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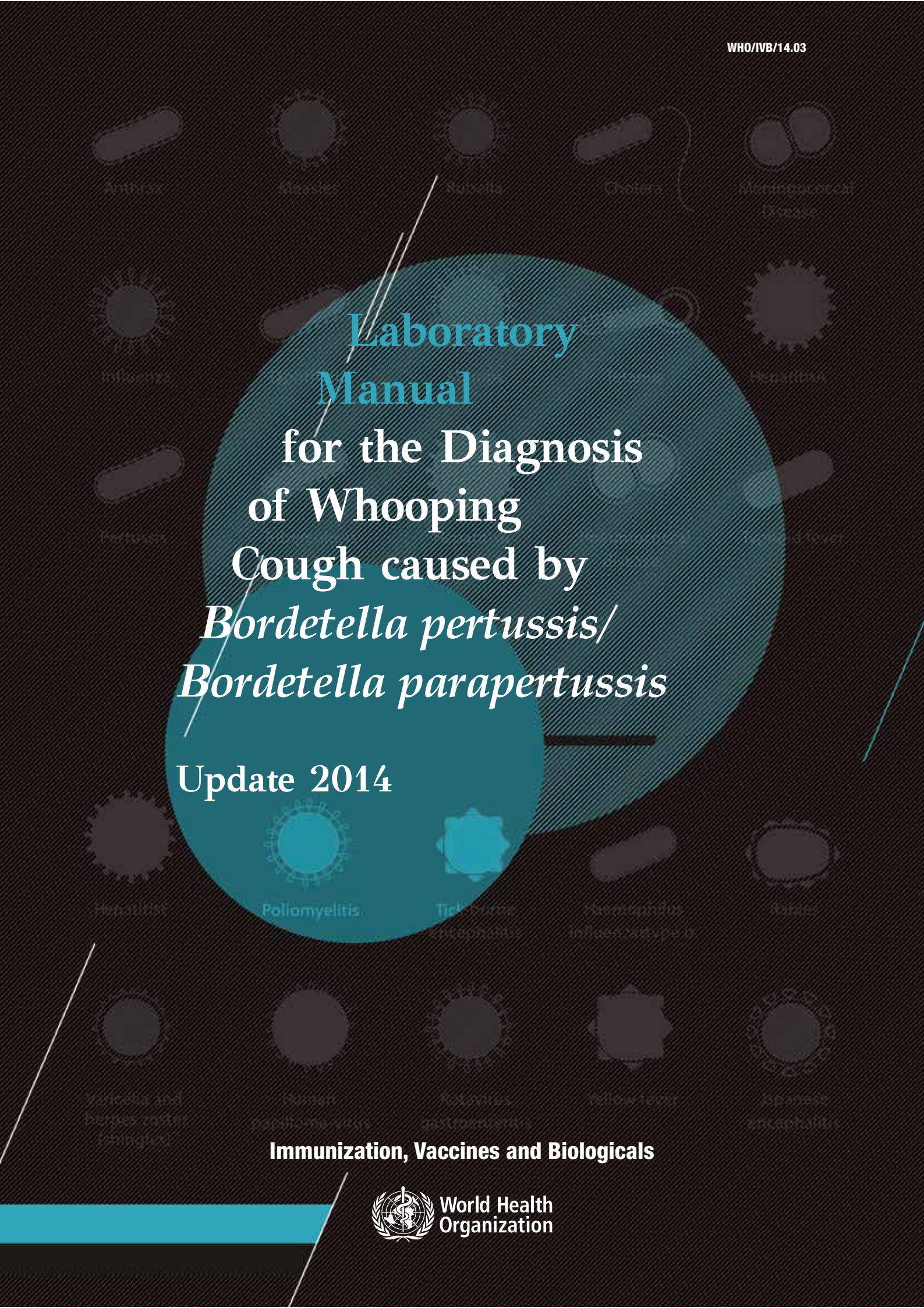

Laboratory Manual for the Diagnosis of Whooping Cough caused by *Bordetella pertussis*/ *Bordetella parapertussis*

Update 2014

Immunization, Vaccines and Biologicals

World Health Organization

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Abbreviations & acronyms

AC-Hly	adenylate cyclase-haemolysin toxin
Ap	acellular pertussis
BG	Bordet Gengou (medium)
BGB	Bordet Gengou with blood
BSA	bovine serum albumin
DFA	direct fluorescent antibody
DNA	deoxyribonucleic acid
DTaP	diphtheria-tetanus acellular pertussis
DTP	diphtheria-tetanus-pertussis
DTwP	diphtheria-tetanus-whole cell pertussis
ELISA	enzyme-linked immunosorbent assay
EQA	external quality assessment
FHA	filamentous haemagglutinin
HPLC	high-performance liquid chromatography
Ig	immunoglobulin
IPC	internal process control
MLD	minimum level of detection
NIBSC	National Institute for Biological Standards and Control
NPA	nasopharyngeal aspirates
NPS	nasopharyngeal swabs
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel-electrophoresis
PRN	pertactin
PT	pertussis toxin

RL	Reagan Lowe (medium)
RSV	respiratory syncytial virus
RT-PCR	real-time polymerase chain reaction
SPG	saccharose–phosphate–glutamate (solution)
Spp.	species
TCF	tracheal colonization factor
TCT	tracheal cytotoxin
UNG	uracil-DNA glycosylase
USA	United States of America
Vag	vir-activated genes
Vrg	vir-repressed genes
WHO	World Health Organization
wP	whole-cell pertussis

1. Introduction

Whooping cough is a worldwide infectious disease caused by the bacteria *Bordetella pertussis* and *Bordetella parapertussis*. It is a respiratory disease occurring after transmission of the bacteria from person- to-person 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 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 also be very severe for children and adults. For over 40 years, whole-cell pertussis vaccines have been very effective, preventing around 760 000 deaths worldwide every year. Nevertheless, pertussis disease continues to impose a high burden — there are still 50 million cases of pertussis disease and 300 000 deaths annually, 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 (whole-cell 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 diphtheria-tetanus-pertussis (DTP) vaccine, and their efficacy, should be assessed by national programmes. In the United States of America (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.

Clinical symptoms. In non-immunized persons and non-immune patients, the classical disease follows three phases. The first, called the catarrhal phase, starts after an incubation period of 7–10 days (range: 4–28 days) with non-specific symptoms, such as rhinorrhoea, sneezing and non-specific coughs. Typically, the patients do not develop fever. The second, paroxysmal phase, is characterized by the specific symptoms of pertussis, such as coughing spasms, inspiratory whooping and vomiting.

During the third, convalescent phase, the coughing attacks slowly decrease in frequency. Cases in neonates and unvaccinated young infants often present with non-specific coughs and apnoea as the only symptoms. In older, vaccinated schoolchildren, adolescents and adults, the symptoms can vary widely. Adult pertussis is often associated with a long illness and the persistent cough is often paroxysmal and has a mean duration of approximately six weeks. It is frequently accompanied by choking, vomiting and by whooping. Various case definitions for pertussis are used; the World Health Organization (WHO) definition requires 21 days or more of paroxysmal coughing, thus defining a severe course of the disease[1]. Various other case definitions exist that have been summarized recently[2] . The disease is most dangerous in infants and most hospitalizations and deaths occur in this age group. Fatal cases of the disease may go undetected in young infants.

Pertussis-like symptoms may also be caused by adenoviruses, respiratory syncytial virus (RSV), human parainfluenza viruses, influenza viruses, *Mycoplasma pneumoniae* and other agents, and so laboratory confirmation is important.

1.2 Bordetella species

Bordetella species are gram-negative coccobacilli bacteria and the species can be distinguished based on a number of biochemical characteristics, as 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[3]. Other Bordetella Spp. rarely isolated in humans include *B.hinzii, B.trematum, B.petrii, B.avium and "B.ansorpii"*.

B. pertussis, *B. parapertussis* and *B. bronchiseptica* are similar species but *B. parapertussis* and *B. bronchiseptica* lack the production of the pertussis toxin due to mutation in the promoter region of the genes encoding this toxin[4]. *B. holmesii* is a species not producing the virulence factors produced by the other three species[5,6]. Differentiation between *B. parapertussis* and *B. pertussis* is based on culture, biochemical and immunologic differences (Table 2).

1 World Health Organization, Department of Vaccines and Biologicals. Pertussis surveillance: a global meeting (WH/V&B/01.19). Geneva: World Health Organization; 2001

2 Cherry JD et al., Clinical definitions of pertussis: Summary of a Global Pertussis Initiative roundtable meeting, February 2011. Clin Infect Dis. 2012;54(12):1756–64.

3 Diavatopoulos et al. Bordetella pertussis, the Causative Agent of Whooping Cough, Evolved from a Distinct, Human-Associated Lineage of B. bronchiseptica PLoS Pathog. 2005

4 Parkhill J. et al. Comparative analysis of the genome sequences of Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Nat Genet, 35(1), 32-40 (2003).

5 Bouchez V. et al. Bordetella holmesii: comparison of two isolates from blood and a respiratory sample. Adv Infect Dis (2013) 3(2):123-133

6 Paul J. et al. Bordetella holmesii: initial genomic analysis of an emerging opportunist Pathogens and Diseases Volume 67, Issue 2, pages 132–135, March 2013

Table 1: Members of the genus *Bordetella*

Species	Host	Transmission	Disease
B. pertussis	Humans	Droplets	Pertussis
B. parapertussis	Humans	Droplets	Parapertussis (pertussis-like disease)
	Sheep	Unknown	Respiratory disease
B. holmesii	Humans	Droplets (?)	Respiratory disease?
	Humans	Unknown	Systemic infection (immunocompromised hosts)
B. bronchiseptica	Animals	Droplets (?)	Respiratory disease
	Humans	Droplets (?)	Respiratory disease
			Systemic infection (immunocompromised hosts)

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. (For reviews: Mattoo, S. et al. *Clin Microb Rev*. 2005[7]; Hegerle, N. et al. *Future Microbiol*. 2013[8]).

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 autotransporter pertactin (PRN) and tracheal colonization factor (TCF). Both proteins are also able to bind phagocytic cells via their Arginylglycylaspartic acid (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.

[7] Mattoo S. et al. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin Microbiol Rev. 2005 Apr;18(2):326-82.

[8] Hegerle N. et al. Epidemiology of whooping cough & typing of Bordetella pertussis. Future Microbiol. 2013 Nov;8(11):1391-403

Table 2: Useful characteristics for differentiating *Bordetella* species

	B. pertussis	B. parapertussis	B. bronchiseptica	B .avium	B. ansorpii	B. hinzii	B. holmesii	B. petrii	B. trematum
Growth on:									
RL medium	+ (3–4d)	+ (2–3d)	+ (1–2 d)	ND	ND	ND	ND	ND	ND
Columbia	-	v	+	+	+	+	+	+	+
MacConkey	-	-	+	+	+	+	(+)	+	+
Oxidase	+	-	+	+	+	+	-	+	-
Motility	-	-	+	-	v	+	-	-	+
Pigment	-	brown	-	-	-	-	-	yellow	yellow

ND: not determined; RL = Regan-Lowe medium.

+ ≥ 90% of the strains are positive.

- ≤ 10% of the strains are positive

v 10–89% of the strains are positive.

1.2.2 Toxins

B. pertussis expresses different toxins in addition to adhesins.

Tracheal cytotoxin (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. This toxin is not expressed by *B. parapertussis*.

The toxin adenylate cyclase-haemolysin (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 *and* Bordetella parapertussis *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 then 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 the absence of external signal, i.e. in the 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 this 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 (PFGE) 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 in France, using microarrays, it was shown that there is a temporal decrease in genetic diversity with a loss of pseudogenes or genes not important for the virulence of the bacterium. However, there is no proof that these changes could affect vaccine efficacy**.** Surveillance must continue in order 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 (Figures 1 & 2). 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 real-time polymerase chain reaction (RT-PCR). Indirect diagnosis is, essentially, by serology and consists of detecting specific antibodies in the serum of an infected individual.

Culture is not very sensitive since the percentage of success is generally no higher than 60%. The highest rates are obtained with infants. It is important to continue to culture in order to analyse the evolution and adaptation of the pathogen and to perform surveillance of the antibiotic resistance. In fact, culture is only successful if samples are collected within the first two to three weeks after the beginning of the cough, but culture is the most specific diagnosis. Among the direct methods, the RT-PCR is more sensitive than bacterial culture. PCR can be performed on the same biological samples as cultures. 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 anti-PT antibodies in the serum of infected individuals after two to three weeks of the cough. However, do not use serology in infants, as their immune system is immature and liable to interference of maternal antibodies, or in patients vaccinated within one year. The presence of a high level of anti-PT 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.

Figure 1: Schematic graph of symptoms and diagnostic procedures in pertussis

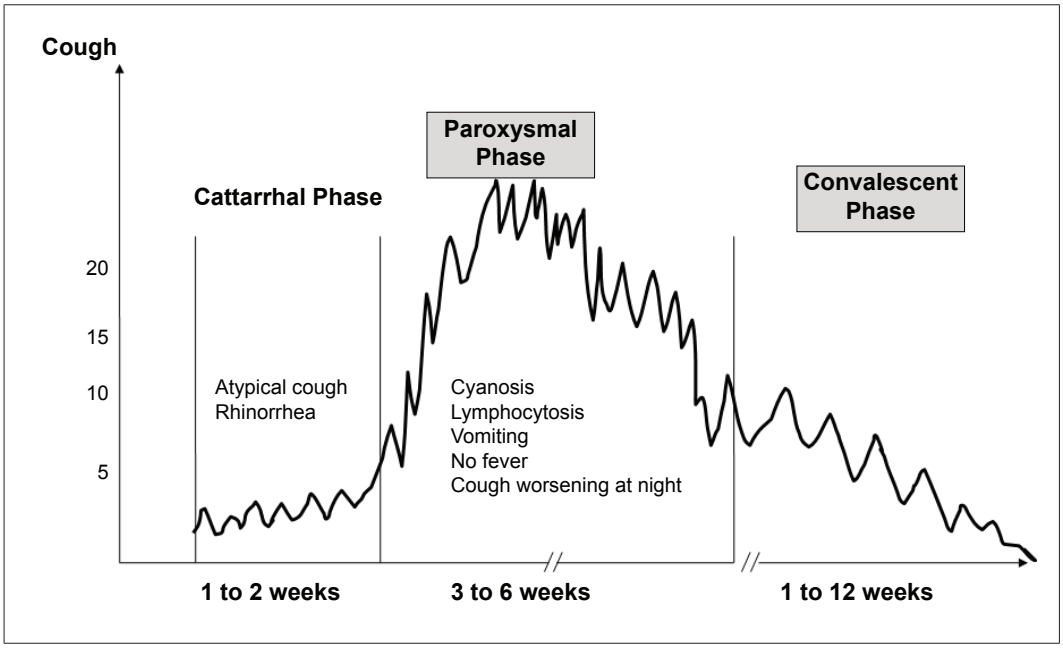
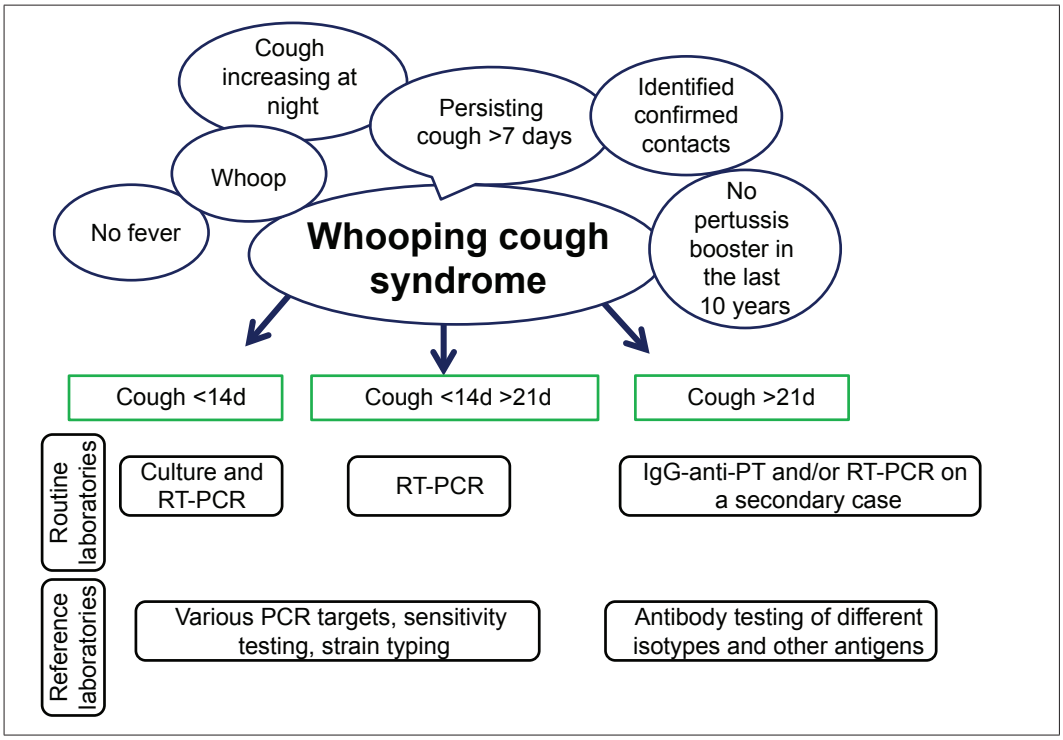


Figure 2: Summary of the recommended diagnosis



3. Direct diagnosis

3.1 Collection and transport of specimen

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) (such as 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. The technique for sampling the NPA or NPS can be seen at http://www.pasteur.fr/recherche/unites/film_cnr/prelev.swf.

Transport of nasopharyngeal swabs or aspirates or sputum

B. pertussis or *B. parapertussis* are fragile bacteria. The NPS or NPA should, after collection at room temperature, be transported quickly to the microbiology laboratory for culture. The swab or the tip of the catheter can also be placed in Reagan Lowe (RL) or Amies medium containing charcoal transport-medium (Annex 2). Isolation rates decrease when transport occurs at 4 °C instead of ambient temperature, or takes longer than 48 hours.

Specimen collection for polymerase chain reaction (PCR) testing

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

3.1.1 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 horse blood (human blood is not an acceptable substitute). For each sample, selective medium i.e. containing cephalexin to inhibit normal flora (40 µg/ml) is used.

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

If culture cannot be performed immediately, it is possible to keep the biological samples at -80 °C and perform the culture later on.

All plates are incubated for seven days at 35–36 °C and inspected at day 3 and 7 after inoculation. If typical colonies appear, they are re-isolated and identified. Plates should be incubated for seven days before being discarded as negative. If *B. parapertussisis* is identified, do not discard plates before day 7 since co-infection can occur. *B. pertussis* grows slower on BG but isolation rates on RL and BG plates are similar after seven days of incubation.

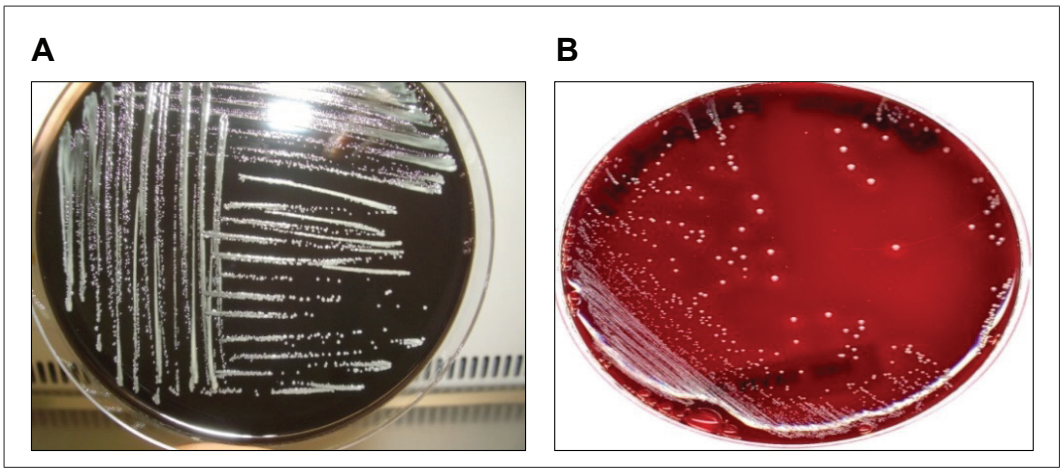
One major advantage of BG is the possibility of characterizing *Bordetella* phases. It has long been known that the expression of virulence properties of *B. pertussis* is unstable. In fact, non-virulent so-called *phase variants* may arise at high frequency. Moreover, the virulent phenotype depends 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 BG 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 (Figure 3).

Figure 3: *Bordetella pertussis* on RL medium (A) ; *Bordetella parapertussis* on BG medium (B)



- On BG plates, both species appear haemolytic. Contrary to *B. pertussis*, *B. parapertussis*is is brown pigmented on BG agar medium, the 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. *B. pertussis* and *B. parapertussis* will appear as typical small gram-negative coccobacilli.

3.1.2 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.
- Check that *B. pertussis* does not grow on Columbia medium.
- Determine biochemical characters, such as oxidase, urease, nitrate-reductase or carbohydrate utilization after subculture on BG or RL agar medium.
- Perform slide agglutination with antibodies to *B. pertussis* and *B. parapertussis*.
- The major characteristics of *B. pertussis* and *B. parapertussis* are listed in Table 2 above.

3.1.3 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 4**). Isolates which cannot be serotyped reproducibly should be sent to an international reference laboratory.

3.1.4 Storage of Bordetella pertussis *and* Bordetella parapertussis

B. pertussis isolates may be frozen in Bovine Serum Albumin (BSA)/glutamate solution (**Annex 5**). These isolates can be stored for at least two years (–40°C) or four years (–80°C).

3.2 Real-time polymerase chain reaction (RT-PCR)

RT-PCR is expensive, but more sensitive and more rapid than culture. RT-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[9]. (**Annex 6 & 7**).

The targets mostly used are listed in Table 3 and the suggested interpretation of the results obtained are listed in Table 4.

Table 3: Possible targets for detection of *Bordetella* DNA by RT-PCR

Target	Present in*	Copy number per genome*
IS481	B. pertussis	50-200
	B. holmesii	8-10
	Some B. bronchiseptica	<5
IS1001	B. parapertussis	~20
	Some B. bronchiseptica	1–7
IS1002	B. pertussis	4–9
	B. parapertussis	9
	B. bronchiseptica	1
h-IS1001	B. holmesii	3–5
ptxP	B. pertussis	1
recA	B. holmesii	1

* Van der Zee et al. JSB. 1996[10]; Tatti et al. JCM. 2011[11]; Loeffelholz. JCM. 2012[12]; Tizolova et al. EJCMID. 2013[13].

[9] Riffelmann M et al. Nucleic Acid amplification tests for diagnosis of Bordetella infections. J Clin Microbiol. 2005;43(10):4925–9

[10] Van der Zee A et al. The differentiation of Bordetella parapertussis and Bordetella bronchiseptica from humans and animals as determined by DNA polymorphism mediated by two different insertion sequence elements suggests their phylogenetic relationship. Int J Syst Bacteriol. 1996 Jul;46(3):640-7.

[11] Tatti KM et al. Novel multitarget real-time PCR assay for rapid detection of Bordetella species in clinical specimens. J Clin Microbiol. 2011 Dec;49(12):4059-66.

[12] Loeffelholz M. Towards improved accuracy of Bordetella pertussis nucleic acid amplification tests. J Clin Microbiol. 2012 Jul;50(7):2186-90

[13] Tizolova A et al. Insertion sequences shared by Bordetella species and implications for the biological diagnosis of pertussis syndrome. Eur J Clin Microbiol Infect Dis. 2013 Jan;32(1):89-96

Table 4: Possible results of *Bordetella* PCR and suggested interpretation

Targets					Report as
IS481	**IS 1001**	**IS1002**	**ptxP**	**h-IS1001**	
Pos	Neg	Pos	Pos	Neg	*B. pertussis*
Pos*	Neg	Pos*	Neg	Neg	*Bordetella spp/B. pertussis*
Pos	Neg	Neg	Neg	Neg	*Bordetella spp*
Neg	Pos	Pos	Neg	Neg	*B. parapertussis*
Neg	Pos*	Neg	Neg	Neg	*Bordetella spp/B. parapertussis*
Pos	Neg	Neg	Neg	Pos	*B. holmesii*

* In rare cases, it can be *B. bronchiseptica*.

Laboratories performing RT-PCR need to perform** external quality assessment **(EQA) regularly.

4. Indirect diagnosis

For a review, Guiso et al. EJCMID. 2011

4.1 Agglutinin detection

Measurement of agglutinins is not a very sensitive technique for diagnosing the disease.

4.2 Antitoxin and adhesin detection

Measurement of anti-PT antibodies by enzyme-linked immunosorbent assay (ELISA) is the technique recommended[16]. Reference sera and antigens can be obtained from the National Institute for Biological Standards and Control (NIBSC) (Annex 8).

[14] Guiso N. et al. What to do and what not to do in serological diagnosis of pertussis: recommendations from EU reference laboratories. Eur J Clin Microbiol Infect Dis. 2011 Mar; 30(3):307-12.

Annex 1:
Collection of nasopharyngeal aspirate (NPA) or swabs (NPS)

A.1.1 Material

Per subject

- Gloves, disposable
- Suction catheter or dacron-flucked nylon swab
- Screwtop container, plastic, 100 ml, sterile
- Syringe, plastic, 5 cc, sterile
- Bag, plastic, zip-lock for holding equipment
- Mask, surgical
- Towelling

Per visit

Bag, plastic, zip-lock for disposing of gloves

A.1.2 Collection of naspharyngeal aspirate or swab

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 their 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 zip-lock bag when the subject is positioned and ready for the tube insertion.

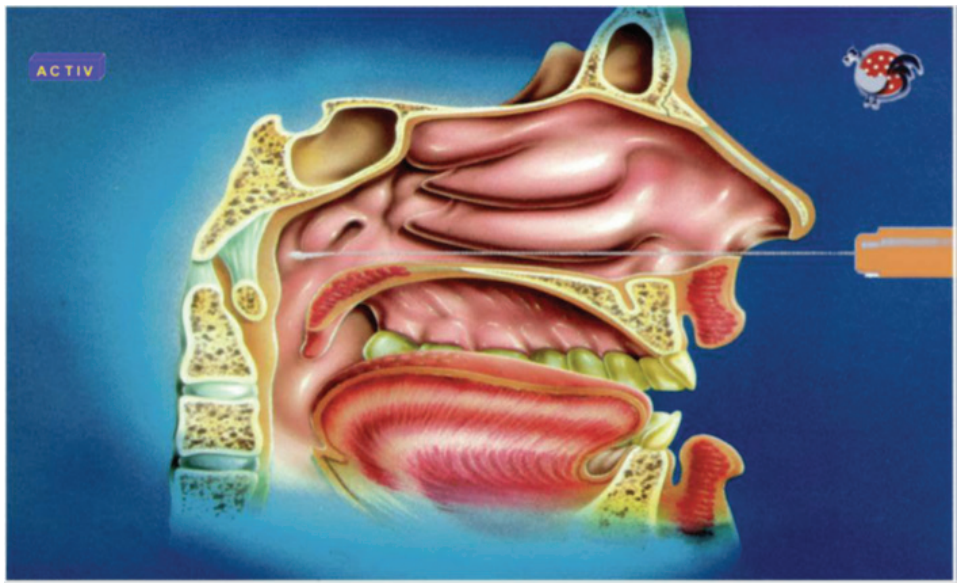
NPA

- 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 has been 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 the 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 inside. 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 all the steps above.
- Transport the NPA specimen.

NPS

- Choose an area for NPS 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 NPS, put on gloves.
- Gently and slowly insert the swab into a nostril rotating the swab, if necessary, to proceed past the back of the nostril. Insert the swab until the back of the throat is reached (approximately 10 cm depending on the age of the volunteer). If gagging occurs, the swab has been inserted too far.
- Once the swab is removed from the nose, put it back inside the sterile container without touching it.
- Label the sterile container. Remove gloves.
- Transport the NPS specimen.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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

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 2–8 °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 –15 °C to –25 °C for six months.

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.

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 RL 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 five 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.

A.2.4 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.2.5 Preparation of Reagan Lowe medium 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 RL medium agar 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.
- Seal the bottles and 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 2–8 °C.
- Prepared Petri dishes and bottles can be stored at 2–8 °C for two weeks and should then be discarded.

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 pipette
- Wheaton medium distributor
- Screw stoppers
- Laminar flow hood.

A.3.2 Reagents

Preparation of specific Bordet Gengou medium

BG agar medium (DIFCO™) ref. 248200: Beckton Dickinson Biosciences, 2350 Qume Drive, San Jose, CA 95131–1807, USA; Tel. 001.408.432.9475; Fax. 001.408.954.2347. Storage period: 1 month. Store at 2–8 °C.

- Weigh 30 g BG medium and dissolve by boiling in a mixture of glycerol 10 ml, adding 5N NaOH until pH = 7.4 and distilled H_2O 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 2–8 °C.
- This medium can be kept for up to 12 weeks at 2–8 °C.

A.3.3 Preparation of cefalexin

- Reconstitute a bottle of 20 mg with 2 ml of double-distilled sterile water.
- Dilute the 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 –15 °C to –25 °C for six months.

Reference strain

Bordetella pertussis Tohama I (CIP 8132 which can be purchased from the Institut Pasteur) for quality control.

A.3.4 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 BG 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 an 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 Bordet Gengou medium with blood (BGB) is cold. Identify batches (in case one part of the blood is contaminated) and leave overnight at room temperature before storage at 2–8 °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.3.5 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 BG 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 2–8 °C. Prepared Petri dishes and bottles can be stored at 2–8 °C for two weeks and should then be discarded.

Annex 4:
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.4.1 Material

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

A.4.2 Reagents

Monoclonal antibodies anti-Fim 2 and anti-Fim 3 reagents can be purchased from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potter Bar, Hertfordshire EN6 30G, United Kingdom; contact point Dr Dorothy Xing).

Stock solution: stored between –15 °C and –25 °C.

For one month: a diluted solution stored at 2–8 °C.

Reference strain: Bp 460 (reagents can be purchased from the NIBSC). This reference strain is expressing Fim 2 and Fim 3 antigens.

A.4.3 Protocol

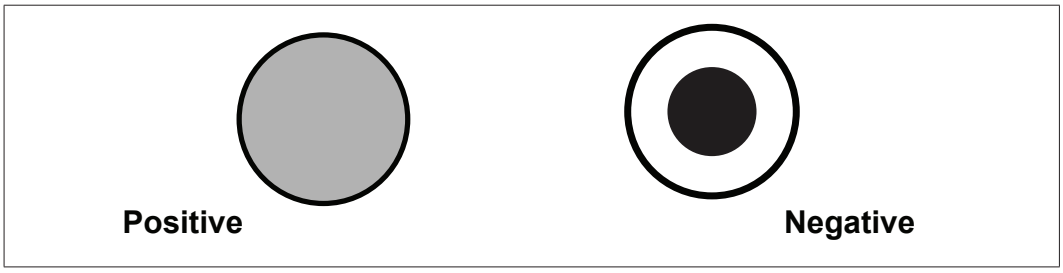
Bacterial strains

- All determinations are performed twice on the same plate.
- 50 µl of bacterial suspension of strain Bp 460 with an OD650 nm = 1 are placed in wells A1 to A6.
- 50 µl of bacterial suspension of strain Bp 460 with an OD650 = 0.5 are 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 OD650 = 1, and wells 7 to 12 are used for the bacterial strain sample with an OD650 = 0.5.
- 50 µl anti-Fim 2 antibodies at the required concentration are distributed in wells 1, 2, 7 and 8 of lines A to H depending on the number of samples.

- 50 µl anti-Fim 3 antibodies at the required 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.4.4 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.



Annex 5:
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.5.1 Material

- Cryotubes volume (2 ml)
- Petri dish
- Spectrophotometer OD 650 nm
- Automatic pipetter, 200 µl and 1000 µl
- 1, 2, 5, 10 ml plastic pipettes
- Tips for 200 µl and 1000 µl automatic pipetters (calibration each year, documented in logbook)
- 1.6 ml polystyrene spectrophotometer cuvettes
- Laminar flow hood (maintenance each year, documented in logbook)
- Plastic or glass rake
- 0.2 µm filter

A.5.2 Reagents

Products

- 90° ethyl alcohol
- Sodium glutamate
- Di-sodium hydrogen phosphate, dihydrous – Na_2HPO_4 -$2H_2O$
- Sodium di-hydrogen phosphate, monohydrous – NaH_2PO_4 -H_2O
- Sodium and potassium phosphate pH 7.2 = PBS 10X: KCl 26.8 mM; KH_2PO_4 14.7 mM; NaCl 1.36 M; Na_2HPO_4 -$7H_2O$ 80.57 mM
- Saccharose
- Bovine serum albumin (BSA)

Solutions

- 25% BSA solution; storage period six months at +4 °C. BSA 25 g – PBS 1X pH 7.2 to make 100 ml. Sterilize by filtering through a 0.2 µm membrane.
- Saccharose–phosphate–glutamate (SPG) solution; storage period six months at 2–8 °C.
  - Saccharose 85.6 g – sodium glutamate 0.94 g – Na_2HPO_4 -$2H_2O$ 1.38 g – NaH_2PO_4 -H_2O 0.39 g.
  - Adjust to pH 7.2.
  - H_2O to make 1000 ml.
  - Sterilize by filtering through a 0.2 µm membrane and store at 2–8 °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.5.3 Protocol

Freezing

- **Day 0**
  - Take bacteria from isolated colonies (72 h growth for *B. pertussis* and *B. parapertussis*) 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 hours.
- **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 five cryotubes identified with the number of the isolate, the species code (see below), the date of freezing and the freezing medium used: BSA/SPG.
  - Freeze the tubes rapidly by placing them in the freezing bath.
  - Inscribe the type and storage site of isolates in the "strains in" logbook 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. Distribute the tubes to different storage sites. Place two cryotubes in the freezer at –80 °C for everyday use, place two cryotubes in the freezer at –80 °C for producing the primary batch, not intended for distribution, and place one cryotube in liquid nitrogen at –196 °C for producing the primary batch, not intended for distribution.

Annex 6:
Real-time PCR using LightCycler® technology for amplification of the insertion sequence IS481

As a basis to develop an in-house PCR method

This protocol is intended for use of RT-PCR on DNA extracted from clinical specimens obtained from patients suspected of having whooping cough i.e. an infection with *B. pertussis*. The protocol is based on the LightCycler® capillary technology (Roche) but can be used as a basis on which to build an in-house method on other real-time formats. The method is based on a detection of the amplification product, a 181pb fragment of IS*481* present in a high copy number in different genome of Bordetella species i.e. *B. pertussis*, *B. holmesii* and some *B. bronchiseptica*.

For information, a list of tested commercial kits for the molecular diagnostic of whooping cough is present at the bottom of **Annex 6**.

A.6.1 Materials

Sample material

Nasopharyngeal swabs or aspirates from patients suspected of having whooping cough.

Apparatus

- LightCycler® instrument using capillaries (LightCycler® 2.0, Roche)
- LightCycler® capillaries and capillary-related tools and adapters (Roche)
- Microcentrifuge
- Sterile Eppendorf 1.5ml tubes
- Automatic pipetters (calibration each year, documented in the logbook)
- Pre-sterilized aerosol resistant pipette tips
- Gloves
- DNA away (MbP)
- Freezers –20 °C (temperature is verified every working day and documented in the logbook)

A.6.2 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 FastStartTaq® DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP) and MgCl2 (store at –15 °C to –25 °C and avoid repeated freezing and thawing);
  - water PCR grade.
- AmpErase® Uracil N-glycosylase (Applied Biosystems).
- Primers and hybridization probes, high-performance liquid chromatography (HPLC) quality:
  - Forward primer (BP-1) 5' – GAT TCA ATA GGT TGT ATG CAT GGT T;
  - Reverse primer (BP-2) 5' – TTC 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).
  - water PCR grade (Rnase and DNase free) for dilution of DNA.

Controls

- Internal control:

  An internal amplification control is recommended to avoid false-negative reporting. Some, based on the separate amplification of a phocine herpes virus, or on an alternative system to the IS*481* PCR, have been described [17,18]. Almost all samples tested so far harboured human gene sequences and thus their amplification can also be used as an inhibition control. [10]

  It is essential to test for the presence of any inhibition to the PCR reaction, to avoid false-negative reporting. If an IPC is not readily available, an alternative is to 'spike' a replicate sample with a dilution of positive control DNA (e.g. 0.01pg for a multiple copy target such as IS*481*). For each test sample reaction mix, a duplicate is prepared, but the spiked sample contains a low dilution of purified positive control DNA in addition to the extracted test sample DNA. These two reactions are then run and analysed in parallel. If the test sample alone does not contain detectable levels of *Bordetella* (i.e. PCR negative), and the spiked reaction produces a signal comparable with that obtained from the same dilution used in the standard curve, then the result can be reported as a valid negative result. If, when compared to the same dilution in the standard curve, the spiked sample yields no signal, then the result should be reported as inhibitory[19] .

[17] Cloud et al. Impact of nasopharyngeal swab types on detection of Bordetella pertussis by PCR and culture. J Clin Microbiol. 2002 Oct;40(10):3838-40.

[18] Fry et al. Laboratory diagnosis of pertussis infections: the role of PCR and serology. J Med Microbiol. 2004 Jun;53(Pt 6):519-25

[19] Fry et al. Role of PCR in the diagnosis of pertussis infection in infants: 5 years' experience of provision of a same-day real-time PCR service in England and Wales from 2002 to 2007. J Med Microbiol. 2009 Aug;58(Pt 8):1023-9

- Positive and negative in-run PCR controls:

  In-run PCR controls should include negative controls (PCR grade water) and positive control. Typically, aliquots of genomic DNA of *B. pertussis* (e.g. reference strain Tohama I; this strain can be purchased from Institut Pasteur as CIP8132) at a concentration of 10 pg/ul, are stored at –15 °C to –25 °C. Avoid multiple cycles of freezing and thawing. For daily use, an aliquot can be stored at 2–8 °C for maximum one month.

Prevention of crossover contamination

The synthetic deoxyuridine triphosphate (dUTP) is used instead of deoxythymidine triphosphate (dTTP) in the PCR mix, allowing the action of uracil-DNA glycosylase (UNG) prior to a new assay to prevent carry-over of amplicons. UNG catalyses the removal of uracil from single- and double-stranded DNA that has been synthesized in the presence of dUTP.

A.6.3 Precaution

Before handling, clean worktop and pipettes with DNA away.

A.6.4 Procedure

DNA sample extraction

Sample material from dry swabs can be re-suspended in sterile phosphate buffered saline (PBS) before DNA extraction by vortexing the swab in 500 µl of PBS in a closed sterile container.

If necessary, respiratory samples such as sputum can be liquefied with the fluidifiant preparation (volume-to-volume) before DNA extraction.

- Fludifiant preparation
  - Sodium citrate (2.9%) 5 ml
  - N-Acetyl L-cysteine 0,05 gr
  - H_2O to make 10 ml

DNA extraction from respiratory samples may be carried out manually using the QIAamp® DNA mini kit (Qiagen), High Pure PCR template preparation kit (Roche) or Chelex®. Automated methods, such as the MagNA Pure Compact (Roche), are also an option.

Prepare a 1:10 dilution of each DNA sample in PCR grade water. Each DNA sample is analysed in both undiluted and 1:10 dilution in order to detect potential inhibition.

Preparation of reagents

- *FastStart DNA Master PLUS preparation:*
  - From LightCycler®FastStart DNA Master PLUS HybProbe kit (store at –15 °C to –25 °C until the expiration date printed on the label); transfer 60 ml from vial 1b into vial 1a, gently mix by pipetting up and down, do not vortex (IMPORTANT). Avoid repeated freezing and thawing.
- *Primers and probes*:
  - Primer BP-1 and BP-2 are diluted to 10 pmol /µl with PCR water grade.
  - Probes BP-FLU and BP-LCR are diluted to 4 pmol /µl with PCR water grade.

Preparation of master mix

Prepare a master mix, in a sterile 1.5 ml tube, according to the components given in the table by multiplying the amount of mix for one test by the number of reactions (including positive and negative control) to be cycled, plus one additional reaction. Remember that each sample is analysed twice (pure and 1: 10 dilution).

Table 1: Master mix (volume for one sample)

Reagents	Volume per capillary	Final concentration
PCR grade water	6.8	
FastStartPLUS® DNA Master 5X	4	1X
BP-1 10 pmol / µl	1	0.5 pmol / µl
BP-2 10 pmol / µl	1	0.5 pmol / µl
BP-FLU 4 pmol / µl	1	0.2 pmol / µl
BP-LCR 4 pmol / µl	1	0.2 pmol / µl
AmpErase® UNG 1unit/ µl	0.2	0.2 unit
Total volume	15	na

- Carefully mix the master mix.
- Pipet 15 µl master mix into each of the pre-cooled capillaries.
- Add 5 µl of the DNA sample. For each sample, two capillaries are needed: one with 5 µl undiluted DNA and one with 5 µl of 1:10 diluted DNA.
- For negative control: pipet 5 µl of PCR grade water into a capillary.
- For positive control: pipet 5 µl of *B. pertussis* DNA (e. g. strain CIP8132) corresponding to a total of 50 pg of DNA into the capillary.
- Seal each capillary with a stopper and place the adapters, containing the capillary, into a standard bench-top centrifuge. Centrifuge at 700 g for 5 sec (or place the capillaries in the rotor and use a LightCycler® Carousel Centrifuge).
- Place the capillaries in the rotor of the LightCycler® instrument.

PCR cycles

Cycle the samples according to the LightCycler® manual, as indicated in Table 2.

Table 2: Cycle programme for PCR

	Segment	Target (°C)	Hold	Slope (°C / sec)	Acquisition mode	Cycles
UNG incubation		50	10 min		None	1
Denature		95	10 min		None	1
PCR	1	95	10 sec	20	None	40
	2	60	10 sec	20	Single	
	3	72	20 sec	20	None	
Cooling		40	30 sec	20	None	1

A.6.5 Results

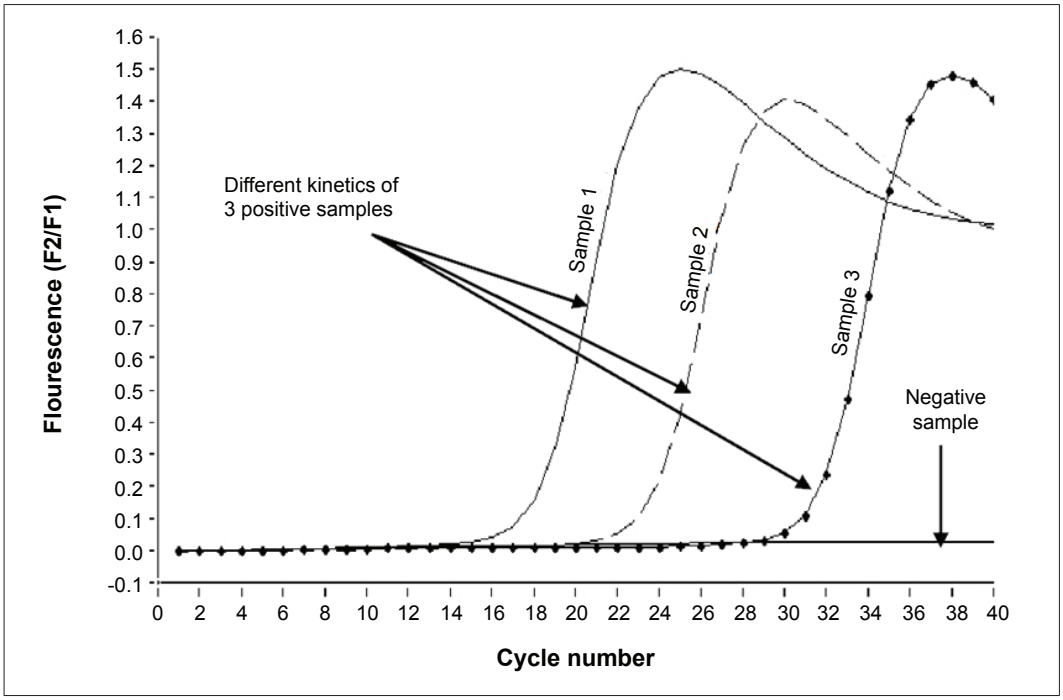
Validation of controls

The used controls (positive and negative in run and IPC) must be validated before doing the analysis of the PCR.

Analyse the amplification curves according to the LightCycler® manual. Use channel 640 for the samples.

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 curve (protocol Figure 4). Samples are regarded as "negative" when they do not fulfil the criteria mentioned above.

Figure 4 : Amplification curve



Interpretation of the results

For practical purposes, a positive IS*481* PCR can be considered as a probable *B. pertussis* infection, when the clinical symptoms are in accordance with this result. In the case of epidemiological studies with unknown clinical data, positive results from an IS*481* PCR should only be regarded as evidence of infection with *Bordetella* spp.

The use of *ptxA*-Pr, single copy number target *B. pertussis*-specific assay but less sensitive than the IS*481* PCR, will therefore be able to confirm *Bordetella pertussis* DNA detection.

A.6.6 Commercial kits

Several commercial kits are available and use the target IS*481* (and/or IS*1001*). They have the advantage of being "ready-to-use" and include an internal control to detect inhibitors. These kits are mostly using hydrolyse probe (Taqman® technology) instead of hybridization probe (Roche development). However, these two types of probe are quite suitable for the molecular diagnosis based on RT-PCR.

Of these kits, the SimplexaBordetella® assay (Focus Diagnostics), the SmartCyclerBordetella® pertussis/parapertussis assay (Cepheid), and the Bordetella R-gene® (ArgeneBioMerieux) have been evaluated and proven suitable, whereas the Bordetella pertussis Real Time PCR® kit (Shanghai ZJ Bio-Tech) was found to be unsuitable. New kits to identify the infecting organism are frequently introduced on to the market. However, using multiplex PCR rather than singleplex, can in some cases reduce the analytical sensitivity. As a general rule, the amplification targets and interpretation of results should follow the guidance below regarding the selection of amplification targets.

Annex 7:
Real-time PCR using LightCycler® technology for amplification of the insertion sequence IS*1001*

As a basis to develop an in-house PCR method

This protocol is intended for use of real-time PCR on DNA extracted from clinical specimens obtained from patients suspected of having whooping cough i.e. an infection with *B. parapertussis*.The protocol is based on the LightCycler®capillary technology (Roche) but can be used as a basis on which to build an in-house method on other real-time formats. The method is based on a detection of the amplification product, a 464 pb, fragment of IS*1001* present in high copy number in the genome of *B.parapertussis* and some *B. bronchiseptica*.

A.7.1 Materials

Sample material

Nasopharyngeal swabs or aspirates from patients suspected of having whooping cough

Apparatus

- LightCycler instrument using capillaries (LightCycler® 2.0, Roche)
- LightCycler Capillaries and capillary related tools and adapters (Roche)
- Microcentrifuge
- SterileEppendorf 1.5ml tubes
- Automatics pipetters (calibration each year, documented in logbook)
- Pre-sterilized aerosol resistant pipette tips
- Gloves
- DNA away (MbP)
- Freezers -20°C (Temperature is verified every working day and documented in the logbook)

A.7.2 Reagents

- LightcyclerFastStart DNA Master PLUS Hybridization Probes kit (Roche):
  - LightcyclerFastStart Enzyme (1a), (store at -15°C to -25°C and avoid repeatedfreezing and thawing)
  - LightcyclerFastStart Reaction Mix Hybridization Probes (1b), containing FastStartTaq DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP) and MgCl2 (store at -15°C to -25°C and avoid repeated freezing and thawing)
  - Water PCR grade.

- AmpErase® Uracil N-glycosylase (Applied Biosystems)
- Primers and hybridization probes (HPLC quality)
  - Forward primer (BPpara-1) 5'– CAC CGC CTA CGA GTT CGA GAT
  - Reverse primer (BPpara-2) 5'– CCT CGA CAA TGC TGG TGT TCA
  - Probe 1 (BPpara-FLU) 5'– GTT CTA CCA AAG ACC TGC CTG GGC-(F)
  - Probe 2 (BPpara-LCR) 5'– (LC-Red 640)-AGA CAA GCC TGG AAC CAC TGG TAC-(P)
  - Water PCR grade (Rnase and DNase free) for dilution of DNA

Controls

- InternalProcess Control (IPC):

  An internal amplification control is recommended to avoid false-negative reporting. Some, based on the separate amplification of a phocine herpes virus or on an alternative system to the IS481 PCR, have been described[14,15]. Almost all samples tested so far harbored human gene sequences, and thus their amplification can also be used as an inhibition control[10].

  It is essential to test for the presence of any inhibition to the PCR reaction to avoid false-negative reporting. If an IPC is not readily available, an alternative is to 'spike' a replicate sample with a dilution of positive control DNA (e.g. 0.01pg for a multiple copy target such as IS481). For each test sample reaction mix a duplicate is prepared, but the 'spiked' sample contains a low dilution of purified positive control DNA in addition to the extracted test sample DNA. These two reactions are then run and analyzed in parallel. If the test sample alone does not contain detectable levels of Bordetella (i.e. PCR negative), and the spiked reaction produces a signal comparable with that obtained from the same dilution used in the standard curve, then the result can be reported as a valid negative result. If, when compared to the same dilution in the standard curve, the spiked sample yields no signal then the result should be reported as inhibitory[16].

- Positive and negative in-run PCR control:

  In-run PCR controls should include negative controls (PCR grade water) and positive control. Typically, aliquots of genomic DNA of B.parapertussis (e.g. reference strain 12822, this strain can be purchased from Institut Pasteur as CIP12822) at a concentration of 10 pg/ul are stored at -15 to -25°C. Avoid multiple cycles of freezing and thawing. For daily use, an aliquot can be stored at 2 to 8°C for 1 month maximum.

Prevention of crossover contamination

The synthetic deoxynucleotidedUTP is used instead of dTTP in the PCR mix allowing the action of Uracil-DNA Glycosylase (UNG) prior to a new assay to prevent carry-over of amplicons. UNG catalyzes the removal of uracil from single- and double-stranded DNA that has been synthesized in the presence of dUTP.

A.7.3 Precaution

Before handling, clean worktop and pipettes with DNAaway.

A.7.4 Procedure

DNA sample extraction

Sample material from dry swabs can be re-suspended in sterile PBS before DNA extraction by vortexing the swab in 500µl of PBS in a closed sterile container.

If necessary, respiratory samples as sputum can be liquefied with the fluidifiant preparation (volume to volume) before DNA extraction.

- Fludifiant preparation
  - Sodium citrate (2.9%) 5 ml
  - N-Acetyl L-cysteine 0,05 gr
  - H_2O to make 10 ml

DNA extraction from respiratory samples may be carried out manually using the QIAamp® DNA mini kit (Qiagen), High Pure PCR template preparation kit (Roche) or Chelex®. Automated methods such as the MagNA Pure Compact (Roche) are also an option.

Prepare a 1:10 dilution of each DNA sample in PCR grade water. Each DNA sample is analyzed in both undiluted and 1:10 dilution in order to detect potential inhibition.

Preparation of reagents

- *FastStart DNA Master PLUS preparation*:
  - From LightCycler®FastStart DNA Master PLUS HybProbe kit (store at -15°C to -25°C until the expiration date printed on the label): transfer 60 ml from vial 1b into vial 1a, gently mix by pipetting up and down, do not vortex (IMPORTANT). Avoid repeated freezing and thawing.
- *Primers and probes*:
  - Primer BPpara-1 and BP-2 are diluted to 10 pmol /µl with PCR water grade.
  - Probes BPpara-FLU and BPpara-LCR are diluted to 4 pmol /µl with PCR water grade

Preparation of master mix

Prepare a master mix in a sterile 1.5 ml tube according to the components given in the table by multiplying the amount of mix for one test by the number of reactions (including positive and negative control) to be cycled, plus one additional reaction. Remember that each sample is analyzed twice (pure and 1: 10 dilution).

Table 1: Master mix (volume for one sample)

Reagents	Volume per capillary	Final concentration
PCR grade water	6.8	
FastStartPLUS DNA Master 5X	4	1X
BPpara-1 10 pmol / µl	1	0.5 pmol / µl
BPpara-2 10 pmol / µl	1	0.5 pmol / µl
BPpara-FLU 4 pmol / µl	1	0.2 pmol / µl
BPpara-LCR 4 pmol / µl	1	0.2 pmol / µl
AmpErase UNG 1unit/ µl	0.2	0.2 unit
Total volume	15	na

- Mix carefully the master mix.
- Pipet 15µl master mix into each of the pre-cooled capillaries.
- Add 5µl of the DNA sample. For each sample, two capillaries are needed: one with 5µl undiluted DNA and one with 5µl of 1:10 diluted DNA.
- For negative control: pipet 5µl of PCR grade water into a capillary.
- For positive control: pipet 5µl of *B. parapertussis* DNA (e. g. strain CIP 12822, corresponding to a total of 50 pg of DNA into the capillary).
- Seal each capillary with a stopper and place the adapters, containing the capillary, into a standard benchtop centrifuge. Centrifuge at 700g for 5 sec. (or place the capillaries in the rotor and use a LightCycler Carousel Centrifuge).
- Place the capillaries in the rotor of the LightCycler instrument.

PCR cycles

Cycle the samples according to the LightCycler manual as indicated in Table 2.

Table 2: Cycle programme for PCR

	Segment	Target (°C)	Hold	Slope (°C / sec)	Acquisition mode	Cycles
UNG incubation		50	10 min		None	1
Denature		95	10 min		None	1
PCR	1	95	10 sec	20	None	40
	2	60	10 sec	20	Single	
	3	72	20 sec	20	None	
Cooling		40	30 sec	20	None	1

A.7.5 Results

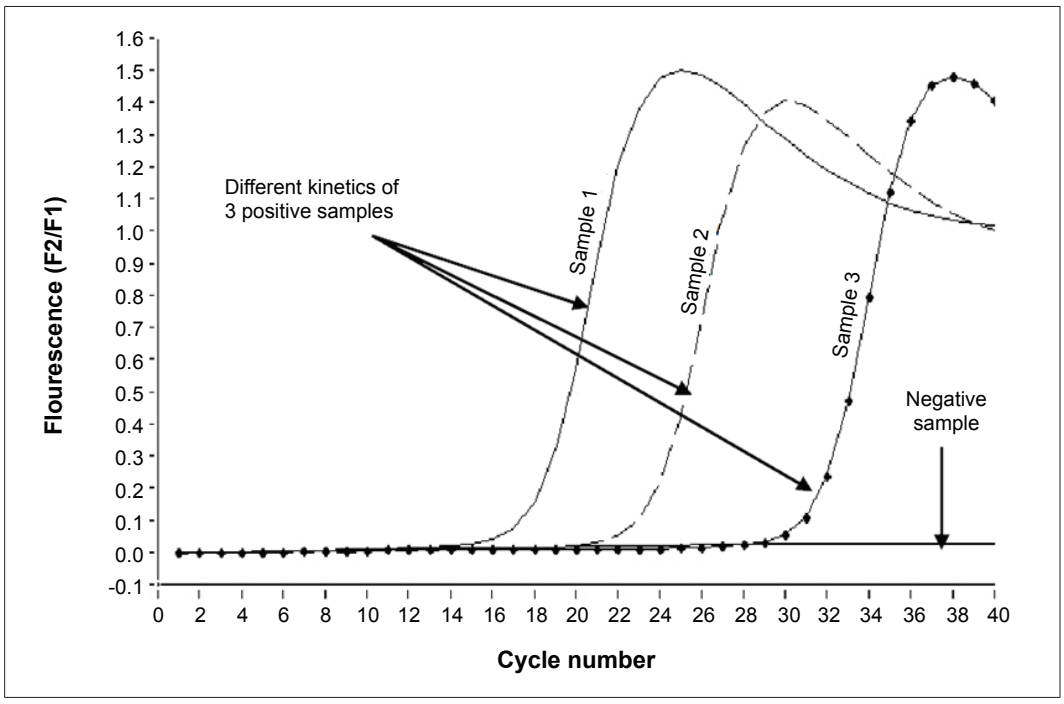
Validation of controls:

The used controls (positive and negative in run and IPC) must be validated before to do the analysis and interpretation of the results.

Analyse the amplification curves according to the LightCycler manual. Use channel 640 for the samples.

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 curve (protocol figure 5). Samples are regarded as "negative" when they do not fulfil the criteria mentioned above.

Figure 5 : Amplification curve



Annex 8:
Measurement of anti-pertussis toxin antibodies by ELISA

In-house test

This ELISA technique allows the detection of anti-pertussis toxin (anti-PT) antibodies in the serum of a patient.

A.8.1 Material

1) Microplate reader.

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

   – measure OD 405 withoutmicroplate: 0.000;
   – measure OD 405 with microtitre plate: 0.030–0.040;
   – measure OD 405 with microtitre plate with water: 0.040–0.050. Document annually in a logbook.

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 six months. Document in a logbook.

4) Incubator temperature is read every working day and documented in the logbook.

5) Microplate washer 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™certified.

10) Enzyme-conjugated goat anti-human IgG antiserum: Kirkegaard & Perry Laboratories, 075–1002. Reference substrate, Sigma, N2765.

A.8.2 Reagents

1) Sera
   - Reference sera can be purchased from the NIBSC.
   - All test reference sera are kept at –80 °C.
2) Antigens
   - PT can be purchased commercially
3) Calibrators
   - Controls: In-house, positive and negative reference human sera from infected individuals.
   - Conjugate goat anti-human IgG (1 mg) labelled with phosphatase alcaline is re-suspended in 1 ml 50% glycerol, and diluted 1/20000 in incubation buffer before use. Substrate of phosphatase alkaline.
4) Buffers and solutions.
   - **Carbonate buffer:** Na_2CO_3 0.05M, pH 9.6 **(to be done every two weeks)**

Na_2CO_3	0.795 g
$NaHCO_3$	1.465 g 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, $NaHPO_4$–H_2O 0.085M, $NaHPO_4$ –7H_2O 0.015M, pH 7.4 or 6.8 **(to be done every month)**

NaCl	85.00 g
Na_2HPO^4 –2H_2O	22.79 g
Na_2HPO^4–H_2O	2.07 g
H_2Od	700 ml
pH	7.4 or 6.8
Distilled H_2O	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_2Od	700 ml
$MgCl_2$ 1M (with $MgCl_2$. 6H_2O)	0.3 ml
pH with HCl 6N	9.8
distilled H_2O	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 two 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
Tween20	50 ml
Distilled H_2O	to make 1000 ml

A.8.3 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 microtitre plate. Seal to prevent evaporation. Incubate at +28 °C overnight (16–24 h).

2) Sample addition. Prepare eight 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 (four 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 ìl of buffer. Seal plates, and incubate for two 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 beforehand). Plates are rinsed with 250 µl of wash buffer. Plates are inverted and taped 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 h).

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 taped on to 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.

7) Criteria for approval of ELISA results. Quantitative values are calculated when the following criteria are met.

   – A line can be drawn from the dose response curve using at least four 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 +/– 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 re-tested.

– If the control of a single plate exceeds cut-off limits, paired sera are re-tested. 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 four 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 re-determined with a 10-fold dilution.

Commercial kits

Used those recommended in Riffelman et al. JCM. 2010[20]; Dinu et al. Diagn Microbiol Infect Dis. 2013[21].

[20] Riffelman et al. Performance of commercial enzyme-linked immunosorbent assays for detection of antibodies to Bordetella pertussis. J Clin Microbiol. 2010 Dec;48(12):4459-63

[21] Dinu et al. Whooping cough in South-East Romania: a 1-year study. Diagnostic Microbiology and Infectious Disease. 2013



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