

Laboratory manual for the diagnosis of whooping cough caused by *Bordetella pertussis*/ *Bordetella parapertussis*

Immunization, Vaccines and Biologicals



World Health Organization

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Abbreviations

AC-Hly	adenylate cyclase-hemolysin
aP	acellular pertussis
BG	Bordet Gengou (medium)
BSA	bovine serum albumin
DTP	diphtheria–tetanus–pertussis vaccine
FHA	filamentous haemagglutinin
MLD	minimum level of detection
NPA	nasopharyngeal aspirates
NPS	nasopharyngeal swabs
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PT	Pertussis toxin
RL	Reagan Lowe (medium)
TDA	tryptophan desaminase
wP	wholecell pertussis

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1. Introduction

Whooping cough is a worldwide infectious disease caused by the bacteria *Bordetella pertussis* and *Bordetella parapertussis*. It is a respiratory disease that occurs after transmission from person to person of the bacteria in airborne droplets. The bacteria are highly infectious and unprotected close contacts are liable to become infected. Incidence is highest in children under five except where infant vaccination programmes have been very effective and a shift has occurred to adolescents.

Whooping cough is not only a childhood disease. It is dramatic for neonates and infants but can be very severe for children and adults. For over 40 years wholecell pertussis vaccines have been very effective, preventing around 760 000 deaths annually worldwide. Nevertheless, pertussis disease continues to impose a high burden, since there are still 50 million cases of pertussis disease and 300 000 deaths every year, mostly among infants.

Even in high-coverage countries, pertussis disease continues to cause severe illness and death among neonates and infants too young to have completed the primary vaccination series.

Active primary immunization against *B. pertussis* infection is recommended with three doses of a vaccine consisting of either a suspension of killed bacteria (wholecell pertussis, wP) or acellular pertussis (aP) preparations that contain 1–5 different components of *B. pertussis*. These are usually given in combination with diphtheria and tetanus toxoids adsorbed on aluminium salts (DTwP or DTaP). In terms of severe adverse effects aP and wP vaccines appear to have the same high level of safety; reactions are less commonly associated with aP vaccines. Similar high efficacy levels (more than 80%) are obtained with the best aP and wP vaccines although the level of efficacy may vary within each group. Protection is greater against severe disease and begins to wane after about three years. Acellular pertussis vaccines do not protect against infection by *B. parapertussis*. The need and timing for additional booster doses of DTP and their efficacy should be assessed by national programmes. In the USA, booster doses are recommended at 15–18 months of age and either at school entry or at adolescence. Formulations of acellular pertussis vaccine for use in adults have been licensed and are available in several jurisdictions.

1.1 Clinical symptoms

The typical form of the disease consists of four phases:

- *The incubation period or silent phase.* This averages 9–10 days (range 6–20 days) with no symptoms.
- *The initial catarrhal phase.* This has an insidious onset with an irritating cough that gradually becomes paroxysmal, usually within 1–2 weeks. Symptoms are similar to those of a common cold with a dry cough. During this phase, *B. pertussis* or *B. parapertussis* can be isolated from nasopharyngeal aspirates at a rate as high as 90%. Unfortunately, in the absence of clinical symptoms physicians never consider attempting collecting samples for isolation of *Bordetella* except for patients with known contacts with a confirmed case.
- *The paroxysmal phase.* Several weeks of dry, non-productive cough evolve to the characteristic paroxysmal cough with mucous production, cyanosis and vomiting. This phase lasts at least 21 days but can last 60 days with typical paroxysmal cough. Paroxysms are characterized by repeated violent coughs; each series of paroxysm has many coughs without intervening inhalation and can be followed by a characteristic crowing or high-pitched inspiratory whoop. Paroxysms frequently end with the expulsion of clear, tenacious mucus, often followed by vomiting. Infants less than six months old, vaccinated children, adolescents and adults often do not have the typical whoop or cough paroxysm.

Patients exhibit leucocytosis with lymphocytosis, weight loss, sometimes hypoglycaemia and very rarely encephalopathy. Two weeks after the beginning of the cough it becomes very difficult to isolate the bacteria, suggesting that most of the symptoms are due to toxins released by the bacteria. The bacteria do not enter the bloodstream and do not spread. When treated with erythromycin, clarithromycin or azithromycin, patients are no longer contagious after five days of treatment.

- *The convalescent phase of at least 20 days.*

The disease is very severe, sometimes life-threatening in infants less than two months of age. These infants and neonates are most likely to experience complications; they are likely to be admitted to hospital and are more likely to die from complications or secondary infections. Complications include pneumonia, atelectasis, seizures, encephalopathy, weight loss, hernias and death. Pneumonia is the most common cause of death; fatal encephalopathy, probably hypoxic, and inanition from repeated vomiting occasionally occur. Case-fatality ratios in unprotected children are less than 1 per thousand in industrialized countries; in developing countries they are estimated at 3.7% for children under 1 and 1% for children 1 to 4 years.

In several developed countries with high rates of infant immunization for many years, an increasing proportion of cases has been reported in adolescents and adults, whose symptoms varied from a mild, atypical respiratory illness to the full-blown syndrome. Many of these cases occur in previously immunized persons and indicate waning immunity following immunization.

WHO has proposed a standard case definition for surveillance (*WHO-recommended standards for surveillance of selected vaccine – preventable diseases* (see section 5, References).

Parapertussis is a similar and milder disease due to *Bordetella parapertussis*, which occurs less frequently.

1.2 *Bordetella pertussis* and *Bordetella parapertussis*

B. pertussis and *B. parapertussis* are gram negative coccobacilli bacteria and the species can be distinguished based on a number of biochemical characteristics shown in Table 1. It is hypothesized that these two species evolved independently to become human pathogens from *B. bronchiseptica*, a pathogenic species found in animals. *B. pertussis* and *B. parapertussis* are similar species but *B. parapertussis* lacks the expression of the gene coding for pertussis toxin. Differentiation between *B. parapertussis* and *B. pertussis* is based on culture, biochemical and immunologic differences.

Table 1: Biochemical characteristics of *Bordetella pertussis* and *Bordetella parapertussis*

Test	<i>B. pertussis</i>		<i>B. parapertussis</i>	
	Phase I	Phase IV	Phase I	Phase IV
Haemolysis	+	-	+	-
Growth on BG medium	+ 3–5 days		+ 2–3 days	
Oxidase	+		-	
Urease	-		+	
Nitrate reductase	-		-	
Citrate	-		-	
Carbohydrate acidification	-		-	
Mobility	-		-	
Pigment	-		+ brown	
G + C%	67.7–68.9		68.1–69	

Both *B. pertussis* and *B. parapertussis* are highly efficient bacterial pathogens that establish infection by the respiratory route and remain localized in the upper respiratory tract. Over the past 30 years many researchers have shown that the pathogenicity of these bacteria involves numerous proteins classified as adhesins and toxins.

1.2.1 Adhesins

The major adhesin is filamentous haemagglutinin (FHA), a 220 kDa filamentous protein able to bind carbohydrate, heparin and the integrin CR3 site. This binding ability of FHA allows the bacterium to bind to a variety of cells such as phagocytic cells and epithelial cells, as well as extracellular structures in the respiratory epithelium.

In addition to FHA, *B. pertussis* produces fimbriae that are composed of the major subunits Fim 2 or Fim 3 and of the minor subunit Fim D located at the tip. Fim D binds to integrin VLA5 and sulfated sugars. Recent studies indicate that fimbriae play a role in infection of the laryngeal mucosa whereas FHA is important for colonization of the entire respiratory tract.

The third class of adhesins is composed of the autotransporters pertactin (PRN) and tracheal colonization factor (TCF). Both proteins are also able to bind phagocytic cells via their Arg-Gly-Asp (RGD) domains. During infection all these adhesins induce synthesis of antibodies. Anti-fim and anti-PRN are agglutinins because they are able to agglutinate bacteria.

1.2.2 Toxins

B. pertussis expresses different toxins in addition to adhesins.

Cyto-tracheal toxin (TCT), a low molecular weight glycopeptide, is a fragment of peptidoglycan secreted by the bacteria. It destroys tracheal ciliated cells by inducing the synthesis of interleukin-1 and nitric oxide and inhibits the regeneration of the respiratory tract epithelium.

Pertussis toxin (PT) is a toxin secreted by the bacteria and composed of five different subunits. It is an A-B toxin. The B part is responsible for binding to the host cell and allows the A part to enter into the cell. The A part disrupts cellular functions *via* its ADP-ribosylating activity.

The toxin adenylate cyclase-hemolysin (AC-Hly) is a secreted trifunctional protein. It expresses a calmodulin-dependent adenylate cyclase activity, a haemolytic activity and an invasive activity. This toxin binds the integrin CR3 of macrophages, enters the cell and induces apoptosis of the cell. PT and AC-Hly toxins induce synthesis of antibodies during infection.

In addition to these well characterized adhesins and toxins, *B. pertussis* expresses a series of other factors which may also be involved in its pathogenicity as revealed by the genome sequence. New tools are being developed to explore the role of these new factors.

1.2.3 Regulation of *Bordetella pertussis* toxins and adhesins

It is well established that the expression of *B. pertussis* toxins and adhesins can be modulated by changes in the environment, a phenomenon called “phase modulation”. In addition, *B. pertussis* can undergo “phase variation” and loses the expression of these factors. Both modulation and phase variation are under the control of a two-component phospho-relay system encoded by the *BvgA/S* operon. BvgS is an inner membrane protein that senses changes in the environment. After receiving the signal BvgS undergoes autophosphorylation and after, phosphorylates BvgA, which will thus be activated. The BvgA will then bind the promoters of the genes encoding the toxins and adhesins which will trigger the transcription of these genes and expression of the virulence factors. For these reasons the genes encoding virulence factors are called vir-activated genes (*vag*). When toxins and adhesins are expressed, *Bordetella* species are called phase I *Bordetella*.

In absence of external signal, i.e. in absence of activated *BvgA/S* or when mutations have occurred in *BvgA/S* genes, the *vag* are not expressed. Instead, a set of other genes called vir-repressed genes (*vrg*) are expressed. The function of the proteins encoded by the *vrg* and their role in the pathogenicity of *B. pertussis* are not yet known. In that case *Bordetella* species are called phase IV *Bordetella*.

This regulation of expression is important for the pathogenicity of *Bordetella* species but also in laboratory diagnosis. In fact, if growth conditions are changing (medium, temperature etc.) the bacteria change (haemolytic versus non-haemolytic). The phases and their aspect on the plates will be different.

1.2.4 Polymorphism of *Bordetella pertussis* factors

Despite the overall genetic similarity, the genome of *B. pertussis* isolates shows a remarkable plasticity. It is significantly smaller than that of *B. parapertussis* and *B. bronchiseptica*, suggesting that the adaptation to humans led to a reduction in the genome size.

Analysis of the *B. pertussis* population suggests that this population is evolving with time as demonstrated by Pulsed Field Gel-Electrophoresis and sequencing of virulence factors’ structural genes such as those encoding PRN and the S1 subunit of PT. One hypothesis to explain this genetic drift is that it is vaccine driven since the isolates circulating before vaccination are different from the isolates circulating now. Recently, using microarrays, it was shown that in France there is a temporal decrease in genetic diversity with a loss of pseudogenes or genes not important for the virulence of the bacterium (Caro et al 2006 *Microbes Infect* 8:2228-2235). However, there is no proof that these changes could affect vaccine efficacy. Surveillance must continue so as to better understand changing molecular epidemiology and its public health implications.

2. General considerations on whooping cough laboratory diagnosis

This manual provides guidelines on laboratory diagnosis of whooping cough. There are two types of approach to diagnosis: direct and indirect. Direct diagnosis consists of identifying the microorganism responsible for the disease either by culture or by polymerase chain reaction (PCR). Indirect diagnosis is essentially by serology and consists of detecting specific antibodies in the serum of an infected individual.

Culture is considered by WHO as the “gold standard” of laboratory case confirmation. This method of diagnosis is not very sensitive since the percentage of success is generally not higher than 60%. The highest rates are obtained with infants and non-vaccinated children. It is important to continue to culture in order to analyse the evolution of the pathogen and perform surveillance of eventual variants that might be antigenically different from vaccine strains. In fact, culture is only successful if samples are collected within the two to three first weeks after the beginning of the cough, but culture is the most specific diagnosis. Among the direct methods, the polymerase chain reaction method is more sensitive than bacterial culture. PCR can be performed on the same biological samples as cultures. However, PCR is difficult to perform and requires more expensive equipment. Direct fluorescent antibody (DFA) staining of nasopharyngeal secretions is not recommended because of frequent false positive and negative results.

Indirect diagnosis (serology) consists of detecting specific antibodies in the serum of infected individuals, collected at the beginning of cough (acute serum) and on serum collected one month later (convalescent serum). The presence of a high level of antibodies in the serum of a non-vaccinated individual indicates infection. Serology cannot be used as a diagnosis during the year following vaccination since it does not differentiate between antibodies due to the vaccine and natural infection.

Furthermore, serology is not sensitive in infants, as their immune system is immature and due to interference of maternal antibodies.

Choice of the diagnosis depends on the age and the immune status of the suspected patients.

- **Infants:** culture is priority or PCR where culture is not available but serology is unreliable
- **Children:** Culture or PCR for non-vaccinated children (first days of coughing) or serology (if vaccination was not performed during the last 3 years)
- **Adults:** Serology (if vaccination was not performed during the last three years)

3. Direct diagnosis

3.1 Culture

3.1.1 *Collection and transport of biological specimens*

The collection and transport of biological specimens is important in the isolation and identification of bacterial agents of whooping cough.

Collection of biological specimens

B. pertussis or *B. parapertussis* can be isolated from nasopharyngeal swabs (NPS) (calcium alginate swabs) nasopharyngeal aspirates (NPA) or sputum taken from infants, children, adolescents and adults (**Annex 1**). It was previously shown that a 15% gain in the isolation rate is obtained using NPA compared to NPS in neonates and infants. NPA is often preferred by nurses or parents and can be divided in aliquots and saved for other investigations. However, for adults, the bacteria can also be isolated from sputum.

Transport of nasopharyngeal swabs or aspirates or sputum

B. pertussis and *B. parapertussis* are fragile bacteria. The NPS or NPA or sputum should be transported within four hours of collection at room temperature to the microbiology laboratory for culture. The swab or the tip of the catheter can also be placed in Reagan Lowe (RL) transport medium (**Annex 2**) and sputum can be transported in phosphate buffered saline (PBS) or a solution of 1% casaminoacids. Isolation rates decrease when transport occurs at 4°C instead of ambient temperature or takes longer than 48 hours.

Specimen collection for PCR testing

When using swabs it is preferred to collect specimens for PCR test using a dacron swab with polystyrene sticks. Cottonwool budded swabs are not recommended for some PCR work.

3.1.2 *Primary culture and presumptive identification*

Inoculation of primary culture media

After transport at ambient temperature (15–30°C), the NPS or the tip of the catheter or the sputum are streaked on to fresh RL medium or Bordet Gengou (BG) medium (**Annex 3**) supplemented with 15% defibrinated sheep or horse blood (human blood is not an acceptable substitute). For each sample, selective medium, i.e. containing cephalixin to inhibit normal flora (40 µg/ml), and non-selective medium

(without cephalixin) should be used. Inhibition of growth is sometimes observed with cephalixin and for this reason it is recommended to use two culture media plates, selective and non-selective.

In the case of NPS, the swab can also be dipped into Stainer Scholte (SS) broth (**Annex 4**), and shaken thoroughly at 36°C for 48 hours before being streaked on selective and non-selective RL or BG plates.

B. pertussis and *B. parapertussis* are strictly aerobic bacteria – i.e. their growth can only occur in aerobic conditions.

All plates are incubated for seven days at 35–36°C and inspected every two days. If typical colonies appear they are re-isolated and identified. Plates should be incubated for seven days before being discarded as negative. *B. pertussis* grows slower on BG but isolation rates on RL and BG plates are similar after seven days of incubation.

One major advantage of BG is the possibility of characterizing *Bordetella* phases. It has long been known that the expression of virulence properties of *B. pertussis* is unstable. In fact, non-virulent so-called *phase variants* may arise at high frequency. Moreover, the virulent phenotype depends on environmental conditions and is reversibly affected by temperature or chemical compounds, a phenomenon called *phenotypic modulation*.

- Expression of virulence gene is called phase I.
- Loss of haemolysis only is called the intermediary phase (phase II or III).
- Loss of expression of all virulence factors is called phase IV (visualized on Bordet Gengou medium by loss of haemolysis and different aspect of colonies i.e. no longer glossy and more spread out).

Macroscopic examination of colonies

- Typical *B. pertussis* or *B. parapertussis* colonies on BG or RL plates are small (1 mm in diameter after three days of culture), like mercury droplets and glistening. *B. parapertussis* may grow faster and appear greyish.
- On BG plates, both species appear haemolytic. Contrary to *B. pertussis*, *B. parapertussis* is brown pigmented on BG agar medium, colour due to the expression of tyrosinase. These types of colony appear between three and seven days and are called phase I colonies. However, depending on the growth conditions, the morphology of the colonies can change and phases II, III, and IV can be observed.
- Intermediate phase II or III colonies present the same aspect as phase I colonies on BG plates but are non-haemolytic.
- Phase IV colonies appear larger (2 mm in diameter after three days of culture), non-haemolytic, whitish and flattened.
- Plates should be examined on days 3 and 7. If suspected colonies are observed on day 3, they have to be re-isolated on fresh RL or BG medium. After seven days, plates may be discarded.

Microscopic examination of colonies

When typical colonies appear, Gram-staining determination can be performed (**Annex 5**). *B. pertussis* and *B. parapertussis* will appear as typical small non-motile gram-negative coccobacilli.

3.1.3 Identification of *Bordetella pertussis* and *Bordetella parapertussis*

The following steps are recommended to identify colonies that morphologically seem to be *B. pertussis* or *B. parapertussis*.

- Check purity of the growth by performing a Gram stain.
- Determine biochemical characters, such as oxidase, urease, nitrate-reductase, carbohydrates utilization (**Annex 6**) after subculture on BG or RL agar medium.
- Confirm the identification using specific anti-*B. pertussis* and anti-*B. parapertussis* immunofluorescence antiserum (**Annex 7**). This last step allows the recognition of some *Bordetella* antigens at the surface of the bacteria using species-specific antisera.
- The major characteristics of *B. pertussis* and *B. parapertussis* are listed in Table 1 above.

3.1.4 Serotyping of *Bordetella pertussis*

Serotyping, i.e. the detection of the expression of the fimbriae Fim 2 and Fim 3 is performed using monoclonal antibodies (**Annex 8**). Isolates which cannot be serotyped reproducibly should be sent to an international reference laboratory.

3.1.5 Storage of *Bordetella pertussis* and *Bordetella parapertussis*

B. pertussis isolates may be frozen in BSA/glutamate solution (**Annex 9**). These isolates can be stored for at least two years (-40°C) or four years (-80°C).

3.2 Polymerase chain reaction

PCR is a direct test currently used during vaccine trials and epidemiological studies to characterize *B. pertussis* or *B. parapertussis*. The target DNA is located in:

- in the insertion sequences 481 (for *B. pertussis* detection) or 1001 (for *B. parapertussis* detection).

It was recently observed that the PCR assay based on insertion sequence supposed to be specific to *B. pertussis* was cross reacting with *B. holmesii*. However, recently, it was shown that *B. holmesii* is not among the causative agents of pertussis like symptoms in Finnish and Dutch patients and thus does not in practice confound IS481 and IS 1001 PCR (Antila et al. 2006 J. Med. Microbiol). However, most laboratories use this sequence since the PCR is very sensitive and the relevance of *B. holmesii* human infection or carriage is still under discussion.

During the last 10 years, the clinical trials which took place around the world showed that for infants or young children, NPA and NPS are the best material for PCR and can be *used directly without DNA extraction*. In the case of adults, because the mucus is not abundant and because of the presence of inhibitors, DNA needs to be purified from the biological samples and if NPA or NPS cannot be obtained sputum can be used.

PCR is expensive but more sensitive and more rapid than culture. PCR may be used as an alternative for a rapid diagnosis of whooping cough but must be performed according to the recommendations of the regulatory agencies (Riffelmann et al 2005 J. Clin Microbiol 43(10):4925-4929). (**Annexes 10 and 11**).

4. Indirect diagnosis

4.1 Agglutinin detection

Although reagents are commercially available, measurement of agglutinins is not a very sensitive technique for diagnosing the disease.

4.2 Antitoxin and adhesin detection

Measurement of anti-PT, FHA and PRN antibodies by ELISA is the technique used during epidemiological studies or vaccine trials. Reference sera and antigens can be obtained from control laboratories such as Institut Pasteur (**Annex 13**).

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Annex 1:

Collection of nasopharyngeal aspirate (NPA)

A.1.1 Material

Per subject

- Gloves, disposable
- Suction catheter with control
- Screwtop container, plastic, 100 ml, sterile
- Syringe, plastic, 5 cc, sterile
- Bag, plastic, ziplock for holding equipment
- Mask, surgical
- Towelling

Per visit

Bag, plastic, ziploc for disposing of gloves.

A.1.2 Reagents

PBS – phosphate buffered saline (0.01 M PO₄) pH = 7.4.

A.1.3 Collection of nasal aspirate

Every effort must be made to prevent contamination of the tubing, container etc. during the procurement of the sample. Equipment may be contaminated by the coughs of other infected family members. The study nurse should avoid direct contact with the subject and family members until after NPA collection to minimize contamination on the nurse's clothes. The PCR test for *B. pertussis* can detect even minute amounts of dead bacteria. Therefore only remove the equipment from the protective ziplock bag when the subject is positioned and ready for the tube insertion.

- Choose an area for NPA collection that is least used by the family. For example, a family room or kitchen may be more contaminated than other rooms.
- Place a clean paper towel on the table which will hold the equipment.
- If the subject is a child and is to be held by the parent, the parent must be masked.

-
- When the subject is situated and ready for the NPA put on gloves. Remove equipment from the bag and place on the clean paper towelling. Loosen the cap of the sterile container but do not open until inserting the catheter tip.
 - Open the syringe and remove the plastic tip.
 - Secure the syringe on the end of the catheter. Test the syringe.
 - Remove the catheter from the wrapper.
 - Gently and slowly insert the catheter into a nostril rotating the catheter if necessary to proceed past the back of the nostril. Insert the catheter until the back of the throat is reached (approximately 10 cm depending on the age of the volunteer). If gagging occurs, the catheter is inserted too far.
 - Once positioned, the catheter should be withdrawn with suction by placing the thumb over the suction control on the side of the catheter while pulling back on syringe plunger.
 - Once the catheter is removed from the nose, and without touching the tip of the catheter open the sterile container and place the tip in. Screw the top on with catheter and syringe still attached. This protects the part of the tubing containing the specimen.
 - Label the sterile container, and place container, catheter and syringe in a plastic bag and seal. Remove gloves and place in a plastic bag for disposal.
 - For each family member repeat steps 1–11.
 - Transport the NPA specimen.

Important: The NPA samples are very prone to drying out, and *B. pertussis* cannot survive in a dry environment. Therefore, if the NPA is to be cultured, it is imperative that the specimen is washed with a phosphate-buffered saline containing 1% casamino acids (PBS/casa) as soon as possible after leaving the subject's home. Ideally, this procedure is performed by a laboratory technician in a bio-containment hood. If this type of hood is not available (i.e. the laboratory is not accessible after hours, or the lab does not have one) the procedure can be performed on a countertop or desktop in an area where pertussis contamination is very unlikely. It cannot be stressed enough how careful one must be to avoid contamination of the NPA equipment or specimen with pertussis organisms (dead or alive) from another individual. The procedure should NOT be performed in the subject's room or area of the lab where other pertussis testing is taking place.

Annex 2:

Reagan Lowe medium

A.2.1 Material

- Petri dish (diameter: 9 cm)
- 4 ml glass bottles
- Pasteur pipette
- Pipetter
- 1, 2, 5, 10 ml plastic pipettes
- 200 µl and 1000 µl tips for automatic pipetter
- Medium distributor
- Screw caps
- Laminar flow hood type PSM.

A.2.2 Reagents

Preparation of specific RL medium: charcoal medium

- Weigh out 51 g oxoid charcoal medium
- Dissolve by stirring in 1 litre double-distilled H₂O
- Dispense 15.3 ml of the medium using a fractionating distributor or conventional pipetting into washed glass tubes
- Seal with single-use screw caps
- Autoclave for 20 minutes at 120°C
- Store at +4°C (cold room) for four weeks.

Preparation of cefalexin

- Reconstitute a bottle of 20 mg with 2 ml of bi-distilled sterile water
- Dilute these 2 ml of solubilized cefalexin with 48 ml of bi-distilled sterile water
- Store the cefalexin solution (0.4mg/ml) in 1.5 ml sterile Eppendorf tubes per aliquot of 850 µl
- Record the preparation data and the validity limit date in the preparation book
- Store at -20°C for 6 months.

Sheep blood or horse blood

For a plate: 1.7 ml of blood.

Reference strain

Bordetella pertussis Tohama I (CIP 8132 which can be purchased from the Institut Pasteur) for quality control in growth support and testing incubation conditions for cultures.

A.2.3 Preparation Reagan Lowe medium agar dishes

- Petri dishes of medium are prepared under clean air conditions using a laminar flow hood or burning Bunsen burner.
- Place the tubes containing Reagan Lowe medium in a water bath at 52°C in order to melt the medium.
- Petri dishes should be removed under sterile conditions from their plastic wrappings within the laminar flow hood and filled under sterile conditions.
- Remove five tubes of medium from the waterbath at 52°C, rinse them and then place them in a holder under the laminar flow hood or close to the burning Bunsen burner.
- Extract 8.5 ml blood (for 5 tubes) using a 10 ml pipette and distribute 1.7 ml blood into each of the five tubes, seal them and mix the contents by gently inverting each tube three times: this will homogenize the preparation.
- Pour the total mixture of 17 ml (15.3 ml + 1.7 ml) into a Petri dish and spread it uniformly using a slow circular motion.
- Remove the dishes from the laminar flow hood when the charcoal agar has solidified, identify individual batches (in case one part of the blood is contaminated) and leave them overnight at room temperature.
- Perform sterility and growth support tests. Plates without bacteria and plates cultured with bacteria are put in the oven for a few days. Those without bacteria must remain sterile, and on those cultured with bacteria, growth must be observed after three days.

Preparation of bottles containing Reagan Lowe medium for transport

- Prepare the exact number of bottles requested.
- Unscrew the caps under the laminar flow hood.
- Pipette in 1.5 ml of the mixture of (blood–charcoal medium into each bottle).

A.2.4 Preparation of Reagan Lowe medium agar dishes supplemented with cephalexin

- Thaw the volume of cephalexin solution required (0.4 mg/ml).
- Add 170 µl of cephalexin (0.4 mg/ml) per tube of Reagan Lowe medium agar after adding blood (2.5 ml). The final concentration of cephalexin is 40 µg per tube.
- Record the batch reference number in the preparation logbook. Identify prepared plates by inscribing a sign on the cover.
- Seal the bottles, tilt them to allow the mixture to solidify. The cap of the sealed bottle can be laid against the side of a petri dish (height: 1 cm).

Store dishes in plastic bags and keep them at 4°C.

Prepared petri dishes and bottles can be stored at 4°C for 2 weeks and should be discarded afterwards.

Annex 3:

Bordet Gengou medium

A.3.1 Equipment and materials

- Plastic or glass sterile plates (diameter: 9 cm)
- Wheaton 4 ml glass bottle
- Pasteur pipette
- Automatic pipetter (200 µl and 1000 µl) or conventional pipettes
- Plastic pipettes of 1, 2, 5 or 10 ml
- 200 µl and 1000 µl tips for automatic pipetter
- Wheaton medium distributor
- Screw stoppers
- Laminar flow hood.

A.3.2 Reagents

Preparation of specific Bordet Gengou medium

Bordet Gengou agar medium (DIFCO) ref. 248200: (Beckton Dickinson Biosciences, 2350 Qume Drive, San Jose, CA 95131-1807, USA; Tel: 408.432.9475; Fax: 408.954.2347). Storage period: 1 month. Storage at 4°C.

- Weigh 30 g Bordet Gengou medium and dissolve by:
 - boiling in a mixture of glycerol 10 ml
 - adding 5N NaOH until pH = 7.4 and
 - distilled H₂O to make 1 litre
- Record batch reference number in the preparation logbook.
- Distribute 14.5 ml of medium per tube.
- Seal with single-use screw stoppers.
- Autoclave for 20 minutes at 121°C.
- Allow to cool and store at 4°C.
- This medium can be kept for up to 12 weeks at 4°C.

Preparation of cefalexin

- Reconstitute a bottle of 20 mg with 2 ml of double-distilled sterile water.
- Dilute these 2 ml of solubilized cefalexin with 48 ml of bi-distilled sterile water.
- Store the cefalexin solution (0.4 mg/ml) in 1.5 ml sterile Eppendorf tubes per aliquot of 850 µl.
- Record the preparation data and the validity limit date in the preparation book.
- Store at -20°C for 6 months.

Reference strain

Bordetella pertussis: Tohama I (this strain can be purchased from the Institut Pasteur as CIP 81.32).

A.3.3 Preparation of Bordet Gengou agar dishes

- Petri dishes are prepared under clean air conditions using a laminar flow hood, burning Bunsen burner or a hood.
- Place the tubes containing Bordet Gengou medium in a water bath at 52°C in order to melt the medium.
- Petri dishes should be removed under sterile conditions (clean air) from their plastic wrappings beneath the laminar flow hood and filled under sterile conditions in batches of five.
- Five plates should be prepared at the same time under the laminar flow hood or close to the burning Bunsen burner.
- Remove five tubes from the waterbath at 52°C, and put them in a holder under the laminar flow hood or close to the Bunsen burner.
- Extract a 8.5 ml sample of blood using a 10 ml sterile pipette and distribute 1.7 ml blood into each of the five tubes; seal them three times by inverting each tube, and gently mix the agar medium and blood in order to homogenize the preparation.
- Pour total mixture of 17 ml (15.3 + 1.7 ml) into a dish and spread it uniformly by using a slow circular motion.
- Remove the plates from the laminar flow hood when the BGS is cold. Identify batches (in case one part of the blood is contaminated) and leave overnight at room temperature before storage at 4°C.
- Perform sterility and growth test. Plates without bacteria and plates cultured with bacteria are put in the incubator for a few days. Those without bacteria must remain sterile and on those cultured with bacteria, growth must be observed after three days.

A.3.4 Preparation of Bordet Gengou agar plates supplemented with cefalexin

- Thaw the volume of cefalexin solution required (0.4 mg/ml).
- Add 170 µl of cefalexin (0.4 mg/ml) per tube of Bordet Gengou agar medium after adding blood (2.5 ml). The final concentration of cefalexin is 40 µg per tube.
- Record the batch reference number in the preparation logbook. Identify prepared plates by inscribing a sign on the cover.

Store dishes in plastic bags and keep them at 4°C.

Prepared petri dishes and bottles can be stored at 4°C for two weeks and should be discarded afterwards.

Annex 4:

Stainer Scholte medium

A.4.1 Material

- Miscellaneous glassware
- Scales
- Magnetic stirrer and magnetic bars
- pH-meter (or litmus paper with pH range 7–8)
- Autoclave
- Plastic bottles and tubes
- 150 ml stericup; 0.22 µm Millipore filter.

Note:

- Use sterile labware.
- Prepare all media with double-distilled sterile water.
- Substances should be weighed following the instructions supplied with the scales.
- The pH-meter is calibrated and the temperature measured with the probe.

A.4.2 Reagents

Solution

1% calcium chloride solution freshly prepared thus:

Calcium chloride	250 mg	
H ₂ O	to make	25 ml

Concentrated 10X stainer

- Reagent
 - Sodium glutamate ($C_5H_8NO_4Na$) 214 g
 - L-proline ($L-C_5H_9NO_2$) 4.8 g
 - Sodium chloride (NaCl) 50 g
 - Potassium dihydrogen phosphate (KH_2PO_4) 10 g
 - Potassium chloride (KCl) 4 g
 - Magnesium chloride ($MgCl_2$) 2 g
 - Tris base ($C_4H_{11}NO_3$) 30.05 g Réf: T1503;
Purity 99.9%; Sigma
 - 1% calcium chloride ($CaCl_2$) 40 ml
 - Distilled H_2O 1000 ml
 - HCl adjust to pH 7.6
 - H_2O to make 2000 ml

Aliquot by 200 ml before storing in $-20^\circ C$.

- Protocol
 - Put a 1 litre beaker on a magnetic stirrer.
 - Place a clean magnetic bar inside the beaker.
 - Pour 700 ml of pure distilled water into the beaker.
 - Regulate the rotation of the magnetic bar in order to allow effective, homogenous and silent mixture of the solutions.
 - Weigh out the various chemical products required to make the medium.
 - Slowly pour the dry products one after the other into the liquid so that dissolution takes place without clumps being formed.
 - Add 20 ml of 1% calcium chloride.
 - Carefully raise the pH to 7.6 with concentrated hydrochloric acid.
 - Exactly make up the final volume to 1 litre after ensuring the magnetic bar has been removed.
 - Homogenize the medium by inverting it several times after first covering it with parafilm.
 - Distribute it into plastic bottles either in 200 ml or 20 ml fractions depending on workload needs.
 - Label the bottles with the preparation date, expiry date and product name.
 - Freeze at $-20^\circ C$.
 - Complete the medium preparation logbook.
 - Store at $-20^\circ C$ for 6 months.

10X supplement

- Dissolve in a beaker:
 - L-cystine 8 g
 - concentrated HCl 20 ml
- Add 120 ml of a solution containing:
 - FeSO₄, 7 H₂O 2 g
 - Ascorbic acid 4 g
 - Nicotinic acid 0.8 g
- Make up to 200 ml
- Distribute into 5 ml tubes
- Freeze at -20°C.

1X Stainer Scholte medium

- Verify that the bottles have not been damaged during freezing.
- Use a waterbath at 37°C to thaw the 10X concentrated medium.
- Rinse all the labware to be used with double-distilled water.
- Use a 2 litre flask.
- Rinse before measuring out 1800 ml of sterile double-distilled water.
- Add 200 ml of thawed Stainer 10X medium.
- Cover with parafilm and homogenize the medium by inverting it several times.
- Distribute 200 ml of 1X medium into pre-rinsed 2 litre Erlenmeyer flasks.
- Sterilize in the autoclave (120°C for 20 minutes).

“Enriched” 1X Stainer: to be prepared freshly

- Enrich sterile 1X Stainer medium by adding 1X culture supplement.
 - **1X supplement:**
 - 100 mg Glutathione
 - 1 ml 10X supplement
 - 9 ml H₂O à filter through a 0.22 µm stericup
- “Enriched” sterile 1X Stainer
- Add 2 ml 1X supplement to 200 ml 1X Stainer and use immediately.

Annex 5:

Gram Stain determination

A.5.1 Material

- Staining vat
- Dissection forceps
- Microscope slides.

A.5.2 Reagents

- Gentian violet solution (**reagents**)
 - Gentian violet 1 g
 - Crystallized phenol acid 2 g
 - Ethanol 95° 10 ml
 - H₂O to make 100 ml
- Lugol solution (**reagents**)
 - Bisublimated iodine 1 g
 - Potassium iodide 2 g
 - H₂O to make 200 ml
- Ziehl Fuchsin solution (**reagents**)
 - Basic fuchsin 1 g
 - Crystallized phenol acid 5 g
 - Ethanol 95° 10 ml
 - H₂O to make 100 ml

A.5.3 Protocol

Staining of bacterial suspension is performed after isolation on agar on appropriate media: BGS or RL.

Working method

- Remove any grease from microscope slides by placing them in an ethanol (100%) bath.
- Wait for the slide to dry.
- Record the reference numbers of stains to be prepared.
- Spot the preparation onto the degreased microscope slide using a loop.
- Allow the smear to dry near the Bunsen flame.
- Fix the preparation in absolute alcohol above a sink.
- Flame the slide and then *allow it to cool*.
- Immerse the slide for 10 seconds in a staining vat containing *gentian solution*.
- Remove the slide from the staining vat using the dissecting forceps.
- Rapidly rinse the slide under running tap water.
- Immerse the slide for 15 seconds in a staining vat containing *Lugol solution*.
- Remove the slide from the Lugol bath.
- Rapidly wash the slide under running tap water.
- Decolorize using an ethanol wash bottle until the ethanol drops are clear of stain to the naked eye.
- As soon as the first stain-free drop appears halt the process by rinsing immediately and copiously with running water.
- Immerse the slide for 30 seconds in a staining vat containing *Ziehl Fuchsin solution (other alternative secondary stain can be used)*
- Remove the slide from this second staining fluid using the dissecting forceps.
- Wash with copious amounts of water as in the first staining procedure.
- Dry on absorbent paper.

A.5.4 Results

- Observe under the microscope with a 100X immersion lens: **Gram-positive** bacteria appear **violet** while **Gram-negative** bacteria appear **pink/red**.
- Record the outcome on the pallet card for the test strain.

<p><i>Bordetella</i> are Gram-negative bacteria and should therefore appear pink/red colour</p>
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Annex 6:

Kovac's oxidase test, nitrate-reductase-test, urease test, carbohydrates utilization test

A.6.1 Material

- Costar 5 ml plastic pipette
- Bacteriology incubator.

A.6.2 Reagents

- Saline solution: 9 g NaCl /litre of bi-distilled water
- Reagent pack API 20 E (ref 09567B – Biomerieux SA, 69280 Marcy l'Etoile, France)
- Tryptophan desaminase (TDA) reagent

Iron trichloride	3.4 g
H ₂ O	to make 100 ml
- Indole (IND) reagent

Paradimethylaminobenzaldehyde	5.0 g
Isoamyl alcohol	75 ml
37 % HCl	25 ml
- Voges Proskauer 1 (VP1) reagent

Potassium hydroxide	6 g
H ₂ O	to make 100 ml
- Voges Proskauer 2 (VP2) reagent

Alpha naphthol	40 g
Ethanol	100 ml
- Nitrate 1 (NIT1) reagent

Sulphanilic acid	0.8 g
5N acetic acid	100 ml
- Nitrate 2 (NIT2) reagent

N-N-dimethyl-1-naphtylamine	0.6 g
5N acetic acid	100 ml

A.6.3 Protocol

Preparation of the bacterial suspension

- Verify that the colonies grown on Bordet Gengou plates are haemolytic in appearance.
- Resuspend the plated bacteria in 4.5 ml of sterile saline solution to obtain a bacterial suspension with an optical density (OD) of 1.0 at 650 nm.

Seeding the API 20 E rack

The micro-tubes consist of a **tube** topped with a **cupule**.

- *Non-labelled* micro-tubes should be filled up to the level of the tube.
- *Boxed* micro-tubes should be entirely filled (tube and cupule).
- *Underlined* micro-tubes should be filled up to the level of the tube: the cupule is then filled with either paraffin oil, vaseline or glycerol in order to create the conditions for anaerobic growth.
- Fill the moat of the rack with 4 ml water in order to create a moist chamber and then close the rack case ensuring, if necessary, that it is properly sealed with adhesive tape in order to avoid evaporation during the incubation period.
- Incubate at 36°C for at least 48 hours in the incubation oven reserved for *Bordetella spp.*
- Add the reagents required for the following specific reactions before starting to read the following micro-tubes:

TDA:

 Add a drop of TDA reagent.

IND:

 Add a drop of IND reagent.

VP:

 Add a drop of reagents VP1 and VP2.

Nitrate NIT:

 Add a drop of reagents NIT1 and NIT2 to the GLU tube: in the event of a yellow colour indicating a negative reaction, add 3 mg of ZN reagent.

A.6.4 Results

Table of results

Tests	<i>B. pertussis</i>	<i>B. parapertussis</i>
ONPG	-	-
4.2.1.1 ADH	+/- (red)	-
4.2.1.2 LDC	-	-
4.2.1.3 ODC	-	-
CIT	-	-
4.2.1.4 H ₂ S	-	-
4.2.1.5 URE	-	+
TDA	-	-
IND	-	-
VP	+ (pink)	+
GEL	-	-
GLU	-	-
MAN	-	-
INO	-	-
SOR	-	-
RHA	-	-
NO ₃ -NO ₂	-	-

If API 20 E is not available, the priority is to test oxydase, urease, nitrate reductase, pigment expression, and the non-acidification of carbohydrates.

Annex 7:

Immunofluorescence

Immunofluorescence is a test that confirms the identification of the bacteria, since it allows the recognition of *B. pertussis* or *B. parapertussis* surface antigens using specific antisera.

A.7.1 Material

- Immunofluorescence slides
- 24 X 60mm coverslip
- Automatic P20 and P200 pipettes
- Spectrophotometer troughs
- Spectrophotometer
- Fluorescence microscope

A.7.2 Reagents

Products

- Acetone – CH_3COH_3 = 58.08
- Evans Blue – $\text{C}_{34}\text{H}_{24}\text{N}_6\text{Na}_4\text{O}_{14}\text{S}_4$ = 760.6 1% stock solution in 1X PBS, kept at +4°C, diluted to 1/100 for use i.e. 1/10 000 final solution
- Powdered milk
- Sodium and potassium phosphate pH 7.2 – PBS 10X (KCl 26.8 mM, KH_2PO_4 14.7 mM, NaCl 1.36 M, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 80.57 mM)
- Anti-*B. pertussis* rabbit antiserum (DIFCO) Becton Dickinson ref 223091
- Anti-*B. parapertussis* rabbit antiserum (DIFCO) Becton Dickinson ref 223101
- FITC labelled goat IgG anti-rabbit IgG (Biorad ref 75561)
- Reference strain *B. pertussis* Tohama I strain (strain that can be purchased from Institut Pasteur as CIP 8132) and *B. parapertussis* strain (that can be purchased from IP as CIP 63.2)
- Bacterial strains which identification have to be confirmed.

Solutions

- Saturation buffer: 1X PBS + 5% milk – prepare at the same time.
 - Powdered milk 5 g
 - PBS 10X 10 ml
 - distilled H₂O to make 100 ml

A.7.3 Protocol

Preparation of bacterial suspensions

After the *Bordetella* have been isolated on agar Bordet Gengou or Reagan Lowe medium, the bacterial suspensions are prepared by measuring the optical density at 650 nm. The optical density must be equal to 0.5 and is adjusted with physiological water.

1. Preparation of the antigen slides for immunofluorescence:

- Control slide:
 - Identify the microscope slides comprising 2 rows of 6 wells.
 - Deposit 5 µl of known reference bacterial suspension (Bp, Bpp) in wells of one slide as indicated in Figure A.1.
 - Wells 1,2,7,8: *Bordetella pertussis* (**Bp**) reference strain
 - Wells 3,4,9,10: PBS
 - Wells 5,6,11,12: *Bordetella parapertussis* (**Bpp**) reference strain

Figure A.1: Preparation of antigen control slide

1		2		3		4		5		6		
	Bp		Bp		PBS		PBS		Bpp		Bpp	
	Bp		Bp		PBS		PBS		Bpp		Bpp	
7		8		9		10		11		12		

- Deposit 5 µl bacterial suspensions, whose identification has to be confirmed as shown below.

Sample slide (see Figure A.2):

Figure A.2: Preparation of antigen sample slide

1		2		3		4		5		6	
	X1		X1		X1		X1		X1		X1
	X2		X2		X2		X2		X2		X2
7		8		9		10		11		12	

- Leave these bacterial suspensions to dry.
 - Fix the preparation by immersing the slide in a bath of cooled acetone (-20°C) for 10 minutes.
 - Withdraw the slide and allow the acetone to evaporate for 15 minutes under a chemical hood.
- NB: It is possible to store the fixed slides for 24 hours at +4°C or to freeze them at -20°C for several months after fixation.
- Wash the slide with 1X PBS pH 7.2 in order to eliminate excess acetone.
 - If the slide has been frozen, it is to be washed only after it has been thawed, i.e. after 15 minutes.

2. Incubation with the *B. pertussis* and *B. parapertussis* specific antisera

- Dilute the *B. pertussis* or *B. parapertussis* rabbit antisera to approximately 1/250 in saturation buffer (1X PBS + 5% milk) prepared at the same time, distribute 30 µl of the solution per well or 30 µl of buffer (PBS + 5% milk) in accordance with the following schema.

Control slide (see figure A.3):

Wells 1, 3, 5: *B. pertussis* rabbit antiserum

Wells 7, 9, 11: *B. parapertussis* rabbit antiserum

Wells 2, 4, 6, 8, 10 and 12: PBS + 5% milk

Figure A.3: Preparation of control slide for incubation with specific antisera

1		2		3		4		5		6	
	Anti-Bp		PBS		Anti-Bp		PBS		Anti-Bp		PBS
	Anti-Bpp		PBS		Anti-Bpp		PBS		Anti-Bpp		PBS
7		8		9		10		11		12	

Sample slide (see Figure A.4):

Wells 1, 2, 7, 8: *B. pertussis* rabbit antiserum

Wells 3, 4, 9, 10: *B. parapertussis* rabbit antiserum

Wells 5, 6, 11, 12: PBS + 5% milk

Figure A.4: Preparation of sample slide for incubation with specific antisera

1		2		3		4		5		6		
	Anti-Bp		Anti-Bp		Anti-Bp		Anti-Bp		PBS		PBS	
	Anti-Bp		Anti-Bp		Anti-Bp		Anti-Bp		PBS		PBS	
7		8		9		10		11		12		

- Leave these antibody solutions to dry.
- Incubate the slides for 30 minutes at 37°C in a damp chamber (a plastic box lined with moistened absorbent paper).
- Then eliminate the antisera with a jet of 1X PBS, directed so as to avoid mixing the contents of two wells.
- Wash twice using a 1X PBS bath for 5 minutes.
- Dry the slides.

3. Incubation with goat antibodies, specific of rabbit IgG, labelled with fluorescein

- Dilute the anti-rabbit IgG goat antibodies labelled with fluorescein to approximately 1/100 (it depends on the lot) in the presence of diluted Evans blue 1/100 in 1X PBS (from the stock).
- Calculate the volume required knowing that 30 µl of the 1/100 dilution has to be added in all slide wells.
- Incubate the slides for 30 minutes at 37°C in a damp chamber.
- Then eliminate the conjugate by absorption using absorbent paper.
- Wash twice in a bath of 1X PBS for 5 minutes.
- Dry the slides.

4. Preparation of the slides for microscope examination

- The mounting medium, 50% glycerol in 1X PBS, allows the slide to be fixed. Add two, three drops on the slides.
- Keep at +4°C in the dark by protecting the slides with an aluminium foil wrapping until microscope examination.
- Observe under the immunofluorescence microscope.

A.7.4 Results

Read the control slide first.

Wells 1 and 11 must be positive and all the other negative as represented below. Positivity means that bacterial suspension is fluorescent (green).

Control slide (see Figure A.5):

Figure A.5: results, control slide

1		2		3		4		5		6		
	+		-		-		-		-		-	
	-		-		-		-		+		-	
7		8		9		10		11		12		

If the control slide is confirmed, the other slides can be examined.

The bacteria fluorescent after incubation with the anti-*B. pertussis* antiserum are confirmed *B. pertussis* and the bacteria fluorescent after incubation with the anti-*B. parapertussis* antiserum are confirmed *B. parapertussis*.

Sample slide:

Fill out the corresponding form.

- Indicate the date on which fluorescence was read.
- Indicate the name or code of the sample.
- Give the experimenter's name and stamp.

Annex 8:

Serotyping of *B. pertussis*

Serotyping is the detection of the expression of the fimbriae 2 or 3 at the surface of the bacteria using monoclonal antibodies.

A.8.1 Material

- 96 well plate (V-bottom)
- Spectrophotometer
- Plate sealers 8.3 x 13.3 cm.

A.8.2 Reagents

Monoclonal antibodies F2β2G8 anti-Fim 2 (reagents can be purchased from the **National Institute for Biological Standards and Control**, Blanche Lane, South Mimms, Pottersbar, Hertfordshire EN6 30G, United Kingdom; contact point Dr Dororthy Xing).

Stock solution: a 15 mg/ml solution in glycerol/PBS 1 X 50% at -20°C.

For one month: a 4mg/ml solution in PBS 1X at 4°C. For agglutination techniques dilute the stock solution in PBS 1X on the day of the experiment according to the provider.

Monoclonal antibodies C10C2D5 anti-Fim 3 (reagents can be purchased from the **National Institute for Biological Standards and Control**, Blanche Lane, South Mimms, Pottersbar, Hertfordshire EN6 30G, United Kingdom; **contact point Dr Dororthy Xing**).

Stock solution: a 24 mg/ml solution in glycerol/PBS 1X 50% at -20°C.

For one month: a 4mg/ml solution in PBS 1X at 4°C. For agglutination techniques dilute the stock solution PBS 1X on the day of the experiment.

Reference strain: Bp 460 (reagents can be purchased *via* WHO). This reference strain is expressing Fim 2 and Fim 3 antigens.

A.8.3 Protocol

- Bacterial strains
 - All determinations are performed twice on the same plate.
 - 50 µl of bacterial suspension of strain Bp 460 with an $OD_{650\text{ nm}} = 1$ is placed in wells A1 to A6.
 - 50 µl of bacterial suspension of strain Bp 460 with an $OD_{650\text{ nm}} = 0.5$ is placed in wells A7 to A12.
 - Unknown samples are allocated to the wells of lines B to H: wells 1 to 6 are used for the bacterial strain sample with an $OD_{650} = 1$, and wells 7 to 12 are used for the bacterial strain sample with an $OD_{650} = 0.5$.
- F2β2G8 antibodies (anti-Fim 2)

50 µl anti-Fim 2 at the right concentration are distributed in wells 1, 2, 7 and 8 of lines A to H depending on the number of samples.
- C10C2D5 antibodies (anti-Fim 3)

50 µl anti-Fim 3 at the right concentration are distributed in wells 3, 4, 9 and 10 of lines A to H depending on the number of samples.
- PBS 1X (negative control)
 - 50 µl of PBS 1X are distributed in wells 5, 6, 11 and 12 of lines A to H.
 - Cover the plate with a plate case and leave overnight at 37°C.

A.8.4 Results

- Positive results are observed after formation of an antigen-antibody complex in the bottom of the well.
- Negative results are observed when bacteria sediment out at the bottom of the well without forming any antigen-antibody complexes.
- The plate should be read by two independent readers in order to validate a correct result.

Positive



Negative



Annex 9:

Storage of *Bordetella* spp. bacteria

When the bacteria have been identified, they must be stored for further analysis. The bacteria are stored in the medium described below at -80°C.

A.9.1. Material

- Cryotubes volume (2 ml)
- Petri dish
- Spectrophotometer OD_{650nm}
- Automatic pipetter, 200 µl and 1000 µl
- 1, 2, 5, 10 ml plastic pipettes
- Tips for 200 µl and 1000 µl automatic pipetters
- 1.6 ml polystyrene spectrophotometer cuvettes
- Laminar flow hood
- Plastic or glass rake
- 0.2 µm filter.

A.9.2 Reagents

Products

- 90° ethyl alcohol
- Sodium glutamate – C₅H₈NaNO₄ · H₂O
- Di-sodium hydrogen phosphate, dihydrous – Na₂HPO₄ · 2H₂O
- Di-sodium hydrogen phosphate, monohydrous – NaH₂PO₄ · H₂O
- Sodium and potassium phosphate pH 7.2 = PBS 10X: KCl 26.8 mM; KH₂PO₄ 14.7 mM; NaCl 1.36 M; Na₂HPO₄ · 7H₂O 80.57 mM
- Saccharose – C₁₂H₂₂O₁₁
- Bovine serum albumin (BSA).

Solutions

- 25 % BSA solution – storage period: 6 months at + 4°C:
 - Bovine serum albumin 25 g
 - PBS 1X pH 7.2 to make 100 mlSterilize by filtering through a 0.2 µm membrane.
- Saccharose–phosphate–glutamate (SPG) solution – storage period: 6 months at + 4°C:
 - Saccharose 85.6 g
 - Sodium glutamate 0.94 g
 - Na₂HPO₄ · 2H₂O 1.38 g
 - NaH₂PO₄ · H₂O 0.39 g
 - Adjust to pH 7.2
 - H₂O to make 1000 mlSterilize by filtering through a 0.2 µm membrane and store at +4°C.
- Solution for use: BSA/SPG – freshly prepare the volume required:
 - Dilute the BSA solution to 2.5 % with SPG buffer (same as SPG solution above).
 - Use this solution under very strict conditions of sterility.

A.9.3. Protocol

Freezing

Day 0

- Take bacteria from isolated colonies (72 h growth for *B. pertussis* and *B. paraptussis*) in order to prepare a bacterial suspension.
- Measure OD_{650 nm}
- With the appropriate medium, and using the same batch of agar medium dishes, perform two layered cultures and an isolation by spreading 100 µl of a bacterial suspension with OD_{650 nm} = 1 using a sterile loop spreader.
- Incubate dishes at 37°C for 24 to 48 h.

Day 1 or day 2:

- Prepare a freezing bath filled with a mixture of dry ice and alcohol.
- Take the first dish of agar medium in its entirety and put it in 5 ml of sterile BSA/SPG buffer.
- Allocate 1 ml to each of 5 cryotubes identified with the number of the isolate, the species code (see below), the date of freezing, the freezing medium used: BSA/SPG.
 - *Bordetella pertussis*: **Bp**
 - *Bordetella paraptussis*: **Bpp**

-
- Freeze the tubes rapidly by placing them in the freezing bath.
 - Inscribe the type and storage site of isolates in the “strains in” log book and in the file for the strains concerned (isolates of *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and other Bordetellae).
 - Note: The following procedure is strongly recommended:
 - **In parallel, remove all of the second layer and put it into 5 ml of sterile saline solution, measure and record the OD_{650 nm}, then adjust to OD = 1.0 in order to obtain X ml which can be used for various analyses such as adenyl cyclase haemolysin assay and immune blot tests.**

Storage

Distribute the tubes to different storage sites.

- Place 2 cryotubes in the freezer at -80°C for everyday use and producing secondary batches.
- Place 2 cryotubes in the freezer at -80°C for producing the primary batch, not intended for distribution.
- Place 1 cryotube in liquid nitrogen at -196°C for producing the primary batch, not intended for distribution.

Annex 10:

Realtime PCR assay using Lightcycler® technology for amplification of the insertion element IS481 of *Bordetella pertussis*

The PCR format, using Lightcycler® technology, is based on a speed realtime detection of the amplification product, a 181 pb fragment of IS481. Two hybridization probes were employed, allowing sequence-specific detection by using fluorescence energy transfer between the fluorophores conjugated to the probes. The amount of fluorescence is directly proportional to the amount of target DNA generated during the PCR process.

This protocol is very sensitive but lacks specificity in terms of *Bordetella* species. In fact, this PCR detects also *Bordetella holmesii*, *Bordetella petrii* and *Bordetella bronchiseptica* isolates.

A.10.1. Material

- Lightcycler instrument (Roche)
- Lightcycler capillaries (Roche)
- Lightcycler centrifuge adapters: 32 adapters in an aluminium cooling block (Roche)
- Standard benchtop microcentrifuge
- 1.5 ml Eppendorf caps, sterile
- Freezer (-20°C)
- Refrigerator +4°C
- Automatic pipetters.

A.10.2. Reagents

- Lightcycler FastStart DNA Master PLUS Hybridization Probes kit (Roche)
 - Lightcycler FastStart Enzyme (1a), (store at -15°C to -25°C and avoid repeated freezing and thawing)
 - Lightcycler FastStart Reaction Mix Hybridization Probes (1b), containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix and 10 mM MgCl₂ (store at -15°C to -25°C and avoid repeated freezing and thawing)
 - H₂O PCR grade.

-
- Primers and probes
 - Forward primer (BP-1) 5' – gAT TCA ATA ggT TgT ATg CAT ggT T
 - Reverse primer (BP-2) 5' – T^TC Agg CAC ACA AAC TTg ATg ggC g
 - Probe 1 (BP-FLU) 5' – TCg CCA ACC CCC CAg TTC ACT CA-F
 - Probe 2 (BP-LCR) 5' – LC-RED-640-AgC CCg gCC ggA TgA ACA CCC-P
 - Uracil-DNA glycosylase (uNG), heat-labile (Roche)
 - QIAamp DNA Mini Kit (Qiagen) or High Pure PCR Template Preparation Kit (Roche)
 - Reference strain: Tohama I (this strain can be purchased from Institut Pasteur as CIP 81.32).

Fluidifiant preparation

- Sodium citrate (2.9%) 5 ml
- N-actyl L-cysteine 0.05g
- H₂O to make 10 ml

A.10.3. Sample treatment

If necessary, respiratory samples are liquefied with fluidifiant preparation volume to volume before DNA extraction.

Nucleic acids from respiratory samples (swabs, nasopharyngeal aspirations, sputum) are purified with QIAamp DNA Mini Kit (Qiagen) or with High Pure PCR Template Preparation Kit (Roche), according to the manufacturer's recommendations.

A.10.4. Experimental protocol

Preparation of reagents

- Lightcycler FastStart DNA Master PLUS Hybridization Probes: transfer 60 µl from vial 1b into vial 1a, gently mix by pipetting up and down (do not vortex).
- Store at -15°C to -25°C for a maximum of 3 months and avoid repeated freezing and thawing.
- Primers BP-1 and BP-2 are diluted to 10 pmol/µl with H₂O PCR grade.
- Probes BP-FLU and BP-LCR are diluted to 4 pmol/µl with H₂O PCR grade.

Preparation of master mix (volumes are given for one sample)

- Prepare a master mix by multiplying the amount of mix for one test by the number of reactions to be cycled, plus one additional reaction.
- In a 1.5 ml reaction tube on ice, add the components given in Table A.1.

Table A.1: Preparation of master mix

Reagent	Volume	Final concentration
H ₂ O PCR grade	6 µl	
Master mix 5X	4 µl	
Primer BP-1 (10 pmol/µl)	1 µl	0.5 pmol/µl
Primer BP-2 (10 pmol/µl)	1 µl	0.5 pmol/µl
Probe BP-FLU (4 pmol/µl)	1 µl	0.2 pmol/µl
Probe BP-LCR (4 pmol/µl)	1 µl	0.2 pmol/µl
UNG (1µ/µl)	1 µl	1 µ
Total volume	15 µl	na

na = not available/not applicable

- Mix carefully.
- Pipet 15 µl master mix into pre-cooled Lightcycler capillaries.
- Add 5 µl of the DNA template.
- For sample, prepare 2 capillaries, in the first one, pipet 5 µl of extracted DNA and in the second one, pipet 5 µl of 1 : 10 diluted extracted DNA (for detecting potential inhibitors).
- For negative control, pipet 5 µl H₂O PCR grade into a capillary and, for positive control, pipet 5 µl of *Bordetella pertussis* strain CIP 8132 DNA (extracted from 400 µl of OD_{650nm} bacterial suspension) corresponding to 50 pg.
- Seal each capillary with a stopper and place the adapters, containing the capillary, into a standard benchtop centrifuge. Centrifuge at 700g for 5 s.
- Place the capillaries in the rotor of the Lightcycler instrument.
- Cycle the samples as described below (tables A.2, A.3 and A.4).

Thermocycler conditions

Table A.2: Program 1, denature

Cycle program data	Value
Cycles	1
Analysis mode	None
Temperature targets	Segment 1
Target temperature (°C)	95
Incubation time (hrs:min:sec)	0:10:00
Temperature transition rate (°C/sec.)	20.0
Second target temperature (°C)	0
Step size (°C)	0.0
Step delay (cycles)	0
Acquisition mode	None

Table A.3: Program 2, amplification

Cycle program data	Value		
Cycles	40		
Analysis mode	Quantification		
Temperature targets	Segment 1	Segment 2	Segment 3
Target temperature (°C)	95	60	72
Incubation time (hrs:min:sec)	10	10	20
Temperature transition rate (°C/sec.)	20.0	20.0	20.0
Second target temperature (°C)	0	0	0
Step size (°C)	0.0	0.0	0.0
Step delay (cycles)	0	0	0
Acquisition mode	None	Single	None

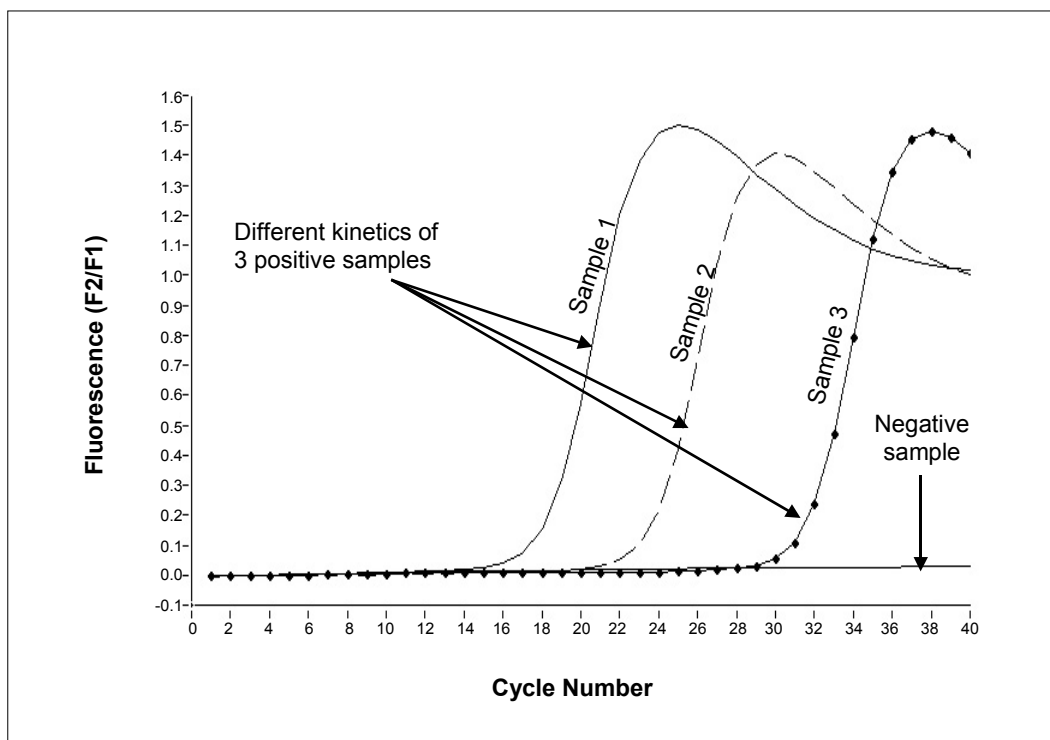
Table A.4: Program 3, cooling

Cycle program data	Value
Cycles	1
Analysis mode	None
Temperature targets	Segment 1
Target temperature (°C)	40
Incubation time (hrs:min:sec)	0:02:00
Temperature transition rate (°C/sec.)	20.0
Second target temperature (°C)	0
Step size (°C)	0.0
Step delay (cycles)	0
Acquisition mode	None

A.10.5. Results

Analysis data are interpreted according to the amplification plot. Samples are regarded as “positive”, when the fluorescence signal increases and shows a typical amplification kinetic. Samples are regarded as “negative” when they do not fulfill the criteria mentioned above. See Figure A.6.

Figure A.6: Amplification kinetics of three positive samples



Annex 11:

Realtime PCR assay using Lightcycler® technology for amplification of the insertion element IS1001 of *Bordetella parapertussis*

The PCR format, using Lightcycler technology, is based on a speed realtime detection of the amplification product, a 464 pb fragment of IS1001. Two hybridization probes were employed, allowing sequence-specific detection by using fluorescence energy transfer between the fluorophores conjugated to the probes. The amount of fluorescence is directly proportional to the amount of target DNA generated during the PCR process.

This protocol is very sensitive but lacks specificity in terms of *Bordetella* species. In fact, this PCR detects also *B. bronchiseptica* isolates.

A.11.1. Materials

- Lightcycler instrument (Roche)
- Lightcycler capillaries (Roche)
- Lightcycler centrifuge adapters: 32 adapters in an aluminium cooling block (Roche)
- Standard benchtop microcentrifuge
- 1.5 ml eppendorf caps, sterile
- Freezer (-20°C)
- Refrigerator +4°C
- Automatic pipetters.

A.11.2. Reagents

- Lightcycler FastStart DNA Master PLUS Hybridization Probes kit (Roche)
 - Lightcycler FastStart Enzyme (1a), (store at -15°C to -25°C and avoid repeated freezing and thawing)
 - Lightcycler FastStart Reaction Mix Hybridization Probes (1b), containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix and 10 mM MgCl₂ (store at -15°C to -25°C and avoid repeated freezing and thawing)
 - H₂O PCR grade.

- Primers and probes
 - Forward primer (BPa-1) 5'– CAC CgC CTA CgA gTT CgA gAT
 - Reverse primer (BPa-2) 5'– CCT CgA CAA TgC Tgg TgT TCA
 - Probe 1 (BPa-FLU) 5'– gTT CTA CCA AAg ACC TgC CTg ggC-F
 - Probe 2 (BPa-LCR) 5' – LC-RED-705-AgA CAA gCC Tgg AAC CAC Tgg TAC-P
- Uracil-DNA glycosylase (UNG), heat labile (Roche)
- QIAamp DNA Mini Kit (Qiagen) or High Pure PCR Template Preparation Kit (Roche)
- Reference strain: Tohama I (this strain can be purchased from Institut Pasteur as CIP 81.32).

Fluidifiant preparation

- Sodium citrate (2.9%) 5 ml
- N-actyl L-cysteine 0.05 g (sigma A7250)
- H₂O to make 10 ml

A.11.3. Sample treatment

If necessary, respiratory samples are liquefied with fluidifiant preparation volume to volume before DNA extraction.

Nucleic acids from respiratory samples (sputum, nasopharyngeal aspirations) are prepared with QIAamp DNA Mini Kit (Qiagen) or with High Pure PCR Template Preparation Kit (Roche), according to the manufacturer's recommendations.

A.11.4. Experimental protocol

Preparation of reagents

- Lightcycler FastStart DNA Master PLUS Hybridization Probes: transfer 60 µl from vial 1b into vial 1a, gently mix by pipetting up and down. (Do not vortex.)
- Store at -15°C to -25°C for a maximum of 3 months and avoid repeated freezing and thawing.
- Primers BPa-1 and BPa-2 are diluted to 10 pmol/µl with H₂O PCR grade.
- Probes BPa-FLU and BPa-LCR are diluted to 4 pmol/µl with H₂O PCR grade.

Preparation of master mix (volumes are given for one sample)

- Prepare a master mix by multiplying the amount of mix for one test by the number of reactions to be cycled, plus one additional reaction.
- In a 1.5 ml reaction tube on ice, add the following components (Table A.5):

Table A.5: Components for preparation of master mix

Reagent	Volume	Final concentration
H ₂ O PCR grade	6 µl	na
Master mix 5X	4 µl	na
Primer BPa-1 (10 pmol/µl)	1 µl	0.5 pmol/µl
Primer BPa-2 (10 pmol/µl)	1 µl	0.5 pmol/µl
Probe BPa-FLU (4 pmol/µl)	1 µl	0.2 pmol/µl
Probe BPa-LCR (4 pmol/µl)	1 µl	0.2 pmol/µl
UNG (1µ/µl)	1 µl	1 µ
Total volume	15 µl	na

na = not available/not applicable

- Mix carefully.
- Pipet 15 µl master mix into pre-cooled Lightcycler capillaries.
- Add 5 µl of the DNA template.
- For sample, prepare 2 capillaries, in the first one, pipet 5 µl of extracted DNA and in the second one, pipet 5 µl of 1 : 10 diluted extracted DNA (for detecting potential inhibitors).
- For negative control, pipet 5 µl H₂O PCR grade into a capillary and for positive control, pipet 5 µl of *Bordetella parapertussis* strain CIP 12822 DNA (extracted from 400 µl of OD_{650nm} bacterial suspension) corresponding to 50 pg.
- Seal each capillary with a stopper and place the adapters, containing the capillary, into a standard benchtop centrifuge. Centrifuge at 700g for 5 s.
- Place the capillaries in the rotor of the Lightcycler instrument.
- Cycle the samples as described below (tables A.6, A.7 and A.8).

Thermocycler conditions

Table A.6: Program 1, denature

Cycle program data	Value
Cycles	1
Analysis mode	None
Temperature targets	Segment 1
Target temperature (°C)	95
Incubation time (hrs:min:sec)	0:10:00
Temperature transition rate (°C/sec.)	20.0
Second target temperature (°C)	0
Step size (°C)	0.0
Step delay (cycles)	0
Acquisition mode	None

Table A.7: Program 2, amplification

Cycle program data	Value		
Cycles	40		
Analysis mode	Quantification		
Temperature targets	Segment 1	Segment 2	Segment 3
Target temperature (°C)	95	60	72
Incubation time (hrs:min:sec)	10	10	20
Temperature transition rate (°C/sec.)	20.0	20.0	20.0
Second target temperature (°C)	0	0	0
Step size (°C)	0.0	0.0	0.0
Step delay (Cycles)	0	0	0
Acquisition mode	None	Single	None

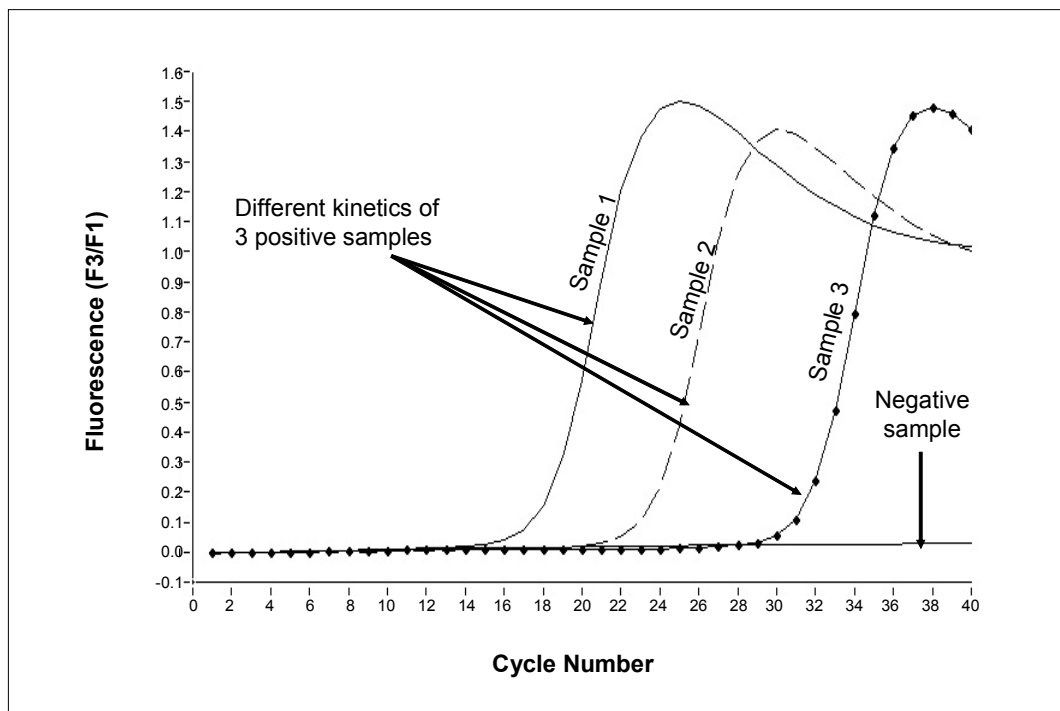
Table A.8: Program 3, cooling

Cycle program data	Value
Cycles	1
Cycles	1
Analysis mode	None
Temperature targets	Segment 1
Target temperature (°C)	40
Incubation time (hrs:min:sec)	0:02:00
Temperature transition rate (°C/sec.)	20.0
Second target temperature (°C)	0
Step size (°C)	0.0
Step delay (Cycles)	0
Acquisition mode	None

A.11.5. Results

Analysis data are interpreted according to the amplification plot. Samples are regarded as “positive”, when the fluorescence signal increases and shows a typical amplification kinetic. Samples are regarded as “negative”, when they do not fulfill the criteria mentioned above. See Figure A.6.

Figure A.7: Amplification kinetics of three positive samples



Annex 12:

Measurement of antitoxin and anti-adhesins by ELISA

This ELISA technique allows the detection of anti-pertussis toxin (anti-PT), anti-filamentous haemagglutinin (anti-FHA) or anti-pertactin (anti-PRN) antibodies in the serum of a patient.

A.13.1. Material

1. Microplate reader

Routine cleaning once a week using the maintenance programme. Document in logbook.

Filter control:

- Measure OD 405 without microplate: 0.000
- Measure OD 405 with microtiter plate: 0.030–0.040
- Measure OD 405 with microtiter plate with water: 0.040–0.050

Document in a logbook once yearly.

2. Scales

External service, calibration and adjustment once yearly by supplier.

3. Automatic pipetters

- 5–40 µl, 0.5–10 µl, 100 µl, multichannel 12: 50–300 µl, multichannel 5–50 µl
- Tips and syringes as recommended by the manufacturer
- Internal maintenance and calibration every 6 months. Document in a logbook.

4. Incubator

Temperature is read every working day and documented in the logbook.

5. Microplatewasher

Maintenance should be according to the instruction manual.

6. Refrigerator

Maintenance should be according to the instruction manual.

7. Freezers

-20°C and -80°C

Temperature is verified every working day and documented in the logbook.

-
8. Tubes
Eppendorf 1.5 ml.
 9. Plates
 - Nunc maxisorp certified
 - Nunc microwell v certified.
 10. References
Reference enzyme-conjugated goat anti-human IgG antiserum: Kirkegaard & Perry laboratories, 075-1002.
Reference substrate, Sigma, N2765

A.13.2 Reagents

1. Sera
All test reference sera are kept at -80°C .
2. Antigens
Pertussis toxin, FHA or PRN can be purchased *via* reference laboratories such as Institut Pasteur, 25–28 rue du Dr Roux, 75724 Paris, cedex 15.
3. Calibrators
Patients' sera are stored at -80°C for long-term storage, and up to one week at 4°C for the experiment.
Human reference serum: Batch 3 FDA (200 EU/ml for PT and FHA antibodies) and batch 4 FDA (90 EU/ml for PRN). These reagents can be purchased via Food and Drug Administration (FDA), 5600 Fishers Lane, Rockville, Maryland 20857, USA.
4. Controls
In house, positive and negative reference human sera from infected individuals.
5. Conjugate
Goat anti-human IgG (1 mg) labelled with phosphatase alkaline is resuspended in 1 ml 50% glycerol, and diluted 1/20000 in incubation buffer before use.
Substrate of phosphatase alkaline.
6. Buffers and solutions
 - **Carbonate buffer:** Na_2CO_3 0.05M, pH 9.6 (to be done every 2 weeks)

Na_2CO_3	0.795 g
NaHCO_3	1.465 g
H_2O	to make 300 ml
pH	9.6
Distilled H_2O	to make 500 ml
20 min at 120°C	

- **Phosphate buffered saline 10X:** NaCl 1.45M, NaH₂PO₄-H₂O 0.085M, NaH₂PO₄-7H₂O 0.015M, pH 7.4 or pH 6.8 (to be done every month)

NaCl	85.00 g
Na ₂ HPO ₄ -2H ₂ O	22.79 g
NaH ₂ PO ₄ -H ₂ O	2.07 g
H ₂ O	700 ml
pH	7.4 or 6.8
Distilled H ₂ O	to make 1000 ml

20 min at 120°C

- **Substrate buffer:** Tris 1M, MgCl₂ 0.3 mM, pH 9.8 (to be done every two weeks)

Tris	121.1 g
H ₂ O	700 ml
MgCl ₂ 1M (with MgCl ₂ · 6H ₂ O)	0.3 ml
pH with HCl 6N	9.8
Distilled H ₂ O	to make 1000 ml

20 min at 120°C

- **Incubation buffer:** SAB 0.5%; Tween 20 0.5%; PPG 0.005%; PBS 1X (to be done every 2 weeks)

PBS 1X	1 litre
Serum albumine bovine	5 g
Tween-20	5 ml
Polyethylene glycol (PEG)	50 µl

- **Wash buffer 10X :** NaCl 1.45 M, Tween-20 5% (to be done every month)

NaCl	85 g
Tween 20	50 ml
Distilled H ₂ O	to make 1000 ml

A.13.3 Protocol

1. Coating of plates

Add 100 µl of diluted antigen (2 µg/ml solution of antigen; concentration depending on the antigen used) to all 96 wells of a microtiter plate. Seal to prevent evaporation. Incubate at +28°C overnight (16–24 h).

2. Sample addition

Prepare 8 two-fold dilutions of sera in incubation buffer. Initial dilution is 1 : 60 for routine sera. After serum dilutions are completed, wash a coated assay plate with wash buffer (4 washes of 250 µl). As soon as possible, transfer 50 µl of diluted serum to the appropriate wells of the coated and rinsed assay plate containing 50 µl of buffer. Seal plates, and incubate for 2 hours at +28°C. Sera are dispensed to plates as follows:

-
- Column 1: Buffer control (incubation buffer)
Column 2: Reference serum
Column 3: Reference serum
Column 4: Control serum positive or negative
Columns 5–12: Test sera

RECORDS: One set of dilutions is made for the reference serum. This set of dilutions is used for all assay plates.

3. Addition of goat anti-human IgG

The labelled goat anti-human IgG antiserum is diluted in incubation buffer (around 1/20000; this depends on the lot and has to be tested before). Plates are rinsed with 250 µl of wash buffer. Plates are inverted and tapped to clean absorbent towels to remove all wash buffer. 100 µl of diluted labelled goat antiserum are added to all 96 microplate wells. Seal and incubate at +28°C overnight (16–24 hours).

4. Substrate addition

Substrate buffer is brought to room temperature prior to use. A 1 mg/ml solution of PNPP (phosphatase alkaline substrate) in substrate buffer is prepared just prior to use. Plates are rinsed with 250 µl wash buffer. Plates are inverted and tapped onto clean absorbent towels to remove all wash buffer. 100 µl of PNPP substrate solution is added immediately after. Time of substrate addition is recorded. Incubate at room temperature (20°–25°C) for exactly 60 min. If desired, colour reaction can be stopped by adding 50 µl of 5N NaOH to each well.

5. Measurement of absorbance

Use spectrophotometer to read absorbance at 405 nm wavelength. Linear range of the instrument is between 0.1 and 2.

6. Quantification of results

ELISA units for each sample are computed, based on comparison of the response curve of the test serum to that of the reference serum. The reference line calculation program developed by R. Mollby and I. Kuhn is currently used (Unit Calc software). This software can be purchased via PhPlate Microplate techniques AB, Nobels väg 12A, SE-171 77 Stockholm, Sweden – Tel: +46 (0)8 318 002, Fax: +46 (0)8 345 704.

7. Criteria for approval of ELISA results

The units are calculated when the following criteria are met:

- A line can be drawn from the dose response curve using at least 4 dilution points.
- The regression coefficient thus obtained is not lower than 0.95.
- The slope of the test serum line is not lower than 0.5 or higher than twice that of the slope of the reference line.

When curves do not meet the above criteria (as with many negative sera), the event is signalled by the computer. The computer also provides a warning if a higher serum dilution generates a higher absorbance value than previous dilution(s). In these cases, points may be excluded manually, after which new calculations can be performed.

8. Criteria for retesting of sera

A control curve is modelled for the reference serum and control serum. Cut-off limits are determined as the total mean \pm 2SD for the mean values of each serum over 15 consecutive experiments.

- If the mean of the controls exceeds cut-off limits the test is repeated. However, if one control diverges from the others in such a way that the mean calculated without it is within limits, only sera paired (or connected to) the diverged plate are retested.
- If the control of a single plate exceeds cut-off limits, paired sera are retested. However, if controls are grouped close to the limit, and the mean lies within limits, all plates are accepted.
- If the reference is outside specified limits, plating is repeated.
- If an obvious technical defect has occurred, plating is repeated.
- If the background is higher than 0.15, plating is repeated.

9. Determination of the minimum level of detection (MLD)

Individual data from 20 separate assays are plotted in order to estimate the MLD.

A positive serum is one containing at least 4 times the MLD in view of the fact that for such samples the coefficient of variation is less than 25%.

10. Interpretation of results for diagnostic

Very low amounts of antibodies (< 5 EU or incalculable by the computer) are considered as 2 EU.

Very high amount of antibodies must be redetermined with a 10-fold dilution.



The World Health Organization has managed cooperation with its Member States and provided technical support in the field of vaccine-preventable diseases since 1975. In 2003, the office carrying out this function was renamed the WHO Department of Immunization, Vaccines and Biologicals.

The Department's goal is the achievement of a world in which all people at risk are protected against vaccine-preventable diseases. Work towards this goal can be visualized as occurring along a continuum. The range of activities spans from research, development and evaluation of vaccines to implementation and evaluation of immunization programmes in countries.

WHO facilitates and coordinates research and development on new vaccines and immunization-related technologies for viral, bacterial and parasitic diseases. Existing life-saving vaccines are further improved and new vaccines targeted at public health crises, such as HIV/AIDS and SARS, are discovered and tested (Initiative for Vaccine Research).

The quality and safety of vaccines and other biological medicines is ensured through the development and establishment of global norms and standards (Quality Assurance and Safety of Biologicals).

The evaluation of the impact of vaccine-preventable diseases informs decisions to introduce new vaccines. Optimal strategies and activities for reducing morbidity and mortality through the use of vaccines are implemented (Vaccine Assessment and Monitoring).

Efforts are directed towards reducing financial and technical barriers to the introduction of new and established vaccines and immunization-related technologies (Access to Technologies).

Under the guidance of its Member States, WHO, in conjunction with outside world experts, develops and promotes policies and strategies to maximize the use and delivery of vaccines of public health importance. Countries are supported so that they acquire the technical and managerial skills, competence and infrastructure needed to achieve disease control and/or elimination and eradication objectives (Expanded Programme on Immunization).

Department of Immunization, Vaccines and Biologicals

Family and Community Health

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Email: vaccines@who.int

or visit our web site at: <http://www.who.int/vaccines-documents>



World Health
Organization