

NATIONAL STANDARD METHOD

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



Association of Medical Microbiologists
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Association of Medical Microbiologists



IDENTIFICATION OF *HAEMOPHILUS* SPECIES AND THE HACEK GROUP OF ORGANISMS

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Reference no: BSOP ID 12i2

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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

Controlled document reference	BSOP ID 12
Controlled document title	Identification of <i>Haemophilus</i> species and the HACEK group of organisms

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.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
2/ 17.04.09	1.1	2	1	Front Page	NIMAG logo inserted
			All	All	Department name changed to DEST
			8	3	Tables amended to include characteristics of all HACEK organisms
			11-12	4	Flowcharts inserted

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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*², *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 analysis³, *Aggregatibacter aphrophilus*, which includes V-factor dependent and V-factor independent isolates. *H. segnis* has been re-classified as *Aggregatibacter segnis*³. There are eight biotypes of *Haemophilus influenzae* (I-VIII) and eight biovars of *Haemophilus parainfluenzae* (I-VIII)³. Pittman described six antigenically distinct capsular types of *H. influenzae*, designated a-f³.

Characteristics⁴

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 paraphrohaemolyticus* 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 [BSOFTP 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.

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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 commensals⁵. 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 cocci are 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 CO₂ 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% CO₂ 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% CO₂. *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% CO₂. 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 CO₂ 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.

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Eikenella corrodens⁶

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**¹⁰

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*¹⁰.

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 CONSIDERATIONS¹¹⁻²¹

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^{4,22-43}

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 ([BSOFTP 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).

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3.3.1 AEROBIC GROWTH CHARACTERISTICS OF HACEK GROUP ORGANISMS

HACEK group organisms	Characteristics of growth on blood agar after aerobic incubation at 35°C - 37°C for 16 - 48 h
<i>A. actinomycetemcomitans</i>	Will not grow in air but grows in air + CO ₂ . 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.
<i>A. aphrophilus</i>	Requires added CO ₂ 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 <i>H. paraphrophilus</i>) whereas others are V-factor-independent (formerly <i>H. aphrophilus</i>). Non-haemolytic.
<i>C. hominis</i>	Some strains will not grow without added CO ₂ . May require X-factor on primary isolation. Colonies smooth, convex and opaque. 1 - 2 mm at 48 h. Slight α-haemolysis.
<i>C. valvarum</i>	Grows best in air +5% CO ₂ . 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.
<i>E. corrodens</i>	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% CO ₂ .
<i>K. kingae</i>	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% CO ₂ .
<i>K. denitrificans</i>	Non-haemolytic. Two types of colony: a spreading, corroding type and a smooth, convex type.
<p>Note 1: For descriptions of <i>Haemophilus</i> species see <i>Haemophilus</i> species (Introduction)</p> <p>Note 2: In some cases it may be possible to use commercial biochemical identification kits</p>	

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3.4 TEST PROCEDURES

Biochemical tests²³

Summary of biochemical tests:

	Catalase	Oxidase	Urease
<i>H. influenzae</i>	+	+	(+)
<i>H. aegyptius</i>	+	+	+
<i>H. haemolyticus</i>	+	+	+
<i>H. parainfluenzae</i>	d	+	d
<i>H. pittmaniae</i>	d	d	-
<i>H. parahaemolyticus</i>	d	+	+
<i>H. paraphrohaemolyticus</i>	+	+	+
<i>A. actinomycetemcomitans</i>	+	+	-
<i>A. aphrophilus</i>	-	-	-
<i>C. hominis</i>	-	+	-
<i>K. kingae</i>	-	+	-
<i>E. corrodens</i>	-	+	-

Growth requirement for X and V factors – Distinguishing among *Haemophilus* species (see [BSOFTP 38 - X and V factor test](#) or porphyrin synthesis test [BSOFTP 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 recent changes in taxonomy

Summary of X and V test results

	X factor	V factor	X + V factor	Porphyrin
<i>H. influenzae</i> ^a	No growth	No growth	Growth	Negative
<i>H. haemolyticus</i> ^b	No growth	No growth	Growth	Negative
<i>H. parainfluenzae</i>	No growth	Growth	Growth	Positive
<i>H. pittmaniae</i>	No growth	Growth	Growth	Positive
<i>H. parahaemolyticus</i>	No growth	Growth	Growth	Positive
<i>H. paraphrohaemolyticus</i>	No growth	Growth	Growth	Positive

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

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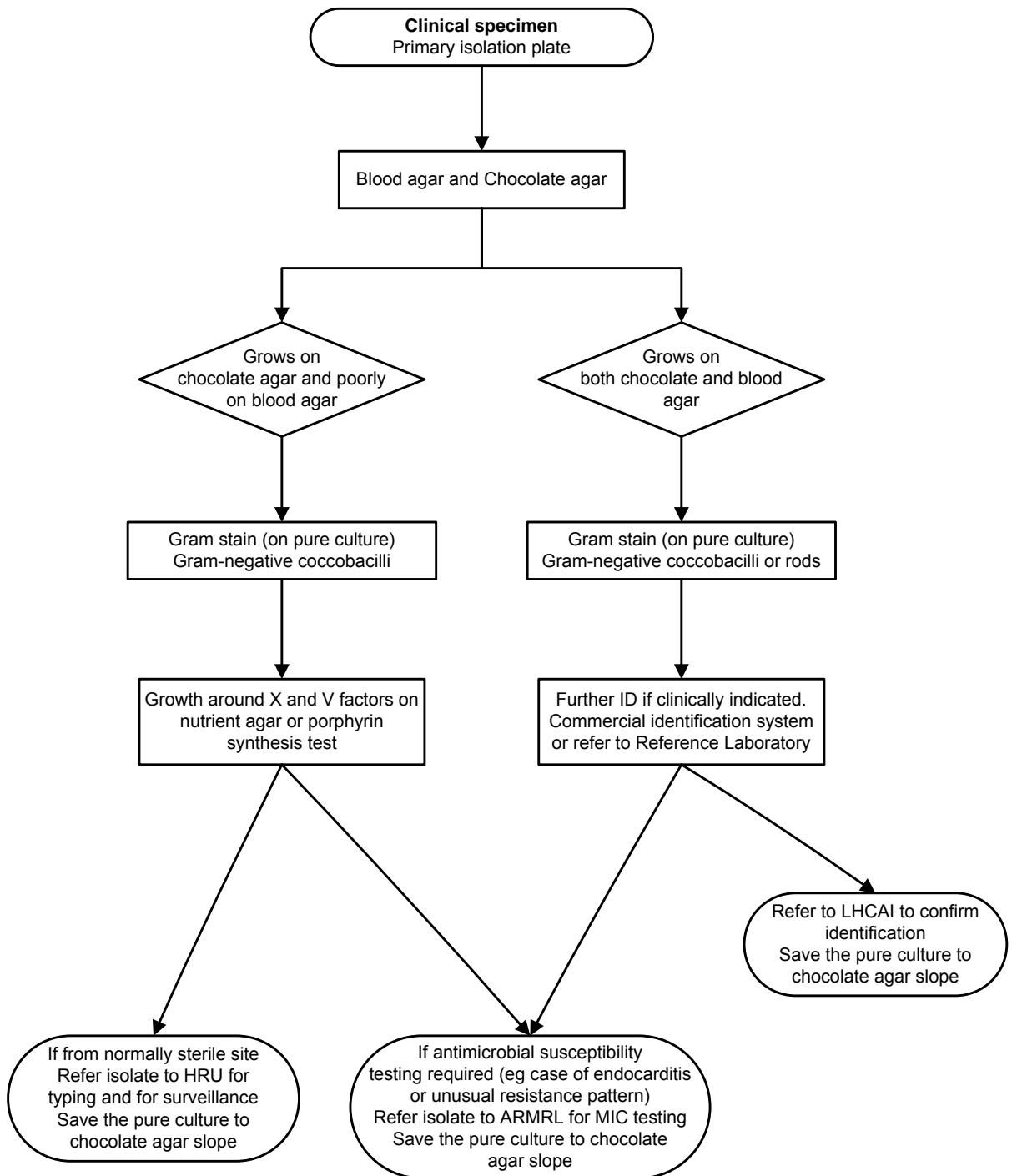
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4 IDENTIFICATION FLOWCHARTS

4.1 HAEMOPHILUS SPECIES



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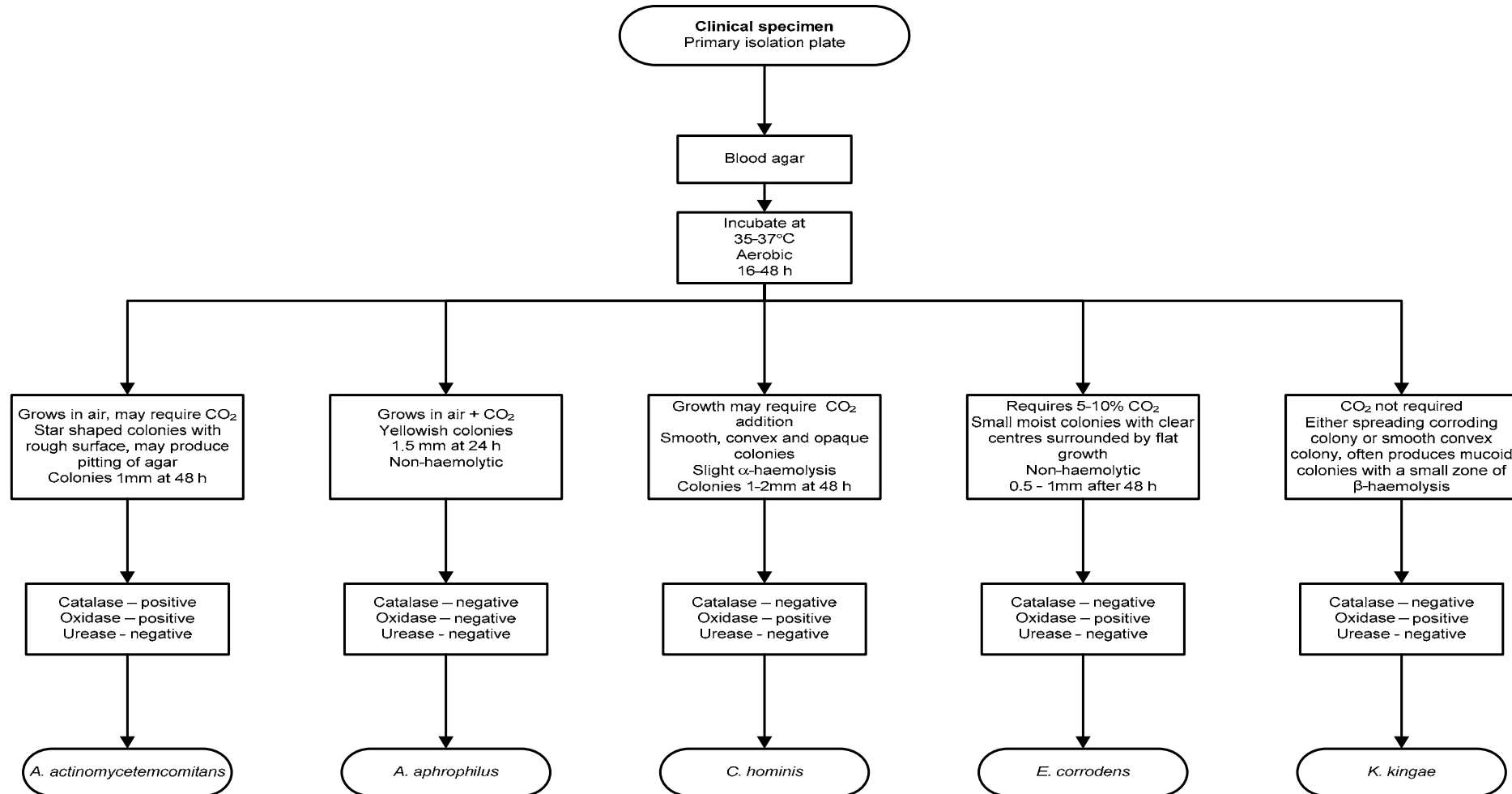
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4.2 HACEK GROUP



5 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

<http://www.hpa.org.uk/cfi/rsil/rsiluser.pdf>

Telephone: +44 (0) 208 327 7331/ 6091/ 7330

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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

http://www.hpa.org.uk/cfi/lhcai/req_single_isolate.pdf

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>

Telephone: +44 (0)208 327 6511

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7 ACKNOWLEDGEMENTS AND CONTACTS

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