UK Standards for Microbiology Investigations
Identification of Haemophilus species and the HACEK group of organisms

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### Amendment table

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New or revised documents should be controlled within the laboratory in accordance with the local quality management system.

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*Reviews can be extended up to five years subject to resources available.
Identification of *Haemophilus* species and the HACEK group of organisms

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 SMI 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. *Haemophilus* are exclusively adapted to human mucosal membranes in the respiratory tract.

This UK SMI includes both biochemical tests and automated methods for the identification of microorganisms. 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.

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

4 Introduction

4.1 Taxonomy/characteristics

There are currently 24 species of the genus *Haemophilus*1. The *Haemophilus* species associated with humans are *H. influenzae*, *H. aegyptius*, *H. haemolyticus*, *H. parainfluenzae*, *H. pittmaniae*, *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. ducreyi* and *H. sputorum*2,3. 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*4.

*H. influenzae* is the type species.

*Haemophilus* species are Gram negative spherical, oval or rod-shaped cells less than 1µm in width, variable in length, with marked pleomorphism, and sometimes forming filaments. The optimum growth temperature is 35–37°C. They are facultatively anaerobic, non-acid-fast, none spore forming and non-motile.

Members of the *Haemophilus* genus are typically cultured on blood or chocolate agar plates as all species require at least one of the following blood factors for growth: haemin (factor X) and/or nicotinamide adenine dinucleotide (factor V). Chocolate agar is an excellent *Haemophilus* growth medium as it allows for increased accessibility to
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these factors. Blood agar contains free V, but not X, factor. *Haemophilus* species requiring X factor can be cultured on blood agar using the "Staph streak" technique: both *Staphylococcus* and *Haemophilus* organisms are cultured together on a single blood agar plate. In this case, *Haemophilus* colonies will grow in small "satellite" colonies around the larger *Staphylococcus* colonies because the metabolism of *Staphylococcus* produces the necessary X blood factor required for *Haemophilus* growth. All *Haemophilus* species grow more readily in an atmosphere enriched with CO₂; *H. ducreyi* and some nontypable *H. influenzae* strains will not form visible colonies on culture plates unless grown in CO₂-enriched atmosphere. *Aggregatibacter aphrophilus* and *Haemophilus paraphrophilus* require CO₂ for primary isolation.

On chocolate blood agar, colonies are small and grey, round, convex, which may be iridescent, and these develop in 24 hours. Iridescence is seen with capsulated strains. Carbohydrates are catabolised with the production of acid. A few species produce gas. Nitrates are reduced to nitrates.

These have been isolated from abscess, respiratory secretions, middle ear fluid, CSF, purulent sputum and blood culture⁵.

The medically important *Haemophilus* species are described as follows;

**Haemophilus influenzae**⁶

They are small, non-motile Gram negative bacterium in the family *Pasteurellaceae*. They are facultatively anaerobic. On chocolate blood agar, colonies are small and grey, round, convex, which may be iridescent, and these develop in 24 hours. Iridescence is seen with capsulated strains. There is no growth on MacConkey or CLED agar and colonies show no β-haemolysis on blood agar (with additional X factor). They require both the X and V factors for growth.

They are positive for oxidase, catalase, nitrate reduction and phosphatase. Eleven to eighty nine percent of strains are positive for indole production and 80-89% of strains are positive for urease and ornithine decarboxylase tests. They are also negative for ONPG, H₂S production and aesculin hydrolysis. Acid is produced from D-Glucose, D-Galactose, Maltose, D-Ribose and D-xylose and not from lactose, D-mannitol, D-Mannose, sucrose, Inulin, Trehalose, Raffinose, L-Rhamnose, L-Sorbose, D-Sorbitol, Fructose and Melibiose⁶.

Pittman described six antigenically distinct capsular types of *H. influenzae*, designated a-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 nontypable. There is also a biotyping scheme for *H. influenzae* based on a series of biochemical reactions (indole, ornithine decarboxylase and urease production). There are 8 biotypes of *H. influenzae* (I-VIII)⁴.

*H. influenzae* has been isolated from respiratory secretions, CSF, sputum and blood culture⁷.

Before the introduction of a vaccine against serotype b (Hib), the majority of infections were caused by serotype b strains which caused meningitis, epiglottitis, orbital cellulitis and bacteraemia (generally biotypes I and II of this species). However, all types of *H. influenzae* (including nontypable strains) can cause systemic infections such as meningitis, bacteraemia, septic arthritis and cellulitis. Most non-typable *H. influenzae* strains fall into biotypes II to VI and can cause acute conjunctivitis, otitis media, sinusitis, tracheobronchitis and pneumonia as well as invasive diseases⁷.
Identification of *Haemophilus* species and the HACEK group of organisms

**Haemophilus parainfluenzae**

They are small, non-motile Gram negative bacterium in the family *Pasteurellaceae*. They are facultatively anaerobic. There is no growth on MacConkey or CLED agar and show no β-haemolysis on sheep cells. They require V factor but not the X factor for growth.

They are positive for oxidase, nitrate reduction and H2S production. Acid is produced from Fructose, D-Galactose, D-Glucose, Maltose, Sucrose and D-Mannose. Eleven to eighty nine percent of strains are positive for catalase, ONPG, Ornithine decarboxylase and Urease. They are negative for indole production and aesculin hydrolysis. Acid is not produced from D-Adonitol, L-Arabinose, Cellobiose, Dulcitol, D-Sorbitol, L-Sorbose, Trehalose, D-Xylose, Glycerol, Inulin, Lactose, D-Mannitol, Melibiose, Raffinose, L-Rhamnose, D-Ribose and Salicin.

There are eight biotypes of *Haemophilus parainfluenzae* (I-VIII) based on a series of biochemical reactions (indole, ornithine decarboxylase and urease production)⁴.

They have been associated with some cases of acute otitis media, sinusitis and chronic bronchitis⁵.

*H. parainfluenzae* has been isolated from clinical specimens – respiratory secretions (from the lower airways, oropharynx, and nasopharynx), abscess and sputum. Although it has been isolated from sputum, it is considered a part of the normal oral flora and so not reported as significant⁸.

**Haemophilus haemolyticus**

They are Gram negative, non-motile and non-spore-forming short to medium length rods. There is no growth on MacConkey or CLED agar. They classically show β-haemolysis on blood agar (with additional X factor), although non-haemolytic isolates have been reported [your ref 69]. They also require the X and V factors for growth.

They are positive for oxidase, catalase, nitrate reduction, phosphatase, urease and H2S production. Some strains of *H. haemolyticus* (11-89%) are positive for Indole production on blood agar (with additional X factor), although non-haemolytic isolates have been reported [your ref 69]. They also require the X and V factors for growth.

They are positive for oxidase, catalase, nitrate reduction, phosphatase, urease and H2S production. Acid is produced from D-Galactose, D-Glucose, Maltose and D-Ribose and about 11-89% of strains produce acid from D-Xylose. They are negative for ONPG, ornithine decarboxylase and aesculin hydrolysis. Acid is not produced from D-Adonitol, L-Arabinose, Cellobiose, Dulcitol, Glycerol, Inulin, Lactose, D-Mannitol, D-Mannose, Melibiose, Raffinose, L-Rhamnose, Salicin, D-Sorbitol, L-Sorbose, Sucrose and Trehalose.

*H. haemolyticus* is a commensal of the respiratory tract, but does occasionally cause invasive disease⁹.

**Haemophilus parahaemolyticus**

These usually differ morphologically from other haemophilic bacteria in that they are larger, stain more heavily and unevenly, and occur in long tangled thread forms with much pleomorphism.

The colonies tend to be larger, less translucent, and on blood agar, they are surrounded by a large colourless zone of haemolysis. In broth, there is stringy floccular sediment with clear supernatant. The V factor but not X factor is required for growth.
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They are positive for oxidase, nitrate reduction, H₂S production and urease tests. Some strains of *H. parahaemolyticus* (11-89%) are positive for catalase, ONPG, Ornithine decarboxylase and produce acid from D-Galactose. Acid is also produced from fructose, D-Glucose, maltose and sucrose. They are negative for indole production and aesculin hydrolysis. Acid is not produced from D-Adonitol, L-Arabinose, Cellobiose, Dulcitol, Glycerol, Inulin, Lactose, D-Mannitol, D-Mannose, Melibiose, Raffinose, L-Rhamnose, Salicin, D-Sorbitol, L-Sorbose, D-Xylose and Trehalose.

The bacteria are associated frequently with acute pharyngitis and occasionally cause sub-acute endocarditis.

**Haemophilus paraphrohaemolyticus**

They are Gram negative, non-motile and non-spore-forming short to medium length rods measuring 0.75-2.5µm and 0.4-.0.5µm. They grow well at 37°C both in air and in air with added CO₂.

On blood agar plate, the colonies are smooth, round and dome-shaped and they also produce large zones of clear haemolysis. Chocolate agar promotes larger colonies than blood agar, irrespective of the presence or absence of CO₂. The V factor but not X factor is required for growth. No growth is observed on inspissated serum or on MacConkey or CLED agar.

They are positive for catalase, oxidase, nitrate reduction, H₂S production and urease tests. Acid is produced from fructose, D-Glucose, Maltose and sucrose. Eleven to eighty nine percent of strains are positive for ONPG and produces acid from D-Galactose. They are negative for ornithine decarboxylase, Indole production and aesculin hydrolysis. Acid is not produced from D-Adonitol, L-Arabinose, Cellobiose, Dulcitol, Glycerol, Inositol, inulin, lactose, D-Mannitol, D-Mannose, Melibiose, Raffinose, L-Rhamnose, D-Ribose, Salicin, D-Sorbitol, L-Sorbose, Trehalose and D-Xylose.

It has been isolated from sputum, throat, pharynx, thumb print and urethral discharge in humans.

**Haemophilus aegyptius**

They are Gram negative, non-motile, non-spore-forming, non-encapsulated bacillus, 0.25-0.5µm by 1.0-2.5µm, with rounded ends and sometimes with a bipolar body. It is a facultative aerobe. It requires both haemin and V factors for growth. The optimum temperature is 35-37°C with a range of 25-40°C. The colonies on blood agar are small and dew-drop-like without haemolysis; on transparent agar, they have a bluish tinge in transmitted light; and in semifluid medium they are granular to fluffy. They are soluble in sodium desoxycholate, reduce nitrates to nitrites, and do not produce indole. Slight acidity is formed from glucose and galactose; reaction on levulose is variable and on xylose negative. It agglutinates human red cells.

It has been reported that *Haemophilus aegyptius* can be differentiated from *Haemophilus influenzae* by serological means and to a certain extent, by growth characteristics and biochemical reactions. However, it has been proposed that this species should be reclassified as a biogroup within the *H. influenzae* species.
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**Haemophilus pittmaniae**

They are non-motile, facultatively anaerobic, Gram negative, small, pleomorphic rods, with occasional long, filamentous forms. Colonies on chocolate agar are greyish white and reach a diameter of 1-2mm after 24hr at 35°C. A distinct β-haemolytic zone is produced around the colonies on horse or sheep blood agar. They depend on V-factor for growth on brain heart infusion agar plates, but are capable of growth on blood plates due to release of V factor from lysed blood cells. They are positive for porphyrin test, negative or weakly positive in catalase and oxidase tests. Acid is produced from D-glucose, D-fructose, sucrose, D-mannose, D-galactose and maltose. A small amount of gas is produced from glucose. They also produce β-galactosidase (ONPG), alkaline phosphatase, acid phosphatase and leucinearylamidase, but not β-glucosidase (NPG), α-glucosidase (PNPG), β-glucosaminidase (GNAC), β-glucuronidase (PGUA) or α-fucosidase (ONPF).

They are negative for the indole, urease, in lysine and ornithine decarboxylase and arginine dihydrolase tests. Acid is not produced from lactose, D-xylose, D-mannitol, D-sorbitol, sorbose, melibiose, inulin, aesculin or amygdalin. *Haemophilus pittmaniae* was originally isolated from human saliva and is part of the normal flora of the oral mucous membranes of man. It is an opportunistic pathogen and has been isolated from various sites of infection, including blood and bile.

**Haemophilus ducreyi**

Cells are Gram negative coccobacilli in “railroad track” arrangement. They grow best in microaerophilic conditions at 33-35°C in a humid atmosphere containing 5% CO₂. The identification of *H. ducreyi* growing from cultured specimens is not easy because the organism often cannot grow in the media used for routine biochemical testing; *H. ducreyi* grows on Mueller-Hinton agar with 5% sheep blood in a CO₂ enriched atmosphere. They produce characteristic tan-yellow colonies that are highly self-adherent and can be ‘nudged’ intact over the surface of the agar. Furthermore, identification is not easy because *H. ducreyi* is asaccharolytic. They require X factor for growth and this can most easily be evaluated using the porphyrin test. They are positive for oxidase and negative for catalase test.

*H. ducreyi* has been isolated from a number of ulcer specimens including leg, foot, perianal and genital (penis).

**Haemophilus sputorum**

Cells are non-motile, small regular rods, 0.3-0.5µm × 2.0-3.0µm, with occasional coccoid forms. Colonies on chocolate agar are convex, whitish, opaque, and reach a diameter of 0.5–1.5mm within 24hr. Zones of β-haemolysis are produced around colonies on horse or sheep blood agar; occasional strains are non-haemolytic and consequently fail to grow on blood agar. Cells are dependent on V factor for growth.

They are positive for oxidase and give variable results on catalase tests. Cells produce β-galactosidase, urease, and leucinearylamidase; the species are negative for indole test, arginine di-hydrolase, lysine decarboxylase, ornithine decarboxylase, and phenylalanine arylamidase. H₂S is not or only weakly emitted (lead acetate test), IgA1 protease is not produced.
Acid is produced from fermentation of d-glucose, fructose, d-maltose and maltotriose; acid is not produced from N-acetyl-β-D-glucosamine, D-xylose, D-ribose, D-mannose, lactose, and D-malate. Variable fermentation is observed with D-galactose.

*H. sputorum* was orginially isolated from a case of human tooth alveolitis and is occasionally involved in human infections and has been isolated from blood, sputum of patients with cystic fibrosis, and tooth alveolitis.

*Haemophilus massiliensis*17

They are Gram negative, non-endospore forming, facultatively anaerobic and non-motile bacterium. Colonies are not haemolytic, round and light with a size of 0.5 to 1 mm on blood enriched Colombia agar. Cells are rod-shaped and growth occurs between 25 and 45 °C, optimal growth temperature is at 37 °C. They are positive for catalase and oxidase. It was isolated from human peritoneal fluid.

**Other 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 commensals18. 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 may be referred to the Antimicrobial Resistance and Healthcare Associated Infections Reference Unit, PHE Microbiology Services, Colindale for confirmation of identification and MIC testing.

**Aggregatibacter species**4

This is a member of the family Pasteurellaceae. The genus *Aggregatibacter* contains 3 species, *Aggregatibacter actinomycetemcomitans*, *Aggregatibacter aphrophilus* and *Aggregatibacter segnis*19.

They are Gram negative, non-motile, facultatively anaerobic rods or coccobacilli. Growth is mesophilic. Several species of the genus are capnophilic and primary isolation may require the presence of 5-10% CO2. There is no dependence on X factor and the requirement for V factor is variable. Granular growth in broth is common. Colonies on sheep and horse blood agar are greyish white and non-haemolytic. Acid is produced from glucose, fructose and maltose, whereas arabinose, cellobiose, melibiose, melezitose, salicin and sorbitol are not fermented. The fermentation of galactose, lactose, mannitol, mannose, raffinose, sorbose, sucrose, trehalose and xylose is variable and may aid in identification to the species level. They are also positive for nitrate reduction and alkaline phosphatase production, but strains are negative in tests for indole, urease, ornithine and lysine decarboxylases and arginine dihydrolase. Oxidase reaction is negative or weak; catalase is variably present.

The species of the genus are intimately associated with man; they are part of the human oral flora and are occasionally recovered from other body sites, including blood and brain, as causes of endocarditis and abscesses.

The type species is *Aggregatibacter actinomycetemcomitans*, originally described as ‘*Bacterium actinomycetemcomitans*’.
**Aggregatibacter actinomycetemcomitans**

(Previously known as Actinobacillus actinomycetemcomitans and then as Haemophilus actinomycetemcomitans).

They are small rods, 0.3-0.5 x 0.5-1.5µm, which may exhibit irregular staining and may appear as cocc in broth or actinomycotic lesions. They may occur singly, in pairs or in small clumps. Small amounts of extracellular slime may be produced. Cells are non-motile.

It grows best under microaerophilic conditions with added CO₂ and is facultatively anaerobic. The optimal growth temperature is 37°C after 24hr incubation. Colonies on chocolate agar are small, with a diameter of ≤0.5mm after 24hr, but may exceed 1-2mm after 48hr. On primary isolation, the colonies are rough, textured and adherent and have an internal, opaque pattern described as star-like or like ‘crossed cigars’. The rough phenotype is related to fimbriation and to the production of hexoseamine-containing exopolysaccharide. Cells from rough colonies grow in broth as granular, autoaggregated cells that adhere to the glass and leave a clear broth. X and V factors are not required. If extracellular slime is produced, cultures may be sticky on primary isolation. Surface cultures have low viability and may die within 5-7 days.

They are positive for catalase, oxidase and acid is produced from glucose, fructose, maltose and mannose, whereas arabinose, cellobiose, galactose, lactose, melibiose, melezitose, trehalose, raffinose, salicin, sorbitol and sucrose are not fermented. Variable fermentation is observed with mannitol and xylose. They are negative for urease and ONPG hydrolysis.

The key tests for discrimination between Aggregatibacter actinomycetemcomitans and V factor-independent strains of Aggregatibacter aphrophilus are catalase and ONPG, plus fermentation of lactose, sucrose and trehalose.

They are indigenous to man, with primary habitat on dental surfaces. Aggregatibacter actinomycetemcomitans has regularly been isolated together with Actinomyces species from human actinomycosis. It has been sometimes found in other pathological processes such as endocarditis, brain abscess and urinary tract infections.

**Aggregatibacter aphrophilus**

The species Haemophilus aphrophilus and Haemophilus paraphrophilus have been reclassified as a single species Aggregatibacter aphrophilus.

These are Gram negative, short regular rods, 0.5 x 1.5-1.7µm with occasional filamentous forms. They require 5-10% CO₂ for primary isolation. Growth may be enhanced by haemin, but porphyrins are synthesized from δ-aminolaevulinic acid and X factor is not required. Some isolates require V factor (formerly H. paraphrophilus) whilst others are V factor independent (formerly H. aphrophilus). The colonies on chocolate agar are high convex, opaque, granular and yellowish and reach a diameter of 1.0-1.5mm within 24hr.

Acid is produced from glucose, fructose, lactose, maltose, mannose, sucrose and trehalose, whereas arabinose, cellobiose, mannitol, melibiose, melezitose, salicin, sorbose, sorbitol and xylose are not fermented. Variable fermentation is observed with galactose and raffinose. H₂O₂ is not decomposed; ONPG is hydrolysed. They are also catalase and urease negative, and oxidase variable.
Key tests for discrimination between V factor-dependent isolates of *Aggregatibacter aphrophilus* and strains of *H. parainfluenzae* biotype V (negative for indole, urease and ornithine decarboxylase) are fermentation of lactose and trehalose.

*Aggregatibacter aphrophilus* is a member of the normal flora of the human oral cavity and pharynx. May cause brain abscess and infective endocarditis and has been isolated from various other body sites including peritoneum, pleura, wound and bone.

*Aggregatibacter segnis*<sup>4</sup>  
**Formerly called Haemophilus segnis**

Cells are small, pleomorphic rods, often showing a predominance of irregular, filamentous forms. Growth on chocolate agar is slow and the colonies are smooth or granular, convex, greyish-white or opaque and 0.5mm in diameter after 48hr incubation. Growth in broth and fermentation media is slow, and reactions are negative or weakly positive. The growth of some strains is enhanced by 5-10% CO<sub>2</sub>. V-factor but not X-factor is required.

Small amounts of acid result from the fermentation of glucose, fructose, galactose, sucrose and maltose. Fermentation of sucrose is usually stronger than fermentation of glucose. Arabinose, cellobiose, lactose, mannitol, mannose, melibiose, melezitose, raffinose, salicin, sorbose, sorbitol, trehalose and xylose are not fermented. Catalase and β-galactosidase (hydrolysis of ONPG) are variably present. They are negative for oxidase, indole, urease and ornithine decarboxylase tests.

*Aggregatibacter segnis* is a regular member of the human oral flora, particularly in dental plaque, and can be isolated from the pharynx. It has occasionally been isolated from human infections including infective endocarditis.

*Cardiobacterium* species<sup>20</sup>

The genus *Cardiobacterium* contains 2 species, *Cardiobacterium hominis* and *Cardiobacterium valvarum*<sup>21,22</sup>. Cells are pleomorphic or straight rods, 0.5–0.75µm in diameter and 1–3µm 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. They do 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<sub>2</sub>. After incubation for 2 days, colonies are 1mm in diameter, smooth, opaque and butyrous and show slight α-haemolysis. Some strains may pit the agar. They are facultatively anaerobic, but CO<sub>2</sub> may be required by some strains on primary isolation. The optimum growth temperature is 30-37°C.

They are positive for oxidase, H<sub>2</sub>S production, indole (weakly), and are negative for nitrate reduction, catalase, urea and aesculin hydrolysis. They utilize dextrose, fructose, maltose, mannitol, sucrose, sorbitol, and mannose but do not utilize galactose, lactose, raffinose and xylose.

*Cardiobacterium hominis* is the type species.

*Cardiobacterium hominis*<sup>20</sup>

They are Gram negative pleomorphic to short, non-motile rods. Growth on blood agar is poor. *C. hominis* does not require X or V factors, but may show an apparent
Identification of *Haemophilus* species and the HACEK group of organisms

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 2 days, colonies are 1mm in diameter, circular, smooth, entire, moist, glistening, opaque and butyrous and show slight α-haemolysis. 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 30-37°C.

They are positive for oxidase, H₂S production, indole (weakly), and are negative for nitrate reduction, catalase, urease and aesculin hydrolysis. They utilize dextrose, fructose, maltose, mannitol, sucrose, sorbitol, and mannose but do not utilize galactose, lactose, raffinose and xylose.

*C. hominis* could be distinguished from other members of the HACEK group and from *Pasteurella, Brucella, Streptobacillus moniliformis* and *Bordetella parapertussis*. The main characteristics of *C. hominis*, distinguishing it from other closely related organisms are absence of catalase activity, positive oxidase reaction, production of indole and absence of nitrate production₂³.

They have been isolated from cerebrospinal fluid, blood as well as from nose and throat in healthy individuals.

**Cardiobacterium valvarum**²⁴

They are fastidious Gram negative regular, pleomorphic to short rods. All strains are facultatively anaerobic and non-motile. Some strains have an acidulous. Its preferred culture medium is sheep blood agar, and visible colonies appear after an incubation period of 3 days. The colonies are round, elevated, opaque, smooth, and glistening. However, the colonies hardly reach 1mm after extended incubation. Therefore, *C. valvarum* is more fastidious than *C. hominis*, whose colonies appear after a two-day incubation and reach a diameter of 2.2mm after 4 days. Microscopically, *C. valvarum* appears readily decolorized by acetone alcohol, and the cellular morphology varies depending on culture medium. When grown on blood agar, it is a fairly large regular rod, measuring 1 by 2 to 4µm. On chocolate agar, it is smaller and pleomorphic.

They are positive for the production of indole, cytochrome oxidase, and H₂S but negative for catalase production, urea hydrolysis, aesculin hydrolysis, and nitrate reduction. It utilizes dextrose, fructose, sorbitol, and mannose, like *C. hominis*, but unlike *C. hominis*, does not utilize maltose, sucrose, or mannitol.

It was first isolated in 2001 from the blood of a 37 year old man with endocarditis. *Cardiobacterium valvarum* is present in subgingival pockets and dental plaques, and all the reported cases of endocarditis have been in persons who had recently undergone a dental procedure or had oral infection²¹.

**Eikenella corrodens**²⁵

The genus *Eikenella* contains only one species, *Eikenella corrodens*. Cells are straight, un-branched, non-sporing, slender Gram negative rods, 0.3-0.4 x 1.5-4µm 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-
Identification of *Haemophilus* species and the HACEK group of organisms

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 35-37°C. *E. corrodens* is non-motile, but ‘twitching’ motility may be produced on some media. Strains are facultatively anaerobic 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.

They are positive for oxidase, ornithine dacrboxylase and nitrate reduction and are negative for acidification of carbohydrates, production of indole, aesculin hydrolysis, catalase and urease tests.

*Eikenella corrodens* exists in dental plaque of both healthy people and periodontitis patients and can cause infections. Other clinical sources include head and neck infections and respiratory tract infections.

**Kingella species**

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

*Kingella* species are straight rods, 1.0µm 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 33-37°C.

They 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.

**Kingella denitrificans**

Previously designated CDC group TM-1. They are Gram negative, non-motile, plump rods 1.0µm in width. Small, translucent non-haemolytic colonies are produced on blood agar after 48hr of incubation at 37°C. Colonies may show pitting of the medium. Growth occurs anaerobically on blood agar. They are positive for oxidase, growth at 30 and 37°C, fermentative result in the O/F test, acid production from glucose, nitrate reduction, nitrite reduction, and production of gas from nitrite.

They are also negative for catalase, growth at 5 and 45°C, growth in the presence of 4 and 6% NaCl, growth on β-hydroxybutyrate in mineral medium, acid production from maltose unless serum was present, starch hydrolysis and urease production. Isolated in the respiratory tract of man.

**Kingella kingae**

The cells are coccoid to medium-sized rods, very much like those of *Moraxella* but slightly smaller, have square ends, and occur in pairs and short chains. They are
Gram negative, with some tendency to resist decolourisation. They are also non-motile, non-encapsulated and no endospores are produced. On blood agar, two types of colonies occur; colonies of freshly isolated strains appear as small depressions, 0.1 -0.5mm in diameter, with a small central papilla initially but after 2 or more days incubation, there is considerable spreading growth and thin granular zones of growth often surround the colonies. Colonies when scrapped shows corrosion marks on the agar surface. The second colony, which often arises in subcultures of the first type, is small, delicate, translucent or slightly opaque, 0.1-0.6mm in diameter after 20hr on blood agar, low hemispherical, and smooth. On further incubation, the colonies increase in size but there is no evidence of corrosion or spreading. Both types of colonies are surrounded by distinct zones of β-haemolysis; their consistencies are soft or coherent and are not pigmented.

They are aerobic and grow at room temperature but their optimal growth is at 33-37°C. They are relatively fastidious and growth on high quality nutrient agar is as good as that on blood agar.

They are negative for catalase and urease tests. No acid is produced from fructose, lactose, saccharose, arabinose, xylose, rhamnose, mannitol, dulcitol, sorbitol, or glycerol. Gelatin and serum are not liquefied. Nitrate are not reduced or slightly reduced.

They are parasitic on human mucous membranes. Strains have been isolated from throat, nose, blood, bone lesions and joints.

**Kingella oralis**

They are Gram negative rods or coccobacilli approximately 0.6-0.7µm in diameter by 1-3µm long with rounded ends. Cells can form pairs or chains. Cells have monopolar fimbriae up to 10µm long. There is a tendency to resist Gram decolourisation. Not motile by means of flagella, but cells form spreading colonies. They are aerobic or facultatively anaerobic. Growth is supported by 5% sheep blood agar supplemented with 5 mg of haemin per litre and 0.5µg of menadione per mL in both anaerobic and aerobic environments with CO₂. They do not grow on MacConkey agar. Colonies are round with slightly irregular borders and flat to umbonate, and each colony has a granular periphery. Colonies appear to corrode the agar surface. They are positive for oxidase test and negative for nitrate, nitrite, indole, urease and aesculin hydrolysis tests. Acid is not produced from lactose, maltose, mannitol, sucrose, and xylose.

The habitat of *K. oralis* appears to be human dental plaque and has been isolated from a supragingival plaque sample from a patient with adult periodontitis.

**Kingella potus**

Cells are gram negative, non-spore-forming, non-motile rods. They are aerobic, DNase positive, oxidase positive, and catalase negative. Colonies are circular, low convex, yellow- pigmented, smooth, entire, approximately 1.5-2mm in diameter, and friable on Columbia blood agar after 48hr of incubation at 37°C. Colonies are non-haemolytic. Non-diffusible yellow pigments are produced. Nitrate and nitrite are not reduced. Aesculin and urea are not hydrolysed. Indole is not produced. Acid is not produced from fructose, glucose, mannose, mannitol, maltose, lactose, or sucrose. No alkaline phosphatase, α-glycosidase, β-galactosidase, or β-glucuronidase activity is detected. This has been isolated from the human wound caused by a bite from a kinkajou.
Identification of *Haemophilus* species and the HACEK group of organisms

Tests that are useful in distinguishing *Kingella potus* from other *Kingella* species and members of the genus *Neisseria* are DNase test and its ability to pigment.

*Kingella negevensis* \(^3^4\)

Cells are coccobacillary, non-spore-forming and non-motile. They are Gram negative, oxidase positive and exhibit aerobic and facultatively anaerobic capnophilic growth. They are catalase, indole, lipase, alkaline phosphatase negative. After incubation at 37°C for 1 day on 5% sheep blood-enriched Columbia agar, colonies are β-haemolytic, round, pale yellow, smooth and 0.5-1 mm in diameter. It was first isolated from the oropharynx of healthy children.

5 Technical information/limitations

**Agar media and X & V factor testing**

The use of chocolate agar is more preferable for X and V factor testing rather than blood agar or blood containing medium because of risk of carryover of X factor. This test 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, usually identifying H. influenzae as H. parainfluenzae. Manufacturers’ instructions should be followed when performing this test.

**Incubation**

The X and V factor tests could sometimes give false V dependent results if incubated in CO₂.\(^3^5\)

For more information on technical limitation for the X and V Factor Test, see [TP 38 – X and V factor test](#).

**Principles of isolation**

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 [TP 29 – Porphyrin Synthesis (ALA) Test](#)) may be used to differentiate haemin producing *Haemophilus* species. Identification is confirmed by commercial biochemical tests, serotyping with type-specific antisera and/or referral to a Reference Laboratory.

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

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), Public Health England, Colindale, for confirmation and typing.

6 Safety considerations\(^3^6-5^3\)

*Haemophilus influenzae* is a Hazard Group 2 organism, and, 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.
Identification of *Haemophilus* species and the HACEK group of organisms

*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. The organism infects primarily by the respiratory route (inhalation), autoinoculation or ingestion in laboratory workers.

Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet. For the urease test, a urea slope is considered safer than a liquid medium. The use of needles, syringes, or other sharp objects should be strictly limited and eye protection must be used where there is a known or potential risk of exposure to splashes.

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

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

Compliance with postal and transport regulations is essential.

### 7 Target organisms

**HACEK group reported to have caused human infection**

*Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Haemophilus haemolyticus*, *Haemophilus parahaemolyticus*, *Haemophilus aegyptius*, *Haemophilus pittmaniae*, *Haemophilus ducreyi*, *Haemophilus sputorum*, *Aggregatibacter aphrophilus* (includes *Haemophilus aphrophilus* and *Haemophilus paraphrophilus*), *Aggregatibacter segnis* (formerly *Haemophilus segnis*), *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*), *Cardiobacterium hominis*, *Cardiobacterium valvarum*, *Eikenella corrodens*, *Kingella kingae*, *Kingella denitrificans*, *Kingella oralis*, *Kingella potus*

### 8 Identification

#### 8.1 Microscopic appearance

**Gram stain** *(TP 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.

#### 8.2 Primary isolation media

Chocolate agar incubated in 5-10% CO₂ at 35-37°C for 24-48hr.

Blood agar incubated in 5-10% CO₂ at 35-37°C for 24-48hr.

#### 8.3 Colonial appearance
Identification of *Haemophilus* species and the HACEK group of organisms

*Haemophilus* species are small, round, convex colonies, which may be iridescent and develop after 24hr 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 subheading “Characteristics” and table below).

**Table 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 35-37°C for 16-48hr</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>Will not grow in air but grows in air + CO₂. Minute colonies at 24hr, 1mm at 48hr. 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><em>A. aphrophilus</em></td>
<td>Requires added CO₂ for primary isolation. Opaque; yellowish colonies 1.0-1.5mm at 24hr. X-factor enhances growth but there is not an absolute requirement for it. 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><em>A. segnis</em></td>
<td>Growth on chocolate agar is slow and the colonies are smooth or granular, convex, greyish-white or opaque and 0.5mm in diameter after 48hr incubation.</td>
</tr>
<tr>
<td><em>C. hominis</em></td>
<td>Some strains will not grow without added CO₂. May require X-factor on primary isolation. Colonies smooth, convex and opaque. 1-2mm at 48hr. Slight α-haemolysis.</td>
</tr>
<tr>
<td><em>C. valvarum</em></td>
<td>Grows best in air +5% CO₂. Slow growing, colonies smooth, round, opaque and glistening, 0.6-0.8mm after 48hr. Some strains show slight α-haemolysis, others are non-haemolytic.</td>
</tr>
<tr>
<td><em>E. corrodens</em></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 0.5-1mm after 48hr. Requires 5-10% CO₂.</td>
</tr>
<tr>
<td><em>K. kingae</em></td>
<td>2 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><em>K. denitrificans</em></td>
<td>Non-haemolytic. 2 types of colony: a spreading, corroding type and a smooth, convex type.</td>
</tr>
<tr>
<td><em>K. oralis</em></td>
<td>Colonies are round with slightly irregular borders and flat to umbonate, and each colony has a granular periphery. Colonies appear to corrode the agar surface.</td>
</tr>
<tr>
<td><em>K. potus</em></td>
<td>Colonies are circular, low convex, yellow-pigmented, smooth, entire, approximately 1.5-2mm in diameter, and friable on Columbia blood agar after 48hr of incubation at 37°C. Colonies are non-haemolytic.</td>
</tr>
</tbody>
</table>
8.4 Test procedures

Tests mentioned below are no longer done routinely in laboratories except in cases where there are doubts, and may be useful.

Catalase Test (TP 8 - Catalase test)
Oxidase Test (TP 26 - Oxidase test)
Urease Test (TP 36 – Urease test)

Table 2: Summary of the biochemical tests:

<table>
<thead>
<tr>
<th>Organism</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. ducreyi</td>
<td>-</td>
<td>+</td>
<td>Unknown</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>H. sputorum</td>
<td>V</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>H. massiliensis</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>C. valvarum</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E. corrodens</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>K. kingae</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>K. denitrificans</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>K. oralis</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>K. potus</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>K. negevensis</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Identification of *Haemophilus* species and the HACEK group of organisms

+ = positive, - = Negative, (+) = 80-89% positive, d= 11-89% positive, V= variable result

**Growth requirement for X and V factors**

This is used to distinguish *Haemophilus* species ([TP 38 - X and V Factor Test](#) or [TP 29 – Porphyrin Synthesis (ALA) Test](#)).

### Table 3: Summary of X and V test results

<table>
<thead>
<tr>
<th>Organism</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>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No growth</td>
<td>No growth</td>
<td>Growth</td>
<td>Negative</td>
</tr>
<tr>
<td><em>H. haemolyticusb</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. paraphrophlaemolyticus</em></td>
<td>No growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Haemophilus ducreyi</em></td>
<td>Growth</td>
<td>No growth</td>
<td>Growth</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Haemophilus sputorum</em></td>
<td>No growth</td>
<td>Growth</td>
<td>Growth</td>
<td>Positive</td>
</tr>
</tbody>
</table>

<sup>a</sup>*H. aegyptius* is indistinguishable from *H. influenzae* biotype III in normal laboratory tests.

<sup>b</sup>Traditionally described as β-haemolytic on horse blood agar, but non-haemolytic strains exist<sup>13</sup>

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

The presence of capsule polysaccharide on typable strains 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<sup>56,57</sup>.

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 to 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).

### Commercial identification systems

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.

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

This has been shown to be a rapid and powerful tool because of its reproducibility, speed and sensitivity of analysis. The advantage of MALDI-TOF as compared with other identification methods is that the results of the analysis are available within a few minutes.
Identification of *Haemophilus* species and the HACEK group of organisms

minutes to hours rather than several days. The speed and the simplicity of sample preparation and result acquisition associated with minimal consumable costs make this method well suited for routine and high-throughput use\(^{58}\).

MALDI-TOF MS has been used to describe and characterise new specie, *Haemophilus sputorum*\(^3\). Members of the species yield a unique MALDI-TOF mass spectrum distinct from other related *Haemophilus* species.

MALDI-TOF MS can be used to accurately identify the HACEK organisms despite their fastidious nature\(^{13,59}\). This technique can also be used for rapid discrimination of *Haemophilus influenzae*, *H. parainfluenzae* and *H. haemolyticus*, although, there are suggestions of misidentifications of commensal *H. haemolyticus* as *H. influenzae*\(^{60}\). This could be resolved by the addition of a suitable *H. haemolyticus* reference spectrum to the system’s database as well as alternative tests being applied in case of ambiguous test results on isolates from seriously ill patients.

This has also been used to rapidly distinguish between *C. hominis* and *C. valvarum*\(^{61}\).

**Nucleic Acid Amplification Tests (NAATs)**

PCRs have been developed to detect *H. influenzae* and *H. parainfluenzae* in clinical specimens and some have been incorporated into commercial multi-pathogen detection systems. PCR methods are also used to confirm the species of *H. influenzae* isolates and serotype them\(^{56,57}\) [REFS]. They have also been developed to help discriminate non-typeable *Haemophilus influenzae* from non-haemolytic *H. haemolyticus*\(^ {62,9}\).

PCR has been used to identify *H. ducreyi* in clinical specimens. Orle et al. reported on the development of a commercial multiplex PCR assay that permits the simultaneous amplification of DNA targets from *H. ducreyi*, *T. pallidum*, and Herpes Simplex Virus types 1 and 2 directly from genital ulcer specimens\(^ {63}\).

16s rRNA PCR assay followed by sequencing and analysis has been used for rapid identification of difficult and serious infections due to fastidious microorganisms – *Cardiobacterium hominis*\(^ {64}\). In addition, this method can also be used to discriminate *C. hominis* from *C. valvarum*, which has recently been found to be responsible for endocarditis.

### 8.5 Further identification

**Rapid molecular methods**

Molecular methods have had an enormous impact on the taxonomy of *Haemophilus*. Analysis of gene sequences has increased understanding of the phylogenetic relationships of *Haemophilus* species and related organisms and has resulted in the recognition of numerous new species. Molecular techniques have made identification of many species more rapid and precise than is possible with phenotypic techniques.

A variety of rapid typing methods have been developed for isolates from clinical samples; these include molecular techniques such as Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), Ribotyping, and 16S rRNA gene sequencing. All of these approaches enable subtyping of unrelated strains, but do so with different accuracy, discriminatory power, and reproducibility.
Identification of *Haemophilus* species and the HACEK group of organisms

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.

**16S rRNA gene sequencing**

A genotypic identification method, 16S rRNA gene sequencing is used for phylogenetic studies and has subsequently been found to be capable of re-classifying bacteria into completely new species, or even genera. It has also been used to describe new species that have never been successfully cultured.

This has been used for better discrimination of closely related species such as *C. hominis* and *C. valvarum*. It has equally been used for identifying *Aggregatibacter* species.

**Ribotyping**

Ribotyping is based on restriction fragment length polymorphisms of rRNA genes, which are highly conserved and are usually present in multiple copies on the genome. Ribotyping does however present some disadvantages; it is labour intensive and requires costly enzymes and materials. Nevertheless, ribotyping provides a highly reproducible and reliable reference typing system.

This has been used to identify and characterise *H. ducreyi* and it was found to be highly reproducible and that it discriminated among strains of *H. ducreyi*. It may be used to study the epidemiology of *H. ducreyi* and chancroid.

This has also been used successfully in the identification of *H. influenzae* and may help to understand the molecular characteristics of outbreaks, endemicity and value of vaccination.

**Pulsed Field Gel Electrophoresis (PFGE)**

PFGE detects genetic variation between strains using rare-cutting restriction endonucleases, followed by separation of the resulting large genomic fragments on an agarose gel. PFGE is known to be highly discriminatory and a frequently used technique for outbreak investigations and has gained broad application in characterizing epidemiologically related isolates. However, the stability of PFGE may be insufficient for reliable application in long-term epidemiological studies. However, due to its time-consuming nature (30hr or longer to perform) and its requirement for special equipment, PFGE is not used widely outside the reference laboratories.

This has been used successfully to identify and discriminate between strains of non-typeable *Haemophilus influenzae*.

**Multilocus Sequence Typing (MLST)**

Multilocus sequence typing (MLST) is a tool that is widely used for phylogenetic typing of bacteria. MLST is based on PCR amplification and sequencing of internal fragments of a number (usually 6 or 7) of essential or housekeeping genes spread around the bacterial chromosome. A scheme was developed for *H. influenzae* and has been extensively used, not only within this species, but also more widely across the *Haemophilus* genus. For example, a version of MLST was used to describe the new species *H. pittmaniae* and to also separate *H. haemolyticus* and *H. influenzae* into distinct clusters using concatenated sequences of multiple genes, including the 16S rRNA gene.
8.6 Storage and referral
If required, save pure isolate on a chocolate agar slope for referral to the Reference Laboratory.

9 Reporting

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

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

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

9.4 Health Protection Team (HPT)
Refer to local agreements in devolved administrations.

9.5 Public Health England
Refer to current guidelines on SGSS reporting.

9.6 Infection prevention and control team
N/A

10 Referral to reference laboratories
For information on the tests offered, turnaround times, transport procedure and the other requirements of the reference laboratory see the Specialist and reference microbiology: laboratory tests and services page on GOV.UK for user manuals and request forms.

Organisms with unusual or unexpected resistance, or associated with a laboratory or clinical problem, or an anomaly that requires investigation should be sent to the appropriate reference laboratory.
Identification of *Haemophilus* species and the HACEK group of organisms

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

England and Wales

Scotland
[https://www.hps.scot.nhs.uk/a-to-z-of-topics/reference-laboratories/](https://www.hps.scot.nhs.uk/a-to-z-of-topics/reference-laboratories/)

Northern Ireland

*Haemophilus influenzae*

Vaccine Preventable Bacteria Section
Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU)
Public Health England
61 Colindale Avenue
London
NW9 5EQ

Telephone: +44 (0) 020 8327 7887

**HACEK group and *Haemophilus* species for identification**

Bacterial Identification Section (BIDS)
Antimicrobial Resistance and Healthcare Associated Infections Reference Unit (AMRHAI)
Public Health England
61 Colindale Avenue
London
NW9 5EQ

Telephone: +44 (0) 20 8327 6511 / 7887
Identification of *Haemophilus* species and the HACEK group of organisms

Appendix 1: Identification of *Haemophilus* species

**Clinical Specimens**

*Primary isolations plate*

Blood or chocolate agar incubated in 5-10% CO₂ at 35-37°C for 24-48 hr

*Haemophilus* species are small, round, convex, colourless to grey colonies and may be iridescent. Some *H. haemolyticus* have β-haemolytic colonies.

**Gram’s stain on pure culture**

Gram- negative spherical, oval or rod shaped cells with marked pleomorphism or filament formation

**Growth for X and V factors**

(TP 29 or TP 38)

- Positive
- Negative

- *H. parainfluenzae*
- *H. haemolyticus*
- *H. paraphrohaemolyticus*
- *H. sputorum*

- *H. influenzae*
- *H. aegyptius*
- *H. ducryi*
- *H. parahaemolyticus*
- *H. massiliensis*

**Oxidase**

(TP 26)

- Positive
- Negative

- *H. influenzae*
- *H. aegyptius*
- *H. ducryi*
- *H. parahaemolyticus*
- *H. massiliensis*

**Catalase**

(TP 8)

- Positive
- Negative

- *H. influenzae*
- *H. aegyptius*
- *H. ducryi*
- *H. parahaemolyticus*
- *H. massiliensis*

**Urease**

(TP 36)

- Positive
- Negative

- *H. influenzae*
- *H. aegyptius*
- *H. ducryi*
- *H. parahaemolyticus*
- *H. massiliensis*

Further ID if clinically indicated. Refer to the appropriate Reference laboratory.

This flowchart is for guidance only.

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UK Standards for Microbiology Investigations | Issued by the Standards Unit, Public Health England
Appendix 2: Identification of HACEK group

Clinical specimens
Primary isolation plate

Blood agar

Incubate aerobically at 35-37°C for 24-48hr

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

Catalase positive
Oxidase positive
Urease negative

A. actinomycetemcomitans

Grows in air + CO₂
Yellowish non-haemolytic colonies
1.5mm at 24hr

Catalase negative
Oxidase negative
Urease negative

A. aphrophilus

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

Catalase negative
Oxidase positive
Urease negative

C. hominis
C. valvarum

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

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

All Kingae species

This flowchart is for guidance only.

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For the information for the evidence grade ratings given, refer to the scientific information link above in section 2.

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17. Lo CI, Sankar SA, Fall B, Sambe-Ba B, Diawara S, Gueye MW et al. High-quality draft genome sequence and description of *Haemophilus massiliensis* sp. nov. Stand Genomic Sci 2016;11:31-.


26. Dewhirst FE, Paster BJ, La Fontaine S, Rood JI. Transfer of *Kingella* indologenes (Snell and Lapage 1976) to the genus *Suttonella* gen. nov. as *Suttonella indologenes* comb. nov.; transfer of *Bacteroides nodosus* (Beveridge 1941) to the genus *Dichelobacter* gen. nov. as *Dichelobacter nodosus* comb. nov.; and assignment of the genera *Cardiobacterium*, *Dichelobacter*, and *Suttonella* to *Cardiobacteriaceae* fam. nov. in the gamma division of *Proteobacteria* on the basis of 16S rRNA sequence comparisons. IntJSystBacteriol 1990;40:426-33.


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


36. European Parliament. UK Standards for Microbiology Investigations (UK SMIs) use the term "CE marked leak proof container" to describe containers bearing the CE marking used for the collection and transport of clinical specimens. The requirements for specimen containers are given in the EU in vitro Diagnostic Medical Devices Directive (98/79/EC Annex 1 B 2.1) which states: "The design must allow easy handling and, where necessary, reduce as far as possible contamination of, and leakage from, the device during use and, in the case of specimen receptacles, the risk of contamination of the specimen. The manufacturing processes must be appropriate for these purposes". 1998. A, V


42. Advisory Committee on Dangerous Pathogens. The Approved List of Biological Agents. Health and Safety Executive 2013. 1-35. A, V


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