Whooping cough (*Bordetella pertussis*)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for *Bordetella pertussis*.

Authorisation: PHLN
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1 PHLN Summary Laboratory Definition

1.1 Condition:

Whooping Cough

1.1.1 Laboratory Definitive Criteria

- Isolation of *Bordetella pertussis*; OR
- Detection of *B. pertussis* by nucleic acid amplification test (NAAT) OR
- Seroconversion in paired sera for *B.pertussis* using whole cell or specific *B.pertussis* antigen(s) in the absence of recent pertussis vaccination

1.1.2 Laboratory Suggestive Criteria

- In the absence of recent vaccination
- Significant change (increase or decrease) in antibody level (IgG, IgA) to *B. pertussis* whole cell or B.pertussis specific antigen(s) OR
- Single high IgG and or IgA titre to Pertussis Toxin (PT) OR
- Single high IgA titre to Whole Cell *B.pertussis* antigen

1.1.3 Special Considerations / Guide for Use

- Although *B.pertussis* PCR is regarded as definitive evidence of infection the majority of laboratories cannot reliably distinguish *B.pertussis* from *B.holmseii* unless dual targets are used. This practice is recommended. Enunciation of the targets selected and limitations of the assay should be included in interpretive comments.
- Whole cell *B.pertussis* IgG is not useful. Positive results should not be considered evidence of current infection.

- International WHO standards for PT, Filamentous Haemagglutinin (FHA) and pertactin (PRN) IgG and IgA are now available which will enable standardisation of appropriate cut-offs for positivity in future serological assays.[1]
- The best choice of test (culture, NAAT or serology or a combination of these) relates primarily to duration of symptoms at the time of presentation. (See section 3.5)

2 Introduction

Pertussis is caused by *Bordetella pertussis*, a fastidious, gram negative cocco-bacillus, which was first isolated in 1906 by Bordet and Gengou. It is restricted to the respiratory tract of humans and is spread by droplets from person to person. *B. pertussis* produces a range of virulence factors including pertussis toxin and pertactin which cause the typical disease symptoms. The clinical spectrum is diverse and is affected by patient age, previous exposure to the organism, immunisation history, antibiotic administration and concomitant infections with other agents. Clinical expression ranges from asymptomatic infection in children and adults with strong residual immunity to more severe and life-threatening disease in unprotected newborns and infants. Although some previously immunized individuals do develop classical clinical symptoms, atypical pertussis, characterized by the absence of typical whoop and a shorter duration of cough, is more common among adolescents and adults. *Bordetella parapertussis* causes a pertussis syndrome similar to but usually less severe than that caused by *B.pertussis.* Symptomatic *B.parapertussis* infections more commonly present as a nonspecific cough illness or bronchitis. *B.bronchiseptica* and *B.holmesii* have been implicated as infrequent causes of pertussis syndrome and other respiratory illnesses.[2,3,4,5]

Definitive laboratory diagnosis is made by isolation of *B. pertussis* from respiratory specimens but culture is insensitive as the disease progresses and there are fewer organisms present after the first 2–3 weeks of symptoms. Nucleic acid amplification testing (NAAT) usually by polymerase chain reaction (PCR) is more sensitive than culture and is accepted as definitive laboratory evidence of disease in cases with an appropriate clinical history. Serology is used extensively in Australia for diagnosis of pertussis, particularly in adults and adolescents. Many other countries including the USA do not accept a diagnosis of pertussis based on serology although with improved standardisation there have been recent calls to incorporate serological testing in the case definition.[6]The direct fluorescence assay (DFA) has been used in the past but has been now superseded by PCR which offers a more sensitive and specific diagnosis.[3]

Recommended laboratory methods for *B. pertussis* are detailed on the Centers for Disease Control (CDC) website http://www.cdc.gov/vaccines/pubs/surv-manual/chpt10-pertussis.htm and the WHO case definition is outlined at http://www.who.int/immunization_monitoring/diseases/pertussis_surveillance/en/

3 Tests

3.1 Culture

Culture should be attempted, if possible, to allow future typing of an isolate. This allows the monitoring of changes in circulating endemic strains including variation in virulence genes, which could affect vaccine efficacy.[7,8,9] Cultures are also important for the regular monitoring of antibiotic susceptibility or in the unusual circumstance of treatment failure. Currently macrolide resistance is rare.

3.1.1 Suitable and unsuitable specimens

B.pertussis exhibits tropism and binds specifically to ciliated respiratory epithelial cells. Therefore nasopharyngeal aspirates, nasopharyngeal swabs (obtained using flexible shafted swabs with a calcium alginate, Dacron™ or flocked but not rayon or cotton) are recommended. Ideally the swab is left in the posterior pharynx for 10 seconds before being withdrawn.. Both left and right nasopharynx may be sampled with the same swab. In young children, nasal wash specimens may be satisfactory. Throat swabs are less sensitive and anterior nasal swabs and sputum are unacceptable specimens for culture.[2],[3]

3.1.2 Transport

Since *B. pertussis* is a fastidious organism, specimens should be plated onto culture media immediately, preferably at the bedside. Amies transport medium with charcoal is suitable if specimens can be plated out within 24 hours of collection. Special transport media (such as Regan-Lowe medium) containing half strength charcoal agar supplemented with horse blood and cephalexin (40mg/L) are useful where the delay is ≤ 3 days. Transport should occur at room temperature.[3]

3.1.3 Media

Bordet and Gengou used a medium containing blood, glycerine and potato extract for their original isolation and variations of this medium, subsequently called Bordet-Gengou medium (BGA), continue to be used worldwide. CDC recommends a variant which uses 10% defibrinated horse blood and cephalexin as a supplementary primary isolation medium.[2] *B. pertussis* is an aerobe and agar plates should be incubated at 35–36°C, usually for a maximum of 7 days, in air (high humidity) rather than CO_2. Plates should not be allowed to dry out. *B. pertussis* colonies take 3 to 4 days to develop characteristic "half pearl" colonies. They are small, gram-negative, catalase positive coccobacilli. *B. pertussis* is urease negative, unlike *B. parapertussis* and *B. bronchiseptica*. The oxidase reaction is also helpful in differentiating *B. pertussis* (positive) from *B. parapertussis* (negative). Most laboratories use specific antisera to identify *B. pertussis* and *B. parapertussis*.

3.1.4 Antibiotic susceptibility testing

While routine MIC testing of *B. pertussis* is not warranted, isolates from patients who appear to have failed an appropriate course of macrolide therapy should be screened for resistance, and those that appear resistant should be tested by the agar dilution reference MIC method on BGA.[11] MIC to erythromycin of < 0.12 mg/L indicate in vitro susceptibility. The mean MIC of Australian isolates collected between 1971 and 2006 remained between 0.035 and 0.045 mg/L.[12]

3.1.5 Test sensitivity

Cultures are variably positive (30%–50%) and may rarely may take as long as 2 weeks although most laboratories hold cultures for 7 days.
Successful isolation depends on:

- Stage of the illness – the highest sensitivity is at the end of the incubation period, during the catarrhal stage and at the beginning of the paroxysmal stage. Lower sensitivity occurs if specimens are collected during paroxysmal stage between 3–6 weeks and negative during convalescence. The organism is rarely recovered after the fourth week of illness. The success rate is low (0–30%) for adults who typically present late in disease. Bacterial load is higher in children.
- Quality of the specimen collection – nasopharyngeal aspirates are marginally more sensitive than nasopharyngeal Dacron ™ swab collections. More recently, flocked swabs have become

available and provide improved specimen collection. Anterior nose and throat swabs and classical "cough plates" are no longer recommended for culture.

- Speed of plating and quality of the medium – specimens should be plated immediately (at least within 4 hours after collection) onto high quality non-selective media designed for *B. pertussis* isolation.
- Prior antibiotic treatment will negatively affect sensitivity.

3.1.6 Test specificity

100% for *B.pertussis*.

3.1.7 Predictive values and relevant populations

Positive predictive value is 100%. Negative predictive is variable but is highest in young, unvaccinated children early in disease. Negative predictive value is low for sporadic cases of disease in adults, who generally present later in disease and in previously vaccinated individuals.

3.1.8 Suitable internal controls

Properly documented, relevant, quality control programme for each type and batch of medium used is required. The use of a well characterised clinical isolate and or a recognized type strain such as *B.pertussis* ATCC 8467 is recommended.

3.1.9 Suitable external quality assurance program (QAP) program

None available.

3.1.10 Special considerations

Care should be exercised when using specific antisera to identify the different species as *B. parapertussis* may weakly agglutinate *B. pertussis* antisera.[3]

3.2 Nucleic Acid Amplification Testing (NAAT)

PCR has been used since the early 1990s to detect *B. pertussis* in respiratory specimens and has generally been found to be more sensitive and provide a faster result than culture.[3] Chromosomal regions targeted by PCR include, but are not limited to, the pertussis toxin promoter region, a region upstream of the porin gene, repetitive insertion sequences IS481 of *B.pertussis*, the adenylate cyclase gene and a region upstream of the flagellin gene. Many laboratories use the target, IS*481* to determine the presence of *B. pertussis* DNA as it is found as multiple copies (as many as 238 copies) and therefore provides a more sensitive assay; this feature however may make it more prone to generate false positive results because contaminating DNA is more readily amplified to give a positive result. IS*481*, a transposable element, is also capable of DNA rearrangement and horizontal transfer and has been shown to be present in *B.holmesii* and may cross react with other *Bordetella* species.[13] [14] [15] [16] [17] [18] To overcome this, it is recommended that laboratories incorporate dual targets[19] to improve specificity of diagnosis of B.pertussis, although currently this is not widely performed. *B.holmesii* can not be distinguished from *B.pertussis* by the majority of laboratories performing testing for *B.pertussis. B.holmesii* is known to cause disease in patients with serious underlying medical conditions and has been detected in nasopharyngeal specimens from patients with a pertussis-like illness in about 1% of specimens. The clinical consequences of misdiagnosing a *B.holmesii* infection as *B.pertussis* is at present unknown. Correct identification of *B.holmesii* is important to understand its role as a human pathogen.

3.2.1 Suitable and unsuitable specimens

Nasopharyngeal aspirates or nasopharyngeal swabs using DacronTM, rayon tips or nylon flocked swabs are optimal. Calcium alginate swabs should not be used.[3] In contrast to culture, dry swabs may be used for PCR. Throat swabs and sputum samples may be also be used for adolescents and adults but the performance characteristics of assays using these samples should be validated by each laboratory. Amies transport medium with charcoal does not significantly interfere with PCR.

3.2.2 Test details

A method for assays that target IS*481* to determine the presence of B. *pertussis* DNA and IS*1001* for *B. parapertussis* can be found in PCR for Clinical Microbiology An Australian and International Perspective.[19] Riffelmann et al [21] have provided a list of primer-probe combinations published prior to 2005. Additional targets have been published although none have yet been fully validated. [19],[22],[23],[24]

3.2.3 Test sensitivity

Sensitivity depends on the age of the patient, stage of disease, specimen collection, nucleic acid extraction procedure and test format.[21] PCR is more sensitive and remains positive until significantly later in disease than culture and is recommended for diagnosis in adolescents and adults, who typically are more difficult to sample than young children, although culture should still be attempted. PCR may remain positive when culture becomes negative after antibiotic treatment. Bidet et al [25] studying real-time PCR measurement of persistence of *B.pertussis* DNA in nasopharyngeal secretions during antibiotic treatment of young children with pertussis showed that PCR was positive for 100% of patients at 5 days and after 14 and 21 days, PCR was still positive for 83% and 66% respectively.

An Australian study[26] demonstrated that throat swabs are useful for diagnosis by PCR but this study was not designed to compare the positivity rate of one specimen type with the other. The authors cautioned that the PCR method employed should be sufficiently sensitive to detect the lower number of organisms likely to be present in throat swabs. A Danish study found no significant difference in sensitivity between peroral nasopharyngeal swabs and pernasal nasopharyngeal swabs.[27]

3.2.4 Test specificity

Specificity is very high for all targets. Low numbers of IS*481*-like elements have been reported in *B.holmesii* and *B. bronchiseptica* genomes so primers designed to detect *B. pertussis* IS*481* sequences could potentially cross react with non *B.pertussis* DNA.[28] The occurrence of IS481 in these non B.pertussis isolates in clinical specimens is thought to be uncommon.[2],[17]

3.2.5 Predictive values and relevant populations

Positive predictive value is high in symptomatic individuals, but low in asymptomatic individuals who have had household or other close contact with a patient or in outbreak situations[29]Negative predictive value depends on the age of the patient, stage of disease, specimen collection, nucleic acid extraction procedure and test format. Infected people who have history of previous vaccination or infection typically have less severe symptoms and may excrete fewer organisms.

3.2.6 Suitable test acceptance criteria

Results for control samples obtained as expected.

3.2.7 Suitable internal controls

As recommended in the National Pathology Accreditation Advisory Committee (NPAAC) guidelines: Laboratory Accreditation Standards and Guidelines for Nucleic Acid Detection and Analysis .[30] Controls should be designed to monitor the extraction process as well as detect sample inhibitory activity and external contamination by *Bordetella* amplicons.

3.2.8 Suitable original test validation criteria

Consistent with NPAAC Guidelines:
Requirements for the Validation of In-House In vitro Diagnostic Devices (2006).[31]

3.2.9 Suitable external QAP program

RCPA QAP P/L nucleic acid detection for B.pertussis. These reports are available at http://www.rcpaqap.com/micro/reportsmenu.cfm

3.2.10 Special considerations

PCR may be positive in asymptomatic individuals who have had contact with pertussis cases.[29]

3.2.11 Antibiotic susceptibility testing

Identification of the resistance mechanism will facilitate development of molecular susceptibility testing methods that can be used directly on clinical specimens in the absence of an isolate.[32]The erythromycin resistance in these strains is likely due to a mutation of the erythromycin binding site in the 23S rRNA gene.

3.3 Detection of Toxin or Product

Direct fluorescent antigen detection from nasopharyngeal secretions has been mostly superceded by the more sensitive and specific PCR technology. Fluorescent antigen detection requires skilled personnel, appropriate equipment and is labour intensive. The advantage is a rapid result in the absence of easy access to PCR, however there is a considerable rate of false positive results due to polyclonal antibodies to normal oral and nasopharyngeal flora.[3]A positive result should be confirmed by PCR. Sensitivity is low, in line with culture. As this test is no longer widely performed in Australian laboratories, it has not been included in the current case definition.

3.4 Detection of Immune response

3.4.1 Introduction

Antibody assays have been used for diagnosis of pertussis since the organism was first isolated. However, there is no consensus internationally on the role of serology in the diagnosis of pertussis. The current CDC laboratory case definition does not include serology because it believes that "no serologic method for diagnosis of pertussis has been validated between laboratories or has been accepted for diagnostic use in U.S". WHO accepts seroconversion or a significant increase in antibody level but not a single high titre.[33] In Australia, single sample serology is used extensively, particularly in adolescents and adults who typically present late in disease when an antibody rise has already occurred and culture and PCR are unlikely to be positive. In a 1995 – 2005 analysis of Pertussis Epidemiology in Australia, serology was the predominant method of diagnosis (74%) followed by PCR (12%) when the test method data field was recorded.[33] Whilst PCR has been increasingly used as a

method of diagnosis over time, and is the most common method for diagnosis in infants, the proportion of cases diagnosed by serology increases with age. (Table 1).[34]

Table 1: Age-specific pertussis diagnostic methods for NSW, Qld, NT 2000 to 2005, by age group

	% by age group					
Diagnostic method	<1	1-4	5-9	10-19	20-59	60+
Culture	9.6	3.1	1.9	1.3	0.9	0.9
Nucleic acid testing	59.7	39.1	21.3	11.1	7.1	4.3
Serology	8.7	26.4	52.5	73.8	81.3	88

The majority of serological pertussis notifications in Australia have been based on elevated IgA antibodies against whole cell antigens (see http://www.rcpaqap.com/serology/diseaseresult.cfm. Currently available commercial EIA kits use a variety of antigens including whole cell lysates, pertussis toxin (PT), or filamentous haemaglutinin (FHA) and may detect IgA, IgM or IgG depending upon the conjugate used. If the precision of the assay is such that the intra-assay coefficients of variation are <10%, a two-fold increase or decrease in titre can be considered significant.2 Immunoblot assays can demonstrate whether the antibody detected is directed against PT or FHA and can be used to further characterise an immune response measure by EIA in cases where significant public health action is contemplated.

3.4.2 Suitable specimens

Serum is the specimen of choice. In Western Australia, pertussis whole-cell IgA assay has been performed on pernasal aspirates.[35]
This specimen type is not routinely recommended unless extensive local validation has been performed.[31],[36] IgA responses to pertussis antigens can also be detected in saliva, but currently there is no accepted role for this in day-to-day laboratory diagnosis of pertussis.[37]

3.4.3 Test sensitivity and specificity

The accuracy of test result varies between different kits, and the variety of antigens used. Due to technical issues associated with a specific EIA resulting in overdiagnosis of pertussis in 2006, there has been a move away from whole cell antigen assays in Australia to those using PT alone or in combination with FHA. Manufacturers add additional antigens such as FHA, pertactin (PRN) and fimbriae types 2 and 3 to boost the sensitivity of their tests but this leads to a loss of specificity. PT

antibodies are specific for pertussis, but antibodies to FHA or whole cell antigens may be raised due to infections with other *Bordetella* species including *B parapertussis*. FHA can cross-react with other common respiratory pathogens – *Mycoplasma pneumoniae, Chlamydia pneumoniae,* and H influenzae.[2],[38]

An elevated anti-PT IgG has been recognised in the literature over the last decade as being a very specific marker for recent pertussis infection[39],[40],[41],[49]. Investigators from the Netherlands found that a single serum sample with an anti-PT IgG > 100 U/mL was indicative of recent infection with *B.pertussis.*[39] Such levels were present in <1% of the general population, and were reached in most pertussis patients within 4 weeks of disease onset and persistence was short lived. Pebody et al reported that a single serum mean anti-PT IgG equivalent to 125 ESEN U/ mL was consistent with pertussis infection within 6 months of sample collection, while a level of > 62.5 ESEN U/ mL was consistent with infection in the past year.[42] A variety of clinical laboratories have adopted proposed threshold cutoffs of 49 to 200 EU/ml and in the US, Menzies et al concluded that the anti PT IgG ELISA they had developed met all assay validation parameters within the range considered most relevant for serodiagnosis.[41] Applying a cut-off for PT IgG of 125 EU/mL to a seroepidemiological study in a population aged 20-65 years the various countries in the European Union resulted in the following percentage of the cohort above the cut-off: Netherlands 1.5%, Finland 1.5%, France 2.3%, and the UK 5%.[42]

In contrast to PT IgG responses to infection, the whole cell IgG antibody response is not useful for differentiating recent infection from past exposure to pertussis. The whole cell IgA response is also less specific than anti-PT IgA response.

3.4.4 Impact of vaccination on serological results

Immunisation of adolescents and adults can induce IgG and IgA responses to all measured pertussis antigens. The literature suggests that PT antibodies are the least affected and most rapidly resolving[43],[49]

3.4.5 Impact of age on serological results

Immune responses to pertussis develop more quickly with repeat infection. All adult pertussis infections are considered likely to be repeat infections, due to prior exposure to pertussis or prior immunisation. In young children, serological responses (especially to IgA) are less reliable, and false negatives may occur.

3.4.4 Suitable test acceptance criteria

Inhouse assays: consistent with NPAAC guidelines: Requirements for the Validation of In House In vitro Diagnostic Devices (2006)[31].

Commercial kits: according to manufacturer's instructions.

3.4.5 Suitable test validation

As a result of recent international collaboration, both a WHO international standard[44]and WHO Reference Reagent[45] have become available for laboratories to purchase, in order to calibrate EIA assays measuring antibody titres for PT, FHA and pertactin (PRN) in international units. This will allow more accurate interpretation and inter-laboratory comparison of quantitated titres[1] and assist laboratories in determining and reporting appropriate threshold cutoffs for serodiagnosis of infection.

3.4.6 Suitable internal controls.

WHO–approved standard preparations are now available for purchase.[1] Specimens of serum with levels of PT IgG antibody >100 IU/ml could be used as positive controls, preferably if obtained from patients with PCR or culture –positive pertussis. Use of the clinical definition of pertussis alone to define positive controls is unacceptable for test validation, due to poor specificity.

3.4.7 Suitable external QAP program

RCPA QAP P/L Serology for Pertussis. Reports are available at http://www.rcpaqap.com/serology/diseaseresult.cfm

3.4.8 Summary

Whilst there is no consensus internationally on the role of serology in the diagnosis of pertussis, single sample serology for pertussis whole cell IgA has been used extensively for diagnosis in Australia. Serological testing for pertussis antigens, particularly pertussis toxin (PT) can be sensitive and specific, although commercial tests are not fully standardised, and there are no universally accepted correlations with protection.[46] Increasingly anti-PT IgG has been recognised as the best serological marker of acute infection although the actual cutoffs still remain to be defined. The availability of WHO standards and reference sera will assist in determining the most appropriate thresholds. Threshold cutoffs that have been proposed range from 49 to 200 EU/ml. In line with the international literature, the current definition has been expanded to include a significant PT IgG response in an appropriately validated test as suggestive evidence of acute infection. It is likely that in the near future, PT IgG will become the serological test of choice for pertussis serological diagnosis, rather than PT or whole cell IgA.

3.5 Overview of Appropriate Test Selection

The diagnosis of pertussis is best made using a combination of methods based on a number of variables. Simplified recommendations adapted from Muller et al [3] summarise these.

Table 2: Tests used for patients with the following duration of cough[3]

	1-2 weeks		**3-4 weeks**		**>4 weeks**
	Untreated	Treated	Untreated	Treated	Untreated or treated
Unvaccinated infants	PCR, Culture	PCR	Culture, PCR	Serology, (PCR)	Serology

Table 2: Tests used for patients with the following duration of cough[3]					
	1-2 weeks		3-4 weeks		>4 weeks
Unvaccinated children	PCR, Culture	PCR	PCR, Serology, (Culture)	Serology, (PCR)	Serology
Vaccinated infants or child	PCR, Culture	PCR	PCR, Serology, (Culture)	Serology, (PCR)	Serology
Adolescents or adults	PCR, Culture	PCR	PCR, Serology, (Culture)	Serology, (PCR)	Serology

In 2007 Andre et al[47] concluded that single serology was the most efficient diagnostic test with relatively high sensitivity (>64%) and high specificity (>90%) in 195 participants (≥7 year olds) in an epidemiological study. Combining single serology with one PCR or paired serology increased the sensitivity with an associated limited decrease in specificity.

4 typing and subtyping methods

4.1 Typing (subtyping) Method

A number of methods have been used to characterize *B.pertussis* isolates. Most are based on known vaccine virulence factors. Serotyping with antibodies against three surface antigens divides isolates into four serotypes. Two of these three antigens are fimbrial and are also known as Fim2 and Fim3.[48] DNA fingerprinting is a second approach, the various techniques used to obtain DNA fingerprints such as ribotyping, RAPD, and pulse field gel electrophoresis (PFGE). Gene typing, the third method used to type B.pertussis is derived from Multi-Locus Sequence Typing (MLST). A multilocus sequence typing (MLST) scheme based on the sequences of *ptx*A, *ptx*C, and *tcf*A has been described. Other methods derived from this include Multiple-Locus Variable number tandem repeat Analysis (MLVA), and Comparative Genomic Hybridization (CGH). DNA sequence-based typing is increasingly being used, and polymorphisms have been found in various genes including the S1 and S3 subunits of pertussis toxin (*ptx*A and *ptx*C), pertactin (*prn*A), tracheal colonization factor (*tcf*A), fimbrial antigens 2 and 3 (*fim*2 and f*im*3). With PCR replacing culture of isolates, the availabilty of culture collections for epidemiological studies may decrease and alternative molecular based typing are required.

4.1.1 Utility

This typing is important to track changes in antigenic structure of virulence factors such as pertussis toxin and pertactin used in subunit vaccines after the historic Australian pertussis WCV containing *ptx* S1A derived proteins was stopped. [13]

4.1.2 Suitable external QAP program

No programme available but isolates may be submitted to Eupertstrain for analysis.

4.2 SNOMED CT concepts

SNOMED CT concept	SNOMED CT Code
Pertussis (Disorder)	27836007
Bordetella pertussis (Organism)	5247005
Bordetella pertussis culture (Procedure)	122206002
Bordetella pertussis antibody level (Procedure)	315073006
Bordetella pertussis antigen assay (Procedure)	122183004

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