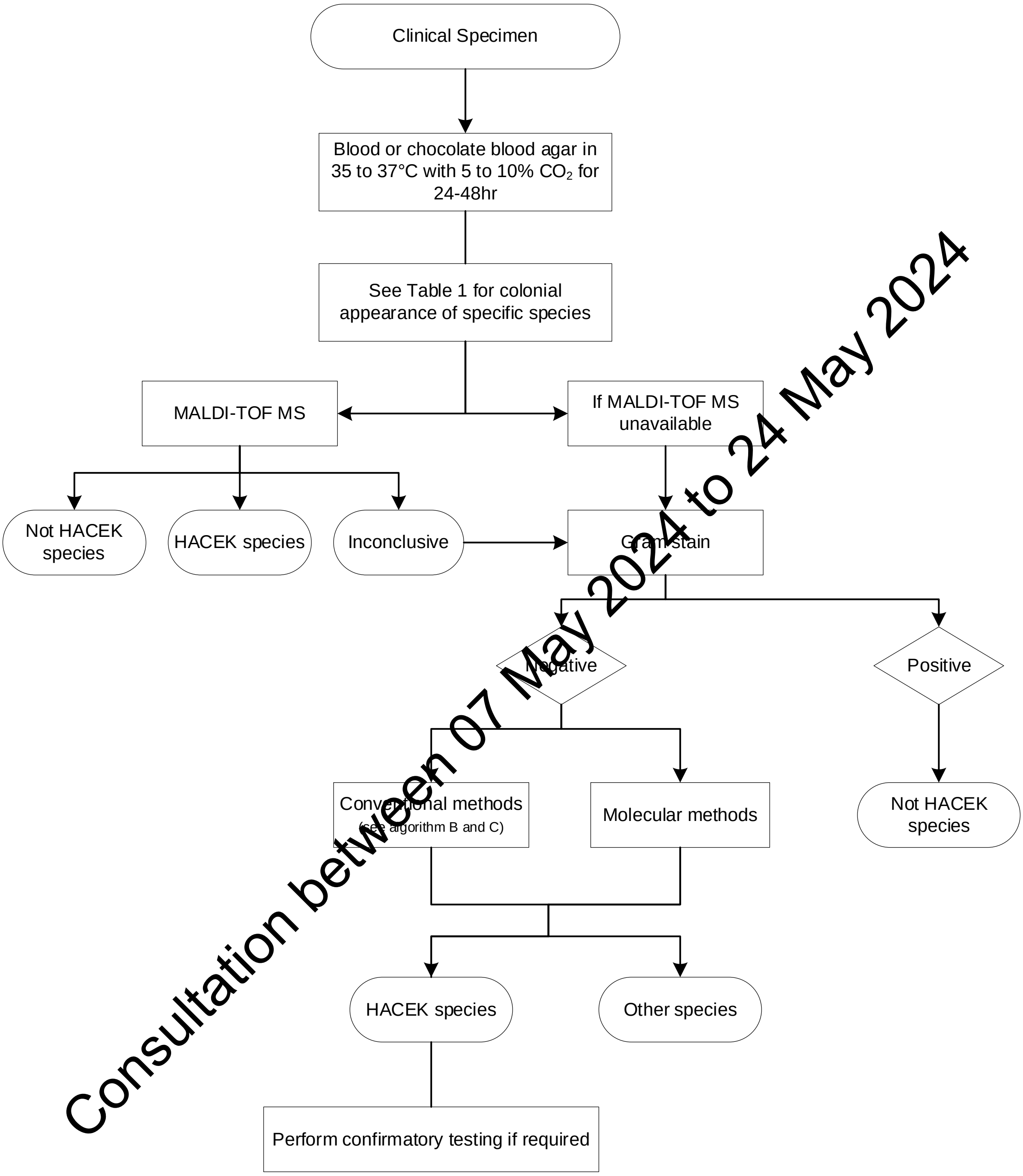
England

Wales

Scotland

Northern Ireland

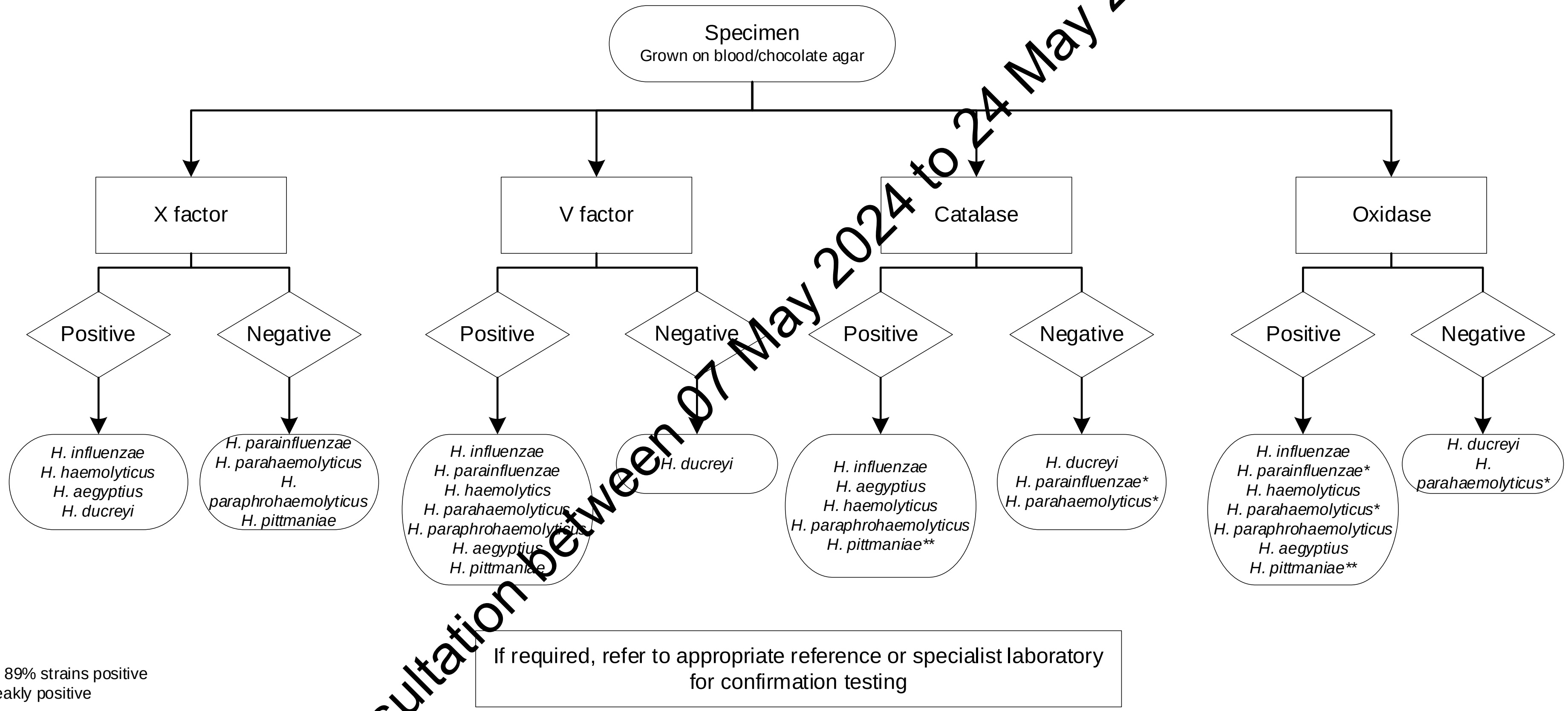
Algorithm A: Identification of HACEK Species



This flowchart is for guidance only.

Algorithm B: Identification of Haemophilus species

This flowchart provides a summary of biochemical tests to supplement algorithm A.

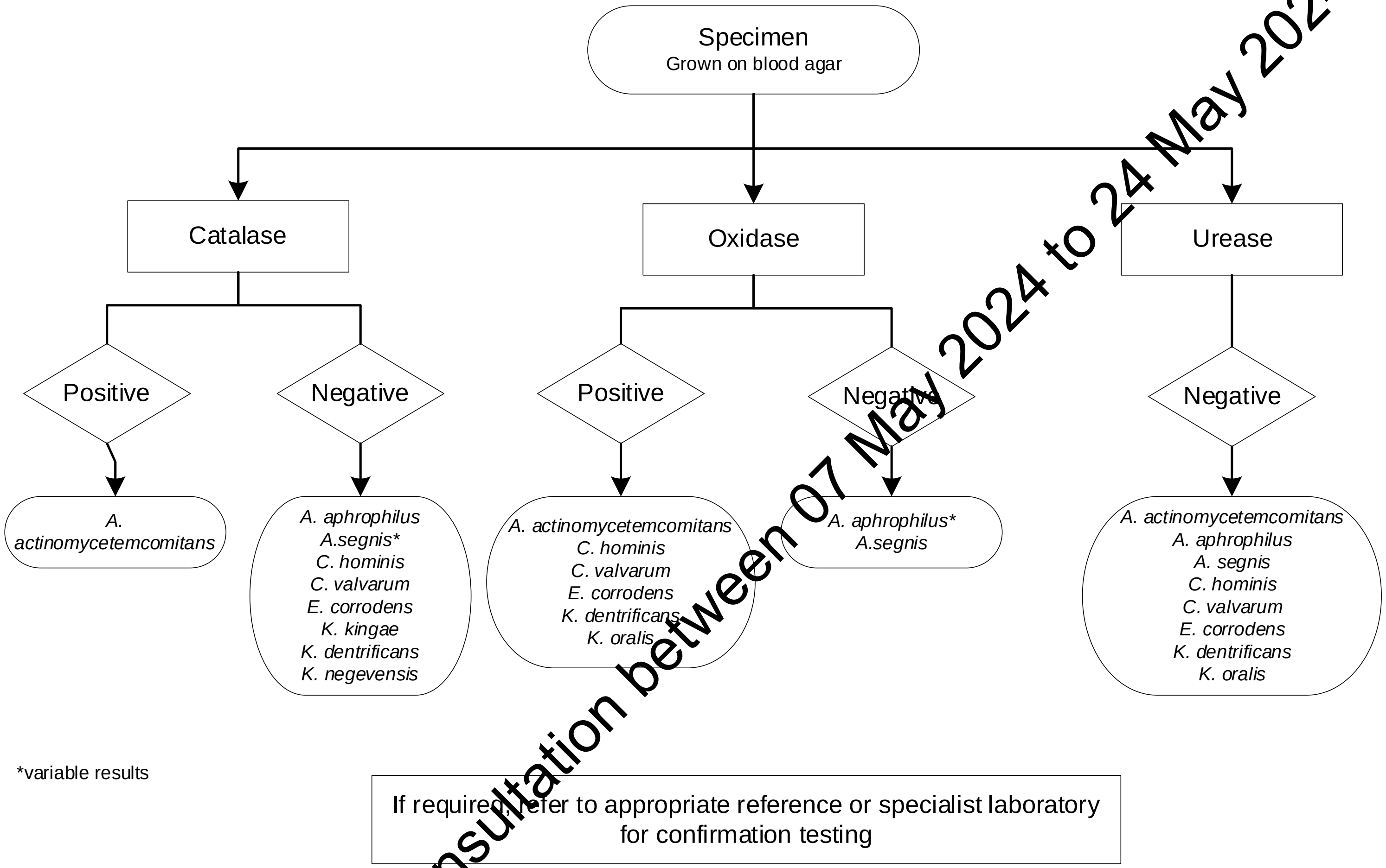


* 11- 89% strains positive
** weakly positive

This flowchart is for guidance only.

Algorithm C: Identification of other HACEK organisms

This flowchart provides a summary of biochemical tests to supplement algorithm A.



This flowchart is for guidance only.

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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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