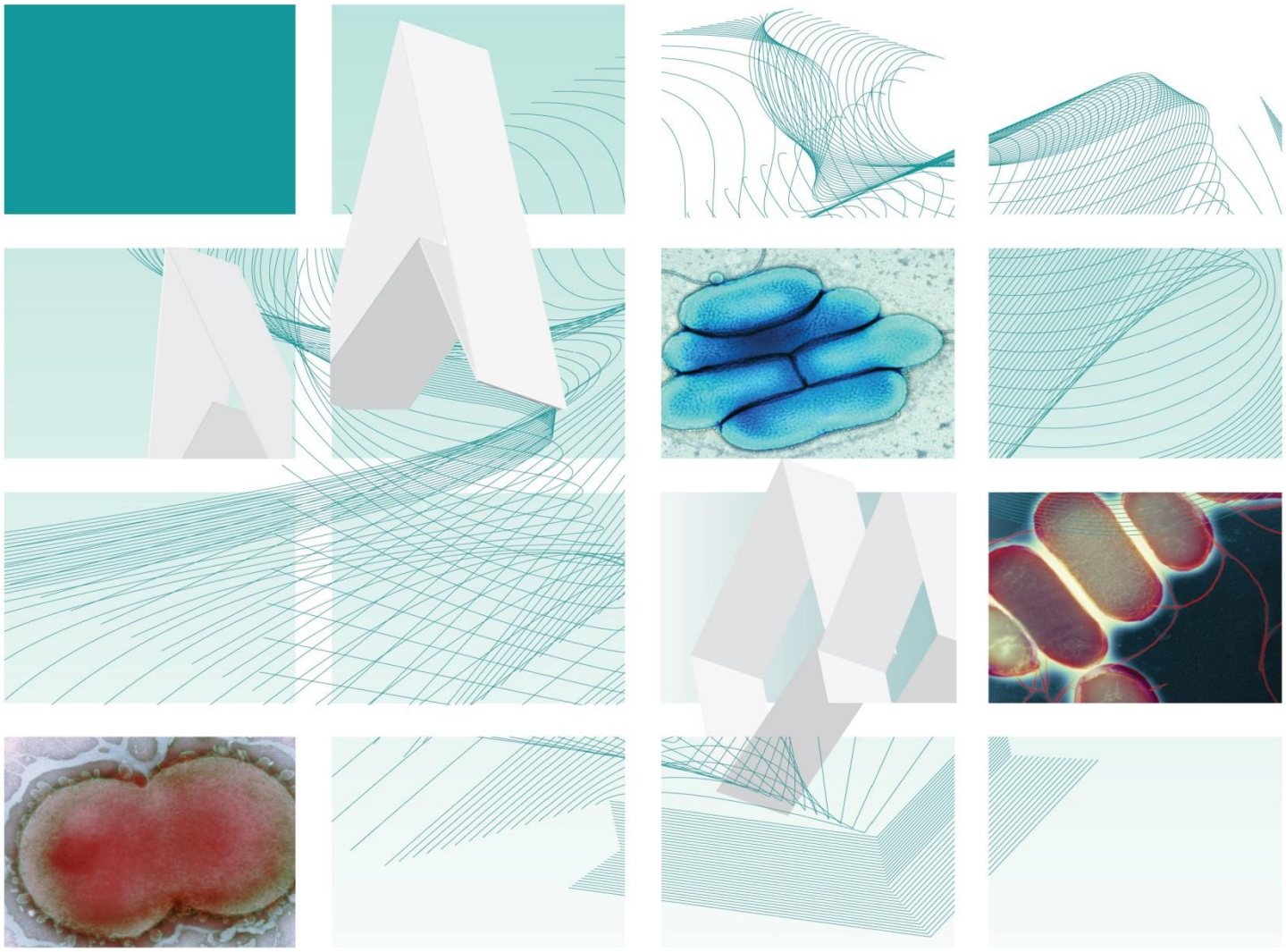
UK Standards for Microbiology Investigations

Identification of *Bordetella* species



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Contents

Amendment table

Each UK SMI method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@phe.gov.uk.

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

Amendment number/date	11/10.08.2020
Issue number discarded	3.1
Insert issue number	4
Anticipated next review date*	10.08.2023
Section(s) involved	**Amendment**
4.1, 7, Appendix	Updated list of *Bordetella* species to include *B. bronchialis, B. flabilis and B. sputigena*
All	Document updated to new Identification template
4.1	Updated information on characteristics of, and antimicrobial resistance in, *B. pertussis*
7.5	Added information on whole genome sequencing; removed reference to pulsed field gel electrophoresis

*Reviews can be extended up to five years subject to resources available.

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 *Bordetella* species and importantly, the two associated with pertussis (whooping cough) in humans: *Bordetella pertussis* and *Bordetella parapertussis,* isolated from clinical specimens to species level. Refer to B 6 - Investigation of specimens for *Bordetella pertussis* and *Bordetella parapertussis* for information.

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 UK SMIs.

4 Introduction

4.1 Taxonomy/characteristics

There are currently sixteen species in the genus Bordetella, twelve of which are associated with potential infection in humans, albeit rarely in some cases[1-3]. Classical Bordetella species are *Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica.* Non-classical Bordetella species include *Bordetella hinzii, Bordetella holmesii, Bordetella trematum, Bordetella avium, Bordetella petrii, Bordetella broncialis, Bordetella flabilis, Bordetalla sputigena* and four other species not reported to be associated with human infection. One further species '*Bordetella ansorpii*' is still awaiting valid publication[1].

Bordetella species are Gram negative coccobacilli 0.2-0.5 x 0.5-2.0µm. Microscopically they appear arranged singly or in pairs and rarely in chains[4]. They often exhibit bipolar staining. Cells may be motile or non-motile. They are strictly aerobic (with the exceptions of *B. petrii*, *B. bronchialis, B. flabilis* and *B. sputigena*) and the optimum temperature is 35-37°C. Colonies on plates appear smooth, convex, pearly, glistening, nearly transparent and surrounded by a zone of haemolysis without definite periphery. The metabolism is respiratory and never fermentative. Species of *Bordetella* require nicotinamide, amino acids and organic sulphur eg cysteine. *Bordetella* species oxidatively utilise glutamic acid, proline, alanine, aspartic acid and serine with production of ammonia and CO_2[5].

Bordetella pertussis

B. pertussis may grow on *Bordetella* selective medium (charcoal blood agar with cefalexin) within three days, but normally 5-7 days incubation is required for primary isolation. Plates should be incubated for 7 days before being discarded as negative[6].

Growth on subculture usually requires shorter incubation (3 days). Colonies are smooth, convex, pearly, glistening, greyish-white and have a butyrous consistency. *B. pertussis* does not grow on nutrient agar or MacConkey agar and grows poorly on blood agar. *B. pertussis* is weakly oxidase positive and is non-motile. They are also urease negative and agglutinate to the *B. pertussis* polyvalent antiserum and weakly or not at all with *B. parapertussis* polyvalent antiserum, depending on how thoroughly it has been cross-absorbed[6].

To date, antimicrobial resistance in Bordetella species has not been reported in the UK, and susceptibility testing is not performed routinely. Although macrolide resistance has been reported in several countries, reported rates vary from high (China[7], Iran[8]) to low and zero in various studies (Finland[9]). A case of acquisition of macrolide resistance during treatment has been reported by Guillot et al[10]. Phenotypic antimicrobial resistance testing of *B. pertussis* is complicated by the slow growth of the organism and poor growth on some media[6]. Molecular methods have been shown as a useful tool for screening *B. pertussis* for macrolide resistance[9].

Isolates of *B. pertussis* should be referred to the National Reference Laboratory, Vaccine Preventable Bacteria Section, Public Health England – National Infection Service, Colindale, London for confirmation, serotyping and further epidemiological studies. *B. pertussis* has three major surface agglutinogens (1, 2 and 3), which are detectable by bacterial agglutination with cross-absorbed antisera. There are three serotypes which can cause human disease (1,2; 1,3; 1,2,3). Currently the least common is 1,2,3[6]. Type 1,3 remains the predominant type and accounts for most isolates[4].

B. pertussis has historically been described as a non-motile and non-flagellated organism; however, it has been reported that laboratory-adapted strains and clinical isolates can be motile, and flagellum-like structures have been found in such strains[11].

Bordetella parapertussis

Colonies of *B. parapertussis* are similar to *B. pertussis* but are larger, duller and become visible sooner. They grow rapidly and can appear on agar plates within 2 to 3 days. Unlike *B. pertussis,* it grows on nutrient agar giving a brown discoloration of the medium after several days.

B. parapertussis is non-motile, oxidase negative and urease positive. They are agglutinated by *B. parapertussis* polyvalent antiserum and slowly, if at all, by *B. pertussis* antiserum[6].

Bordetella bronchiseptica

Colony morphology of this organism ranges from smooth to rough when grown on agar plates. On agar media containing blood, it exhibits glistening β-haemolytic colonies and develops an average diameter of 2.0mm in 1 to 2 days. Colonies equally grow well on MacConkey agar. They are oxidase positive and motile by peritrichous flagella. They are also nitrate and urease positive (usually within 4hr) which is a distinguishing factor from *B. pertussis*[12].

Bordetella ansorpii

They grow on both blood and MacConkey agar. They are negative for oxidase, urease, nitrate reduction, esculinase, mannitol and arginine dihydrolase but positive for citrate, adipate, malate, gelatinase activity and motility[1].

Bordetella trematum

B. trematum cells are motile by means of peritrichous flagella. Motility does not differ significantly when cells are grown at 25, 30, or 37°C. In 16-24hr cultures on blood agar, the average cell is 0.5 to 0.6µm wide and 1 to 1.8µm long; the longest rods are up to 2.4µm long. They produce convex, circular, and greyish cream white colonies with entire edges on blood agar. They do not require special growth factors and grow on conventional media. Growth is not inhibited at an incubation temperature of 42°C, but is reduced markedly at 25°C. Strains grow microaerobically, but not anaerobically. Colonies grown on transparent Diagnostic Sensitivity Test agar at 37°C for 16 to 24hr exhibit greenish yellow to yellow-red iridescence in obliquely transmitted light under a stereomicroscope[13].

They are negative for oxidase, urease activity, glucose fermentation, but give variable results when tested for nitrate reduction and this depends on the strain[13].

Bordetella holmesii

They are small coccoid and short rods, with medium-width longer rods occasionally observed. On blood agar, colonies are punctuate, semiopaque, convex, and round with complete edges. A zone of browning or greening of the media is observed. They are oxidase negative, non-motile, asaccharolytic, fastidious and they produce a brown soluble pigment. They do not grow on Simmons Citrate agar but grow on MacConkey agar plates at 3-7 days after incubation of 35°C[14]. They are negative for motility, aerobic growth at 25°C and at 42°C, urease activity, glucose fermentation but positive for arginine, proline and leucyl glycine[13].

Bordetella hinzii

The cells are motile by means of peritrichous flagella. Two distinct colony types occur. Some strains on blood agar plates show round, convex, glistening, greyish colonies about 1 to 2mm in diameter after 24-48hr of incubation at 37°C in air containing 5% CO_2. Under the same conditions, other strains produce flat, dry, crinkled colonies that are up to 5mm in diameter[15]. *Bordetella hinzii* also grows on MacConkey agar, and are positive for catalase, oxidase and assimilation of citrate adipate, L-malate and phenylacetate. They give variable results for urease production and do not reduce nitrates[1]. They are also negative for glucose fermentation and they grow aerobically at 25°C and 42°C[13].

Bordetella petrii

These are characterized by an ability to grow in aerobic, microaerophilic and anaerobic conditions. Cells possess fimbriae of different diameters. The organism can be cultured on MacConkey agar and appears as creamy white non-haemolytic colonies on blood agar. They are asaccharolytic, non-fermenting bacteria. They are positive for oxidase and tetrazolium reduction tests; and have negative reaction for urease production, nitrate reduction and motility. They can assimilate citrate, adipate, L-malate and D-Gluconate[16].

They are susceptible to erythromycin, gentamicin, ceftriaxone, and piperacillin/tazobactam and are resistant to amoxicillin, co-amoxiclav, tetracycline, clindamycin, ciprofloxacin and metronidazole[17].

Bordetella avium

These are non-lactose fermenting, small rods that are characterized by the ability to grow in aerobic conditions. They can grow on trypticase soy agar supplemented with 5% sheep blood, chocolate agar and MacConkey agar incubated at 35°C in 5% CO_2. They appear as non-haemolytic colonies on blood agar[18]. They are positive for motility, oxidase, catalase and tetrazolium reduction tests; and negative for nitrate reduction and urease production. They can assimilate L-malate, adipate and phenylacetate although some strains may exhibit a weak reaction for L-malate and adipate[13,16].

Bordetella bronchialis[19]

Small motile rods. Oxidase and catalase positive showing an aerobic respiratory metabolism. Leucine arylamidase activity is present and growth is observed at 30 and 37 °C on blood agar. Strains are non-haemolytic on blood agar.

B. bronchialis will grow in the presence of 3.0% NaCl, but does not grow on cetrimide agar. Strains exhibit alkaline phosphatase, acid phosphatase and phosphoamidase activity, but do not exhibit C4-lipase activity. Anaerobic growth occurs at 28 °C.

Weak assimilation of adipate and D-glucose may be seen when using commercial identification systems, but not when using a traditional biochemical test. Assimilation of L-malate and phenylacetate is not observed.

Bordetella flabilis[19]

Cells are Gram-stain-negative, small, motile rods (about 0.2 µm wide and 1.2 µm long) with rounded ends that occur as single units or in pairs. They are oxidase and catalase positive showing an aerobic respiratory metabolism. Leucine arylamidase activity is present and growth is observed at 30 and 37 °C on blood agar. *B. flabilis* does not show haemolysis on blood agar and does not grow on cetrimide agar or in the presence of 3.0 % NaCl. Anaerobic growth occurs at 28 °C.

After 72 h of incubation on trypticase soy agar at 28 °C, colonies are slightly convex, translucent and non-pigmented, with smooth margins, and 0.5 to 1.0 mm in diameter.

Assimilation of adipate is seen when analysed using a traditional biochemical test but may not be seen when using commercial identification systems. Strains do not assimilate L-malate, phenylacetate or D-glucose. Weak acid phosphatase activity is seen, but no alkaline phosphatase, phosphoamidase or C4-lipase activity.

Bordetella sputigena[19]

Cells are Gram-stain-negative, small, motile rods (about 0.2 µm wide and 1.2 µm long) with rounded ends that occur as single units or in pairs. They are oxidase and catalase positive showing an aerobic respiratory metabolism. Leucine arylamidase activity is present and growth is observed at 30 and 37 °C on blood agar. *B. sputigena* shows weak haemolysis on blood agar, grows weakly on cetrimide agar and grows in the presence of 3.0 % NaCl. Anaerobic growth occurs at 28 °C.

After 72 h of incubation on trypticase soy agar at 28 °C, colonies are slightly convex, translucent and non-pigmented, with smooth margins, and 0.5 to 1 mm in diameter.

Traditional biochemical testing shows assimilation of adipate, L-malate and phenylacetate. Commercial identification systems may show assimilation of adipate,

but not assimilation of L-malate and phenylacetate. In both cases D-glucose is not assimilated. C4-lipase and weak acid phosphatase activity is seen; no alkaline phosphatase or phosphoamidase activity is seen.

4.2 Principles of identification

Colonies isolated on *Bordetella* selective agar are identified preliminarily by colonial appearance, Gram stain and slide agglutination with polyvalent antiserum. Biochemical and other additional tests are used to distinguish between species of the genus *Bordetella* and to differentiate *Bordetella* from similar organisms.

Presumptive and confirmed positive isolates of *B. pertussis and B. parapertussis* should be referred to the National Reference Laboratory, Vaccine Preventable Bacteria Section, Public Health England – National Infection Service, Colindale, London.

Full molecular identification using for example, PCR and Matrix Assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) can be used to identify isolates to species level.

All PHE laboratories offering *B. pertussis* PCR testing should refer all positive samples to PHE Colindale for surveillance purposes.

For information, contact the laboratory or see the Bacteriology Reference Department user manual for details: https://www.gov.uk/government/publications/bacteriology-reference-department-brd-user-manual.

5 Technical information/limitations

Agar media

Plates should be incubated aerobically in a moist chamber for 5 to 7 days at 35 to 37°C. Do not incubate in an aerobic atmosphere enriched with carbon dioxide.

Cefalexin is included into the Charcoal media as an inhibitor of many Gram positive and certain Gram negative bacteria present in the normal throat flora, but is not completely inhibitory to all organisms. Growth of *B. pertussis* is slightly retarded on cefalexin-containing media. Some strains of *B. pertussis* are said to be inhibited by cefalexin; therefore, the use of both selective and non-selective media has been advocated[20].

Another selective media, modified Cyclodextrin Solid Medium (MCS) with cefdinir can be used. It has shown to improve the selective isolation of *B. pertussis* from clinical specimens, exhibit higher sensitivity and greater inhibition of nasopharyngeal flora than the media with cefalexin. A long shelf life is another benefit of this medium as most clinical microbiology laboratories are infrequently required to culture specimens from pertussis patients[21]. The cost of the MCS medium is similar to that of other media used.

6 Safety considerations[22-36]

Hazard Group 2 organisms.

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

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

If a swab is used to harvest growth from a plate and emulsify it in saline for agglutination tests, a risk of infection may result, and should be included in local risk assessments.

In the case of sputa, or other lower tract respiratory material, where there is a risk that these samples may contain viable *Mycobacterium tuberculosis* (MTB), all work must be carried out in a Containment Level 3 facility.

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

Compliance with postal and transport regulations is essential.

7 Target organisms

***Bordetella* species reported to have caused pertussis[4] -** *Bordetella pertussis, Bordetella parapertussis*

Other *Bordetella* species reported to have caused infections in humans - *Bordetella bronchiseptica*[37], *Bordetella trematum*[13], *Bordetella hinzii*[15], *Bordetella holmesii*[14], *'Bordetella ansorpii'*[1], *Bordetella petrii*[17], *Bordetella avium*[18], *Bordetella bronchialis*[19], *Bordetella flabilis*[19], *Bordetella sputigena*[19].

7.1 Microscopic appearance

Gram stain (TP 39 - Staining procedures)

Gram negative, thin coccobacilli occurring singly or in pairs, rarely in chains. Some strains may be capsulated.

7.2 Primary isolation media

Charcoal selective agar, incubated aerobically with high humidity and good circulation of air, for 7 days at 35°C-37°C is used for primary isolation. However, extending plate incubation up to 12 days has shown improved recovery of *Bordetella* species from clinical specimens[38].

7.3 Colonial appearance

Colonies of *B. pertussis* on charcoal blood agar with cefalexin are smooth, convex, pearly and glistening, greyish-white and butyrous and appear in 3 days on subculture, longer on primary isolation. Colonies of *B. parapertussis* are similar but larger, duller and become visible within two days. On subculture to nutrient agar, *B. parapertussis* colonies produce a brown pigment, which diffuses into the medium. *B. pertussis* does not grow on nutrient agar.

7.4 Test procedures

7.4.1 Biochemical tests

Oxidase test (TP 26 - Oxidase test)

B. parapertussis is oxidase negative, *B. pertussis* is oxidase positive.

Agglutination (slide) with specific antiserum

Follow manufacturer's instructions and kits should be validated and be shown to be fit for purpose prior to use. A suspension of the suspect colony should be prepared in saline on a microscope slide. Specific *B. pertussis* antiserum, *B. parapertussis* antiserum or saline should be added to the suspensions and mixed.

A positive result is indicated by agglutination with one specific antiserum and no agglutination with saline. If the agglutination result is equivocal, refer the isolate.

Refer isolates of suspected *B. pertussis* and *B. parapertussis* to the Respiratory and Vaccine Preventable Bacteria Reference Unit for further characterisation.

7.4.2 Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS)

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 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[39].

This technique has been used to produce rapid and reliable species-level identification for the non-classical *Bordetella s*pecies, as in the case of endocarditis on a prosthetic homograft aortic valve caused by *Bordetella holmesii* where routine laboratory testing initially misidentified the strain as *Acinetobacter* species but 16S rRNA gene and outer membrane protein A (*ompA*) gene sequencing and identification by MALDI-TOF MS were all consistent with *B. holmesii*[40].

7.4.3 Nucleic Acid Amplification Tests (NAATs)

PCR is usually considered to be a good method as it is simple, sensitive and specific. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by amplification of specific genetic targets. Careful interpretation of PCR results may be required to identify a specific *Bordetella* species, as frequently used amplification targets are present in multiple *Bordetella* species[41].

This is an invaluable tool both for enhanced epidemiological surveillance and for the provision of a rapid diagnosis of pertussis where results can affect patient (and contact) management. This has been used successfully in the identification of *Bordetella pertussis*[41,42]. This service is offered for patients of all ages (including those in primary care) by PHE Specialist Microbiology Network laboratories.

7.5 Further identification

Rapid molecular methods

Molecular methods have had an enormous impact on the taxonomy of *Bordetella*. Analysis of gene sequences has increased understanding of the phylogenetic relationships of *Bordetella* species and this has resulted in the recognition of 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 Multiple-locus Variable-Number Tandem Repeat Analysis (MLVA) and16S rRNA gene sequencing. All of these approaches enable subtyping of unrelated strains, but do so with different accuracy, discriminatory power, and reproducibility.

However, some of these methods remain accessible to reference laboratories only and are difficult to implement for routine bacterial identification in a clinical laboratory.

Whole Genome Sequencing (WGS)

This is also known as full genome sequencing, complete genome sequencing, or entire genome sequencing. It is a laboratory process that determines the complete DNA sequence of an organism's genome at a single time. There are several high throughput techniques that are available and used to sequence an entire genome such as pyrosequencing, nanopore technology, IIlumina sequencing, Ion Torrent sequencing, etc. This sequencing method holds great promise for rapid, accurate, and comprehensive identification of bacterial transmission pathways in hospital and community settings, with concomitant reductions in infections, morbidity, and costs

Multiple-locus Variable-Number Tandem Repeat Analysis (MLVA)

MLVA is a robust, simple, and portable method which can be used to create strain profiles that are easily electronically exchanged. MLVA has been successfully used to type several different bacterial species and proven to be an excellent method with high resolution, particularly useful for organisms with a low level of sequence diversity.

This new approach, MLVA Typing was introduced and this is used to analyse the number of tandem repeat sequences in the *B. pertussis* genome[43,44]. This technique does not require culturing and can be applied directly to nasal or pharyngeal swabs. Variable-number tandem repeat (VNTR) analysis has revealed considerable heterogeneity of the *B. pertussis* genome and clonal expansion during epidemic periods.

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 method has been used recently for the accurate identification of the non-classical *Bordetella* species (ie not including *B. pertussis*, *B. parapertussis* or *B. bronchiseptica*) as in the first case of fatal sepsis caused by *Bordetella hinzii*[45].

The greater mutational variation of the *Bordetella* outer membrane protein A gene (*ompA*) gene compared to the 16S rRNA gene allows unambiguous identification of

the non-classical *Bordetella* species. However, it should be noted that the 16S rRNA gene sequences of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are identical so this method cannot be used to provide a species identification within these 3 species. This is also the case for the *ompA* sequence of these 3 species[46].

7.6 Storage and referral

Save pure isolates on a charcoal blood agar slope for referral to the Reference Laboratory. The slope may require several days incubation before adequate growth is achieved.

8 Reporting

8.1 Infection Specialist

Certain clinical conditions must be notified to the laboratory associated infection specialist, including presumptive and confirmed *B. pertussis* or *B. parapertussis* isolates in accordance with local protocols.

Follow local protocols for reporting to the patient's clinician.

8.2 Preliminary identification

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

8.3 Confirmation of identification

Following the Reference Laboratory report.

8.4 Health Protection Team (HPT)

Refer to local agreements in devolved administrations.

8.5 Public Health England

Refer to current guidelines on SGSS reporting.

"Whooping cough" is a Notifiable disease, for public health management of cases, contacts and outbreaks, all suspected cases should be immediately notified to the local Public Health England Centres.

All clinically significant isolates should be notified by the diagnostic laboratories to ensure urgent initiation of proper procedures.

8.6 Infection prevention and control team

Inform the hospital infection prevention and control team of presumptive and confirmed *B. pertussis* or *B. parapertussis* isolates from hospital inpatients. Other isolates should be reported to the relevant Infection Control Staff in accordance with local protocols, notably if an outbreak is suspected.

9 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.

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

https://www.gov.uk/specialist-and-reference-microbiology-laboratory-tests-and-services

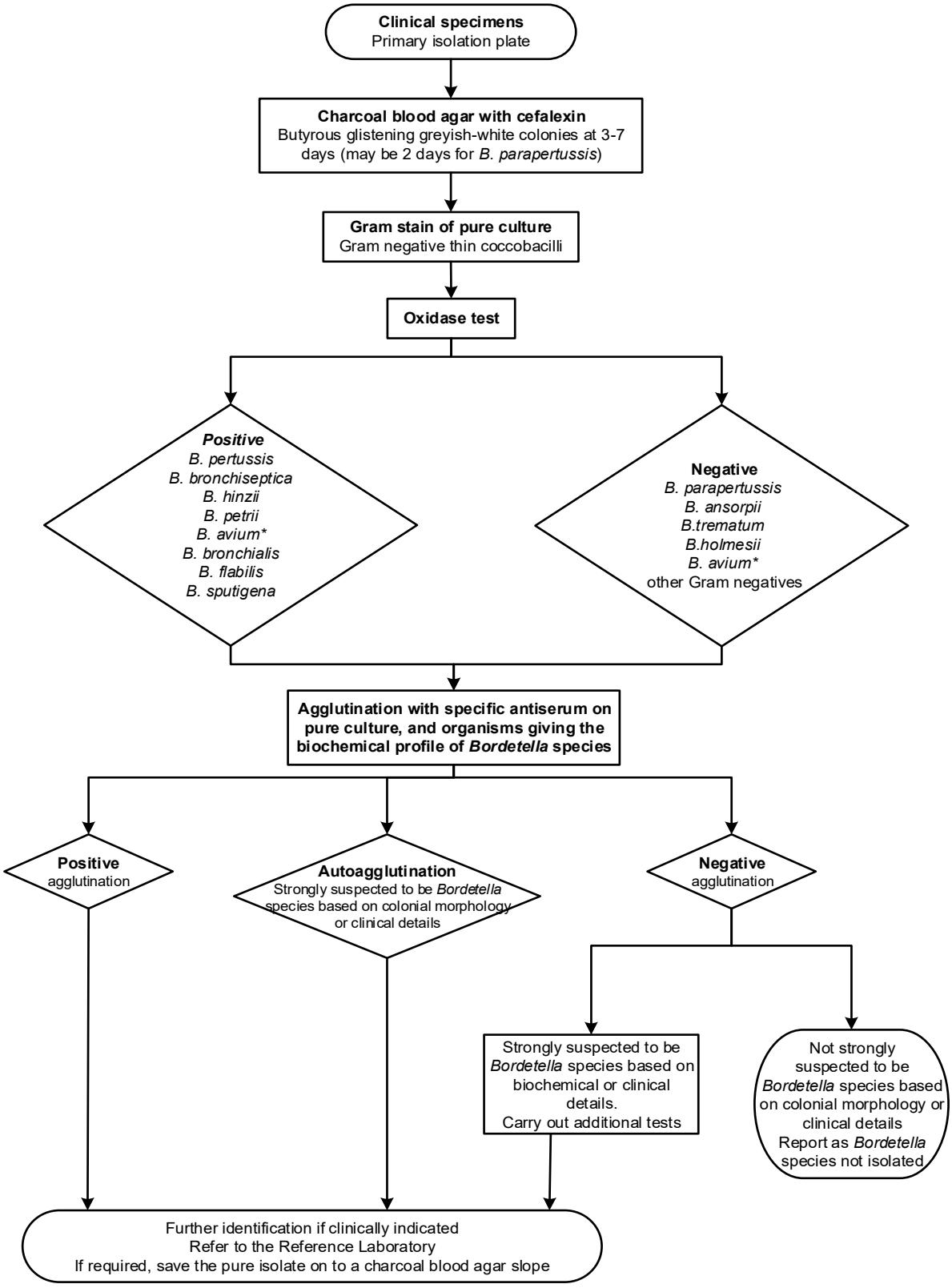
Scotland

https://www.hps.scot.nhs.uk/a-to-z-of-topics/reference-laboratories/

Northern Ireland

http://www.publichealth.hscni.net/directorate-public-health/health-protection

Appendix: Identification of *Bordetella* species



*_B. avium_ is positive when Kovac's oxidase reagent is used and negative when Gaby and Hadley reagents are used.

The flowchart is for guidance only.

References

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

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