IDENTIFICATION OF HAEMOPHILUS SPECIES AND THE HACEK GROUP OF ORGANISMS

BSOP ID 12

Issued by Standards Unit, Department for Evaluations, Standards and Training
Centre for Infections
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The reader is informed that all taxonomy in this document was correct at time of issue.

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AMENDMENT PROCEDURE

<table>
<thead>
<tr>
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<th>BSOP ID 12</th>
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<tbody>
<tr>
<td>Controlled document title</td>
<td>Identification of <em>Haemophilus</em> species and the HACEK group of organisms</td>
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</tbody>
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Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@hpa.org.uk.

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

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<td>Tables amended to include characteristics of all HACEK organisms</td>
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IDENTIFICATION OF
HAEMOPHILUS SPECIES AND
THE HACEK GROUP OF ORGANISMS

SCOPE OF DOCUMENT

This NSM describes the identification of Haemophilus species and other members of the HACEK group (Haemophilus species, Aggregatibacter actinomycetemcomitans (formerly Actinobacillus actinomycetemcomitans), Aggregatibacter aphrophilus (formerly Haemophilus aphrophilus and Haemophilus paraphrophilus), Cardiobacterium hominis, Eikenella corrodens and Kingella species.

INTRODUCTION

Taxonomy

There are thirteen species of Haemophilus. The Haemophilus species associated with humans are H. influenzae, H. aegyptius, H. haemolyticus, H. parainfluenzae, H. pittmaniae, H. parahaemolyticus, H. paraphrohaemolyticus and H. ducreyi. Nucleic acid hybridisation studies and 16S rRNA sequence homologies suggest H. ducreyi does not belong in the genus Haemophilus, though it does seem to be a valid member of the family Pasteurellaceae. Haemophilus aphrophilus and H. paraphrophilus have been re-classified as a single species on the basis of multilocus sequence analysis, Aggregatibacter aphrophilus, which includes V-factor dependent and V-factor independent isolates. H. segnis has been re-classified as Aggregatibacter segnis. There are eight biotypes of Haemophilus influenzae (I-VIII) and eight biovars of Haemophilus parainfluenzae (I-VIII). Pittman described six antigenically distinct capsular types of H. influenzae, designated a-f.

Characteristics

Haemophilus are Gram-negative spherical, oval or rod-shaped cells less than 1 µm in width, variable in length, with marked pleomorphism, and sometimes forming filaments. Small, round, convex, colonies, which may be iridescent, develop in 24 hours on chocolate blood agar. Iridescence is seen with capsulated strains.

All species require preformed growth factors present in blood, particularly X factor (protoporphyrin IX or protoheme) and/or V factor (nicotinamide adenine dinucleotide (NAD) or NAD phosphate (NADP)). On blood agar H. influenzae exhibits satellitism around colonies of Staphylococcus aureus (a source of V factor). Aggregatibacter aphrophilus and Haemophilus parahaemolyticus require CO₂ for primary isolation. Carbohydrates are catabolised with the production of acid. A few species produce gas. The optimum growth temperature is 35°C – 37°C. They are facultatively anaerobic and non-motile. Nitrates are reduced to nitrites.

Principles of identification

Colonies on blood or chocolate agar may be presumptively identified by colonial morphology, Gram stain, haemolysis and requirement for X and V factors and CO₂. The porphyrin synthesis test (see BSOP TP 29 - Porphyrin synthesis (ala) test) may be used to differentiate haem producing Haemophilus species. Identification is confirmed by commercial biochemical tests, serotyping with type-specific antisera and/or referral to a Reference Laboratory. Isolates of H. influenzae from normally sterile sites should be sent to the Haemophilus Reference Unit, Respiratory & Systemic Infection Laboratory, Health Protection Agency, Centre for Infections, Colindale, for confirmation and typing.
HACEK group of organisms

For the identification of *Haemophilus* species in the HACEK group see above.

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\(^6\). The identification is considered together with the clinical details and the isolates may be identified further if clinically indicated. Isolates of clinically significant HACEK organisms from cases of endocarditis and normally sterile sites should be referred to the Laboratory of Health Care Associated Infections, HPA Centre for Infections (Colindale) for confirmation of identification and to the Antibiotic Resistance Monitoring and Reference Laboratory, HPA Centre for Infections, Colindale for MIC testing.

*Aggregatibacter actinomycetemcomitans*\(^6,7\)

*Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) is a Gram-negative coccobacillus or short rod, 0.3 - 0.5 x 0.5 - 1.5 µm, which may exhibit irregular staining. *A. actinomycetemcomitans* is mostly bacillary, but cocc is interspersed. Occasional longer forms up to 6 µm may occur. Cells are arranged singly, in pairs, or (more rarely) in chains. Small amounts of extracellular slime may be produced.

*A. actinomycetemcomitans* does not require X or V factors. It grows best under microaerophilic conditions with added CO\(_2\) and is facultatively anaerobic. The optimal growth temperature is 37°C after 24 hours incubation; colonies on blood or chocolate agar may be less than 0.5 mm and enlarge to 1 mm after several days incubation. The colonies on blood or chocolate agar may be firm, adherent, star-shaped, sometimes with rough surfaces and pitting, and may be difficult to remove from the agar surface. If extracellular slime is produced, cultures may be sticky on primary isolation. Surface cultures have low viability and may die within 5 - 7 days. Cells are non-motile. It is catalase and oxidase positive and urease negative.

*Aggregatibacter aphrophilus*

The species *Haemophilus aphrophilus* and *Haemophilus paraphrophilus* have been reclassified as a single species *Aggregatibacter aphrophilus*\(^8\). These are Gram-negative, short regular bacilli, 0.5 x 1.5-1.7 µm with occasional filamentous forms. They require 5-10% CO\(_2\) for primary isolation. Growth may be enhanced by haemin, but X-factor is not an absolute requirement. Some isolates require V-factor (formerly *H. paraphrophilus*) whilst others are V-factor independent (formerly *H. aphrophilus*). The colonies on chocolate agar are opaque, granular and yellowish, catalase and urease negative, and oxidase variable.

*Aggregatibacter segnis*

Formerly called *Haemophilus segnis*. Cells are small and pleomorphic with a preponderance of filamentous forms. Growth on chocolate agar is slow and the colonies are smooth, greyish-white or opaque and 0.5 mm in diameter after 48 h incubation. The growth of some strains is enhanced by 5-10% CO\(_2\). *A. segnis* requires V-factor but not X-factor.

*Cardiobacterium hominis*\(^4\)

The genus *Cardiobacterium* contains two species, *Cardiobacterium hominis* and *Cardiobacterium valvarum*\(^9\). Cells are pleomorphic or straight rods, 0.5 – 0.75 µm in diameter and 1 – 3 µm in length with rounded ends, and long filaments may occur. Cells are arranged singly, in pairs, in short chains and in rosette clusters. They are Gram-negative, but parts of the cell may stain Gram-positive.

Growth on blood agar is poor. *C. hominis* does not require X or V factors, but may show an apparent requirement for X factor on first isolation. Very small colonies are produced unless incubated in a humid aerobic or anaerobic atmosphere with 5% CO\(_2\). After incubation for two days, colonies are 1 mm in diameter, smooth, opaque and butyrous and some strains may pit the agar. *C. hominis* is facultatively anaerobic, but CO\(_2\) may be required by some strains on primary isolation. The optimum growth temperature is 30°C - 37°C. It is non-motile, oxidase-positive and catalase and urease-negative.
Eikenella corrodens

The genus Eikenella contains only one species, Eikenella corrodens. Cells are straight, unbranched, non-sporing, slender Gram-negative rods, 0.3 - 0.4 x 1.5 - 4 µm in length.

Colonies may be very small on blood agar after overnight incubation or may not be visible for several days. The colonies have moist, clear centres surrounded by flat, and sometimes spreading, growth. Pitting of the medium may occur and yellow colouration may be seen in older cultures due to cell density. There may be colonial variation and spreading growth may vary between colonies of the same isolate. E. corrodens is non-haemolytic but a slight greening may occur around the colonies. Haemin is usually required for aerobic growth and rare strains remain X-dependent after further subculture. The optimum growth temperature is 35°C - 37°C. E. corrodens is non-motile, but ‘twitching’ motility may be produced on some media. Strains are facultatively anaerobic, oxidase-positive, catalase-negative, urease-negative and capnophilic. It may be confused with Bacteroides ureolyticus, which also exhibits pitting or corroding, but unlike E. corrodens is an obligate anaerobe and urease-positive.

Kingella species

The genus Kingella comprises three species, Kingella kingae, Kingella denitrificans and Kingella oralis. Kingella indologenes has been transferred to a new genus and classified as Suttonella indologenes.

Kingella species are straight rods, 1.0 µm in length with rounded or square ends. They occur in pairs and sometimes short chains. Endospores are not formed. Cells are Gram-negative, but tend to resist decolourization.

Two types of colonies occur on blood agar; a spreading, corroding type and a smooth, convex type. It does not require X or V factors. Growth is aerobic or facultatively anaerobic. The optimum growth temperature is 33°C - 37°C. Kingella kingae colonies are surrounded by a distinct zone of β-haemolysis on blood agar. Kingella species are non-motile, oxidase-positive, catalase-negative and urease-negative. Glucose and other carbohydrates are fermented with the production of acid but not gas.

Kingella species may grow on Neisseria selective agar and therefore may be misidentified as pathogenic Neisseria species. They 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.

TECHNICAL INFORMATION/ LIMITATIONS

N/A
1 SAFETY CONSIDERATIONS

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

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

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

Compliance with postal and transport regulations is essential.

2 TARGET ORGANISMS

HACEK group reported to have caused human infection

- *Haemophilus influenzae*
- *Aggregatibacter aphrophilus* (includes *H. aphrophilus* and *H. paraphrophilus*)
- *Haemophilus parainfluenzae*
- *Aggregatibacter segnis* (formerly *H. segnis*)
- *Haemophilus parahaemolyticus*

- *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*)
- *Cardiobacterium hominis* and *Cardiobacterium valvarum*
- *Eikenella corrodens*
- *Kingella kingae*

3 IDENTIFICATION

3.1 MICROSCOPIC APPEARANCE

Gram stain (BSOPTP 39 - Staining Procedures)

*Haemophilus* species are small coccobacilli or longer rod-shaped Gram-negative cells, variable in length with marked pleomorphism and sometimes forming filaments. Other HACEK organisms produce spherical, oval or rod-shaped Gram-negative cells which may be variable in length with marked pleomorphism or filament formation.

3.2 PRIMARY ISOLATION MEDIA

- Chocolate agar incubated in 5 - 10% CO₂ at 35°C - 37°C for 16 - 48 h
- Blood agar incubated in 5 - 10% CO₂ at 35°C - 37°C for 16 - 48 h

3.3 COLONIAL APPEARANCE

*Haemophilus* species are small, round, convex colonies, which may be iridescent and develop after 24 hours incubation on chocolate agar. Satellitism of *H. influenzae* may be seen around colonies of *S. aureus* on blood agar.

Colonial morphology of other HACEK organisms varies with species and isolation medium (see Introduction and below).
### 3.3.1 Aerobic Growth Characteristics of HACEK Group Organisms

<table>
<thead>
<tr>
<th>HACEK group organisms</th>
<th>Characteristics of growth on blood agar after aerobic incubation at 35°C - 37°C for 16 - 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. actinomycetemcomitans</strong></td>
<td>Will not grow in air but grows in air + CO₂. Minute colonies at 24 h, 1 mm at 48 h. Firm, adherent, star-shaped colonies with rough surface and which may produce pitting of the agar. Some strains may be sticky. Non-haemolytic.</td>
</tr>
<tr>
<td><strong>A. aphrophilus</strong></td>
<td>Requires added CO₂ for primary isolation. Opaque, yellowish colonies 1.0-1.5 mm at 24 h. Haemin (X-factor) enhances growth but there is not an absolute requirement for X-factor. Some isolates require V factor (formerly <em>H. paraphrophilus</em>) whereas others are V-factor-independent (formerly <em>H. aphrophilus</em>). Non-haemolytic.</td>
</tr>
<tr>
<td><strong>C. hominis</strong></td>
<td>Some strains will not grow without added CO₂. May require X-factor on primary isolation. Colonies smooth, convex and opaque. 1 - 2 mm at 48 h. Slight α-haemolysis.</td>
</tr>
<tr>
<td><strong>C. valvarum</strong></td>
<td>Grows best in air +5% CO₂. Slow growing, colonies smooth, round, opaque and glistening, 0.6-0.8 mm after 48 h. Some strains show slight α-haemolysis, others are non-haemolytic.</td>
</tr>
<tr>
<td><strong>E. corroden</strong></td>
<td>Colonies very small, moist, clear centres surrounded by flat growth. Pitting may occur. Spreading is rare and usually confined to a very small area around the colony. Non-haemolytic. Colonies 0.5 - 1 mm after 48 h. Requires 5 - 10% CO₂.</td>
</tr>
<tr>
<td><strong>K. kingae</strong></td>
<td>Two types of colony: a spreading, corroding type and a smooth, convex type. Small zone of β-haemolysis. Cells are often capsulate, producing mucoid colonies. Does not require 5 - 10% CO₂.</td>
</tr>
<tr>
<td><strong>K. denitrificans</strong></td>
<td>Non-haemolytic. Two types of colony: a spreading, corroding type and a smooth, convex type.</td>
</tr>
</tbody>
</table>

**Note 1:** For descriptions of *Haemophilus* species see *Haemophilus* species (Introduction)  
**Note 2:** In some cases it may be possible to use commercial biochemical identification kits
3.4 TEST PROCEDURES

Biochemical tests

Summary of biochemical tests:

<table>
<thead>
<tr>
<th>Species</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. influenzae</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>H. aegyptius</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H. haemolyticus</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H. parainfluenzae</td>
<td>d</td>
<td>+</td>
<td>d</td>
</tr>
<tr>
<td>H. pittmaniae</td>
<td>d</td>
<td>d</td>
<td>-</td>
</tr>
<tr>
<td>H. parahaemolyticus</td>
<td>d</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H. paraphrohaemolyticus</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A. aphrophilus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. hominis</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>K. kingae</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E. corrodens</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Growth requirement for X and V factors – Distinguishing among *Haemophilus* species (see BSOPTP 38 - X and V factor test or porphyrin synthesis test BSOPTP 29 - Porphyrin synthesis (ala) test).

Serotyping *H. influenzae* with commercial type-specific antisera.

Commercial identification kit.

**Note:** In many cases the commercial identification kit may not reflect recent changes in taxonomy

**Summary of X and V test results**

<table>
<thead>
<tr>
<th>Species</th>
<th>X factor</th>
<th>V factor</th>
<th>X + V factor</th>
<th>Porphyrin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. influenzae</em></td>
<td>No growth</td>
<td>No growth</td>
<td>Growth</td>
<td>Negative</td>
</tr>
<tr>
<td><em>H. haemolyticus</em></td>
<td>No growth</td>
<td>No growth</td>
<td>Growth</td>
<td>Negative</td>
</tr>
<tr>
<td><em>H. parainfluenzae</em></td>
<td>No growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Positive</td>
</tr>
<tr>
<td><em>H. pittmaniae</em></td>
<td>No growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Positive</td>
</tr>
<tr>
<td><em>H. parahaemolyticus</em></td>
<td>No growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Positive</td>
</tr>
<tr>
<td><em>H. paraphrohaemolyticus</em></td>
<td>No growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Positive</td>
</tr>
</tbody>
</table>

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

β-haemolytic on horse blood agar

3.5 CONFIRMATION

Following serotyping of *H. influenzae*, appropriate X and V and/or commercial identification kit results and/or Reference Laboratory report.

3.6 STORAGE AND REFERRAL

If required, save pure isolate on a chocolate agar slope for referral to the Reference Laboratory.
4 IDENTIFICATION FLOWCHARTS

4.1 HAEMOPHILUS SPECIES

Clinical specimen
Primary isolation plate

Blood agar and Chocolate agar

Grows on chocolate agar and poorly on blood agar

Gram stain (on pure culture)
Gram-negative coccobacilli

Growth around X and V factors on nutrient agar or porphyrin synthesis test

If from normally sterile site
Refer isolate to HRU for typing and for surveillance
Save the pure culture to chocolate agar slope

Grows on both chocolate and blood agar

Gram stain (on pure culture)
Gram-negative coccobacilli or rods

Further ID if clinically indicated.
Commercial identification system or refer to Reference Laboratory

If antimicrobial susceptibility testing required (eg case of endocarditis or unusual resistance pattern)
Refer isolate to ARMRL for MIC testing
Save the pure culture to chocolate agar slope

Refer to LHCAI to confirm identification
Save the pure culture to chocolate agar slope

This NSM should be used in conjunction with the series of other NSMs from the Health Protection Agency

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Email: standards@hpa.org.uk
4.2 HACEK GROUP

Clinical specimen
Primary isolation plate

Blood agar

Incubate at 35-37°C
Aerobic
16-48 h

Grows in air, may require CO₂
Star shaped colonies with rough surface, may produce pitting of agar
Colonies 1 mm at 48 h

Catalase – positive
Oxidase – positive
Urease - negative

A. actinomycetemcomitans

Grows in air + CO₂
Yellowish colonies
1.5 mm at 24 h
Non-haemolytic

Catalase – negative
Oxidase – negative
Urease - negative

A. aphrophilus

Growth may require CO₂ addition
Smooth, convex and opaque colonies
Slight α-haemolysis
Colonies 1-2 mm at 48 h

Catalase – negative
Oxidase – negative
Urease - negative

C. hominis

Requires 5-10% CO₂
Small moist colonies with clear centres surrounded by flat growth
Non-haemolytic
0.5 - 1 mm after 48 h

Catalase – negative
Oxidase – positive
Urease - negative

E. corrodens

CO₂ not required
Either spreading corroding colony or smooth convex colony, often produces mucoid colonies with a small zone of β-haemolysis

Catalase – negative
Oxidase – positive
Urease - negative

K. kingae

Identification of Haemophilus Species and the HACEK Group of Organisms

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Reference no: BSOP ID 12/2
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REPORTING

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

5.2 CONFIRMATION OF IDENTIFICATION
N/A

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

According to local protocols, the medical microbiologist should also be informed of presumptive or confirmed Haemophilus species or other member of the HACEK group of organisms when the request card bears relevant information eg

- Meningitis or brain abscess
- Facial cellulitis
- Septic arthritis
- Osteomyelitis
- Epiglottitis, pneumonia, mastoiditis or empyema thoracis
- Septicaemia or endocarditis

Follow local protocols for reporting to clinician

5.4 CCDC
Refer to local Memorandum of Understanding.

5.5 CENTRE FOR INFECTIONS
Refer to current guidelines on CDSC and COSURV reporting.

5.6 INFECTION CONTROL STAFF
N/A

6 REFERRALS

6.1 REFERENCE LABORATORIES

Haemophilus influenzae from cases of invasive disease (isolates from normally sterile sites)

Haemophilus Reference Unit
Respiratory & Systemic Infection Laboratory
Health Protection Agency Centre for Infections
61 Colindale Avenue
London
NW9 5EQ

Telephone: +44 (0) 208 327 7331/ 6091/ 7330
HACEK group and *Haemophilus* species for identification

Laboratory of Health Care Associated Infections (LHCAI)
Health Protection Agency Centre for Infections
61 Colindale Avenue
London
NW9 5EQ

Telephone: +44 (0) 208 327 7241

Antibiotic Resistance Monitoring Reference Laboratory (ARMRL)
Health Protection Agency Centre for Infections
61 Colindale Avenue
London
NW9 5EQ

[http://www.hpa.org.uk/cfi/armrl/default.htm](http://www.hpa.org.uk/cfi/armrl/default.htm)
Telephone: +44 (0)208 327 6511
7 ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been developed, reviewed and revised by the National Standard Methods Working Group for Clinical Bacteriology (http://www.hpa-standardmethods.org.uk/wg_bacteriology.asp). The contributions of many individuals in clinical bacteriology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor are acknowledged.

The National Standard Methods are issued by Standards Unit, Department for Evaluations, Standards and Training, Centre for Infections, Health Protection Agency, London.

For further information please contact us at:

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REFERENCES


