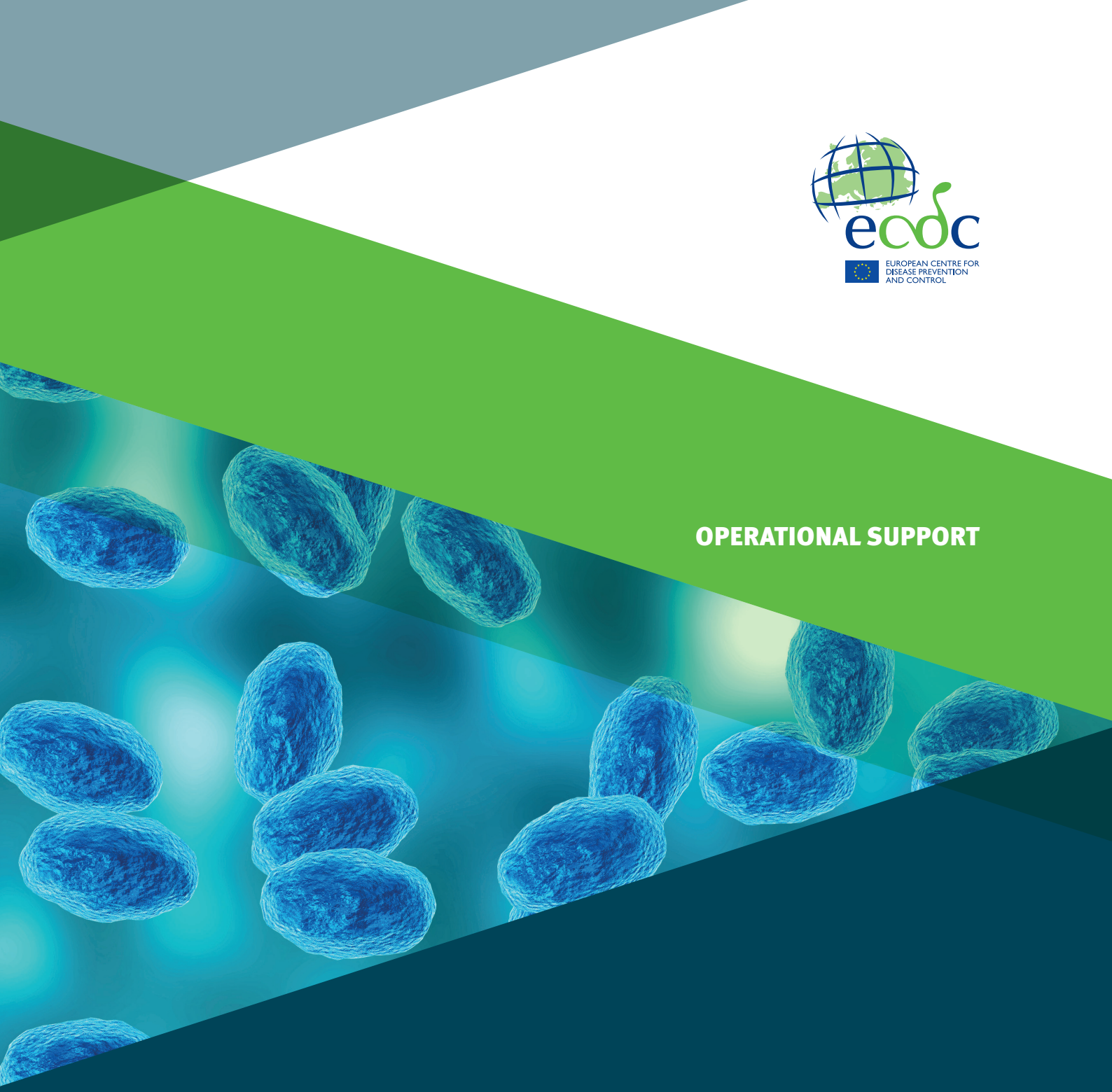


OPERATIONAL SUPPORT

Laboratory diagnosis and molecular surveillance of *Bordetella pertussis*

June 2026

ECDC OPERATIONAL SUPPORT

Laboratory diagnosis and molecular surveillance of *Bordetella pertussis*

Funded by the European Union

This report was funded by EU4Health Programme (Grant ref 101194675), coordinated by Maria Keramarouand Andreas Hoefer, European Centre for Disease Prevention and Control (ECDC), and produced by Alex-Mikael Barkoff, Qiushui He, University of Turku (UTU) (Turku, Finland); Sylvain Brisse, Carla Rodrigues, Valérie Bouchez, Dr Chiara Crestani, Institut Pasteur (IP) (Paris, France); Dr Eveline Van Honacker, Dr Helena Martini, Prof Denis Pierard, Universitair Ziekenhuis Brussels (UZB) (Brussels, Belgium); Isabelle Desombere, Sciensano (SCI) (Brussels, Belgium) and Anja Berger, Andreas Sing, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (LGL) (Oberschleißheim, Germany). This is an updated version, based on the report published by ECDC in 2022 ('Laboratory diagnosis and molecular surveillance of *Bordetella pertussis* - Recommendations from ECDC' doi: 10.2900/35054).

The report was reviewed by Maria Keramarou, Sabrina Bacci and Andreas Hoefer (ECDC).

Acknowledgements
We acknowledge all colleagues who contributed to the previous version of this handbook.

Suggested citation: European Centre for Disease Prevention and Control. Laboratory diagnosis and molecular surveillance of *Bordetella pertussis.* Stockholm: ECDC; 2026.

Stockholm, June 2026

ISBN: 978-92-9498-894-2
doi: 10.2900/8197958
Catalogue number: TQ-01-26-039-EN-N

Contents

Figures

Tables

Abbreviations

ACV	Acellular pertussis vaccine
AST	Antimicrobial susceptibility testing
AZT	Azithromycin
BG	Bordet-Gengou
BIGSdb	Bacterial Isolate Genome Sequence Database
BSA	Bovine serum albumin
CDC	US Centers for Disease Control and Prevention
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunoassay
ERY	Erythromycin
EUCAST	European Committee on Antimicrobial Susceptibility
EUpertstrain	European *Bordetella* expert group
Fim	Fimbrial protein/Fimbriae
FHA	Filamentous hemagglutinin
IS	Insertion sequence
IU	International unit
LAMP	Loop-Mediated Isothermal Amplification
LF	Lateral flow
MAbs	Monoclonal antibodies
MALDI-TOF	Matrix-Assisted Laser-Desorption-Ionization Time-of-Flight
MAST	Multi-Locus Antigen Sequence Typing
MIA	Multiplexed immunoassay
MIC	Minimum inhibition concentration
MLVA	Multiple-Locus Variable-Number Tandem Repeat Analysis
MRBP	Macrolide resistant *Bordetella pertussis*
NIBSC	National Institute for Biological Standards and Control
NPA	Nasopharyngeal aspirate
NPS	Nasopharyngeal swab
NSS	Normal sheep sera
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
POC	Point of Care
PRN	Pertactin
PT	Pertussis toxin
qPCR	Quantitative Polymerase Chain Reaction
RL	Regan-Lowe
SNP	Single nucleotide polymorphism
SPG	Saccharose/phosphate/glutamate
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TMP-SMZ	Trimethoprim-sulfamethoxazole
TSB	Trypticase soy broth
VNTR	Variable-Number Tandem Repeat Analysis
WCV	Whole cell pertussis vaccine
WGS	Whole genome sequencing
WHO	World Health Organization

Executive summary

Pertussis or whooping cough is a highly contagious respiratory disease mainly caused by the bacterium *Bordetella pertussis*. Despite childhood immunisations having been widely implemented, pertussis remains one of the world's leading causes of vaccine-preventable deaths, with epidemics occurring every three to five years in many countries. In 2024, around 210 000 cases of pertussis were reported by 29 EU/EEA countries. This clearly highlights the importance of standardised rapid diagnosis of the disease and continuous surveillance of circulating bacteria in Europe for data reporting/comparison and evaluation of the effectiveness of current vaccination policies.

This laboratory handbook, which is an update of a previous version, provides an overview of recommended laboratory diagnostic and molecular surveillance methods for the detection, identification and typing of *B. pertussis*, including key aspects of laboratory safety. It highlights the appropriate use of different approaches throughout the course of the disease, which is essential for accurate diagnosis. The handbook has been developed by members of the European Reference Laboratory for Public Health on Diphtheria and Pertussis (EURL-PH-DIPE) consortium in collaboration with counterparts at ECDC.

For the purposes of diagnosis, the handbook describes the core methodology for nasopharyngeal sampling, culture, PCR, and antibody detection, including clearly defined targets, cut-offs, and guidance for interpretation. In addition, novel diagnostic techniques, such as point-of-care assays, are introduced.

For the purposes of bacterial surveillance, the handbook outlines fundamental approaches, with particular focus on genes encoding vaccine antigens. It also includes an easy-to-use protocol for measuring vaccine antigen expressions of the bacteria. Given the global increase in macrolide-resistant *B. pertussis*, special emphasis is placed on the rapid detection of such resistance in clinical samples and retrospective analysis of existing isolates. To support this, both PCR- and sequencing-based methods, and culture-based susceptibility testing are presented, along with possible cut-off values for macrolide resistance, although no such recommendations have been established in Europe to date.

Whole genome sequencing (WGS) is currently widely used for bacterial typing. Therefore, this handbook includes a dedicated chapter covering the methodology, from DNA extraction and instrumentation to data analysis and comparison of the results using the BIGSdb-Pasteur *Bordetella* database.

The handbook also provides scientific context for the various approaches described, guiding laboratories in their decision-making processes. The correct application and interpretation of different diagnostic methodologies, and WGS will be crucial for standardised diagnosis and bacterial surveillance, and to enable meaningful comparisons of pertussis data within and between European countries.

It should be noted that it is not possible to cover all existing methods and protocols within the scope of this handbook. In addition, although the protocols included in the appendices are detailed, they may need to be adapted for implementation in different laboratory settings.

What's new in the 2026 edition

This updated edition of the laboratory handbook introduces several important revisions reflecting the evolving epidemiology of pertussis and advances in diagnostic and molecular surveillance technologies. Following the substantial resurgence of pertussis cases, the appearance of macrolide resistant *Bordetella pertussis* after the COVID-19 pandemic and the emergence of new laboratory needs across Europe, the handbook has been revised to incorporate more timely, sensitive and scalable diagnostic approaches.

Readers will find a clear transition away from several legacy methods—such as routine MLVA and PFGE typing—towards modern genomic platforms, including expanded use of WGS, updated antigen-genotyping schemes, and improved PCR-based and point-of-care diagnostic solutions. The new version also introduces strengthened guidance on sampling quality, updates to PCR targets, expanded protocols for macrolide-resistance detection, and enhanced biosafety and biosecurity considerations. Together, these updates provide a more robust, harmonised framework to support accurate diagnosis, high-resolution molecular surveillance, and rapid detection of evolving *B. pertussis* lineages across EU Member States.

Description of EURL-PH-DIPE

The aim of the European Reference Laboratory for Public Health on Diphtheria and Pertussis is to strengthen the preparedness of National Reference Laboratories (NRLs) across Europe for these two diseases, and to provide guidance and support to ECDC and the NRL network. The EURL activities are organised in seven work packages (WP) with clearly defined objectives over the course of seven years. This includes three network meetings; two updates of the laboratory handbook for pertussis; 10 rounds of External Quality Assessment (EQA) (seven for pertussis and three for diphtheria); a European Union/European Economic Area (EU/EEA) seroprevalence study of anti-PT, anti-DT and anti-TT antibodies; two Gap analyses for diphtheria; four wet-lab trainings; 14 twining training visits and seven on-line scientific seminars (one per year).

The EURL-PH-DIPE consortium is led by the University of Turku (UTU), Finland, which manages network coordination, the organisation of EQA studies, training activities, and seroprevalence studies. Other members include Institut Pasteur (IP), which leads the activities related to *B. pertussis* and *C. diphtheriae* whole genome sequencing (WGS) analysis; Vrije Universiteit Brussel (VUB) and Sciensano (SCI) which jointly contribute to both pertussis and diphtheria activities, particularly in diagnostic methodologies and seroprevalence studies, and Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (LGL) – a WHO Collaborating Centre for Diphtheria which contributes to most diphtheria-related activities.

1. Introduction

Despite childhood immunisations having been widely implemented, pertussis remains one of the world's leading causes of vaccine-preventable deaths, with epidemics occurring every three to five years in many countries. Like many other respiratory infectious diseases, pertussis incidence declined sharply during the COVID-19 pandemic, probably due to non-pharmaceutical interventions including lockdown, social distancing and wearing masks. However, with the return to normal activities pertussis outbreaks have been widely reported across the world. According to the World Health Organization (WHO), a total of 941 565 cases were reported in 2024, the highest number since 2000, and around six times as many as those reported in 2023 and in 2019, before the COVID-19 pandemic [1]. In Europe, the average number of reported cases per year during the period 2012–2019 was 38 145, during 2021–2022 it was 2 100 and during 2023 it was 26 042 [2]Click or tap here to enter text.. In 2024, multiple European countries experienced pertussis outbreaks, with a total of 209 674 reported cases—the highest level observed in the past decade.

Any outbreak of pertussis results in the questioning of what may have changed: population immunity, vaccination coverage, or the pathogen itself. Several of factors were involved in the recent pertussis epidemics, and without surveillance of circulating *Bordetella pertussis*, it is difficult to establish the causes of these epidemics. Furthermore, immunity induced by vaccination or natural infection is not life-long and individuals may have several episodes of pertussis in a lifetime. In addition, despite widespread vaccination, worldwide control of pertussis has proved to be difficult to achieve, meaning that it is difficult to avoid the frequent epidemics.

Acellular pertussis vaccines (ACVs) were introduced in many developed countries in the late 1990s or early 2000s to replace whole-cell pertussis vaccines (WCVs) due to a high prevalence of adverse effects with the WCVs. In Europe, pertussis vaccines and vaccination schedules vary among countries. At present, all European countries (except Poland) have switched from WCVs to ACVs. ACVs consist of one to five antigens (in different formulations) of *B. pertussis* [3]. Vaccine-induced immunity is reported to only last for up to 10 years [4,5], and increasing numbers of pertussis cases are reported in adolescents and adults [6]. During the outbreaks in 2023 and 2024, the highest incidences were observed in adolescents aged 10 to 14 years in several countries [2]. In many developed countries, differences in the virulence-associated protein components included in the ACVs (including pertactin (PRN), pertussis toxin (PT) and fimbriae (Fim)) have been found between *B. pertussis* vaccine strains and circulating isolates [7]. Furthermore, since 2000 *B. pertussis* isolates with the *ptxP3* allele for the pertussis toxin promoter have become prevalent in many countries, a genotype that is reported to confer enhanced virulence [8–13]. Since 1994, *B. pertussis* isolates resistant to macrolides have been identified in the USA, France, Iran and China [14–19], and these have increased considerably in China over the past ten years [15,16,20]. The emerging macrolide-resistant *B. pertussis* strain in several European countries was very alarming [21,22]. A point mutation changing nucleotide A to G at position 2047 (A2047G) in domain V of the 23S rRNA gene of *B. pertussis* has been associated with macrolide resistance [16,18]. Transmission of macrolide resistant *B. pertussis* from countries where macrolides are overused may have already occurred [23,24], as shown by a recent study in which 3 344 whole genome sequences of *B. pertussis* from 23 countries were compared and the time-intervals of intra- and international spread of *B. pertussis* were identified [25]. Before the COVID-19 pandemic *B. pertussis* isolates which did not express some of the ACV antigens, such as PRN, had been increasingly reported globally [12,26–31]. However, since the pandemic most recent isolates have been found to express this antigen [21,22].

The current methods for laboratory diagnosis of pertussis include culture, polymerase chain reaction (PCR) and immunological assays (e.g. enzyme-linked immunosorbent assay (ELISA) and multiplexed immunoassays (MIA)) for serology. A study carried out in Europe found that there is high heterogeneity in methods used for the laboratory confirmation of pertussis among national pertussis reference laboratories [32]. To evaluate the effects of different pertussis immunisation programmes in Europe, standardisation and harmonisation of the laboratory methods is clearly needed. In addition, commercial real-time quantitative PCR kits (qPCR) for respiratory pathogen panels are now widely used in diagnostics, allowing many respiratory pathogens to be detected simultaneously. However, this may lead to false negative results for pertussis as the sensitivities of these kits for individual pathogens are likely to be compromised due to multiplexing and/or choice of target [33,34]. New rapid point-of-care (POC) tests for pertussis diagnosis have also been developed recently [35,36].

For more effective national immunisation programmes to prevent pertussis in Europe, it is important to apply standardised diagnostic methods which will make country-based incidence of pertussis comparable. It is also important to monitor changes in bacterial populations and to study the impact of these changes on the prevention and incidence of disease. The aim of this work area was to develop a guidance document for laboratory diagnosis and molecular surveillance of *B. pertussis* for European countries. The document includes an updated summary of diagnostic PCR and serology, together with detailed information on isolation, culture, identification and epidemiological typing of *B. pertussis* to help users choose the best methods, in accordance with their local technical and financial circumstances. In addition, a description of methods for rapid identification and detection of macrolide resistant *B. pertussis* (MRBP) has been included. Some laboratory protocols have been published previously and these are cited in the appropriate sections.

2. Diagnostics

2.1 Nasopharyngeal sampling for culture and PCR

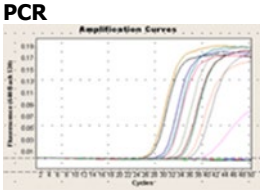
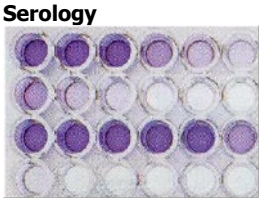
B. pertussis can be isolated from a nasopharyngeal swab (NPS) or nasopharyngeal aspirate (NPA). It has previously been shown that a 15% gain in isolation rate is obtained using NPA compared to NPS in neonates and infants. For small children, NPAs are often preferred by medical staff and parents, and NPAs can easily be divided into aliquots and saved for other investigations. However, for older children, adolescents and adults, NPSs are mostly used as NPAs are difficult to obtain in this population [37]. It should be borne in mind that *B. pertussis* bacteria usually grow in the posterior nasopharynx, and NP swabs should reach the right place. A video of the correct sampling technique, produced by Nicole Guiso at the Institut Pasteur, Paris, France can be found on YouTube at the following link: prelevcoqueluche-desktop.m4v - YouTube[1].

Since *B. pertussis* mainly grows in the human nasopharynx, throat swabs are considered suboptimal for culture and PCR and are not recommended. However, general practitioners sometimes use these due to wider availability of this swab type and easier sampling.

Sampling, isolation, culture and recommended diagnostics are summarised in Table 1 and Figure 1.

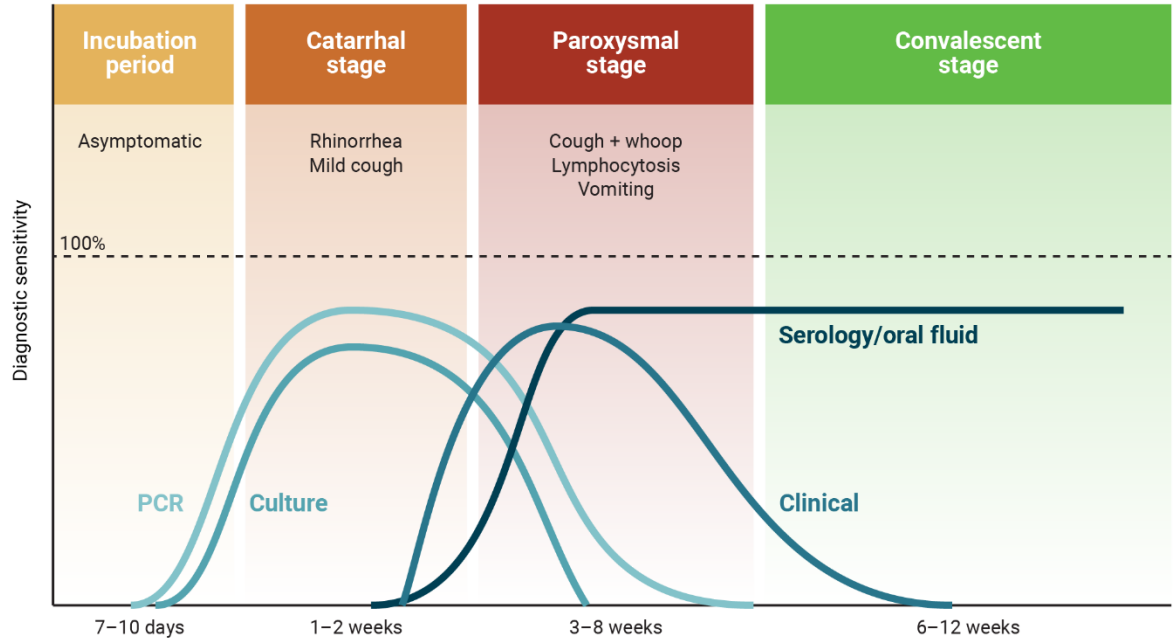
[1] *Prelevcoqueluche-desktop.m4v* - Youtube, ACTIVINFOVAC, Institut Pasteur, 2010 (accessed 05.12.2025) [38].

Table 1. Main tests used in *B. pertussis* diagnostics

Test method	Patient criteria	Sample
Culture 	Suspected cases in all age groups with cough <21 days of duration	NPS/NPA
PCR 	Suspected cases in all age groups with cough <21 days of duration	NPS preferred; throat swab acceptable for community patients
Serology 	Suspected cases in older children/adults with cough >14* days of duration	Serum**

** 'Recent' vaccination with pertussis-containing vaccine potential confounder for serum/OF assays (within the past year, or longer).*
*** Oral fluid also used in the UK for serology*
NPS = nasopharyngeal swab; NPA = nasopharyngeal aspirate.

Figure 1. Pertussis diagnostic guidelines and timing



This figure was adapted from Fry et al. [39].

2.2 Transport of swabs for culture and PCR

After sampling, an NPS or NPA intended for detection of *B. pertussis* or other *Bordetella* (e.g. *B. parapertussis*) should be transported quickly. Prior to transportation, the NPS/NPA should be stored at room temperature to avoid bacterial loss [40]. The collected swabs (or only the tip) can also be placed in Reagan Lowe (RL) or Amies medium containing charcoal, or in universal transport medium (UTM) [40,41]. To avoid bacterial loss transport should also be at room temperature and it should be arranged with minimal delay as a transportation time of more than 48 hours will have a negative effect on viable bacteria [40]. Dry swabs are adequate for PCR testing and either nylon, dacron or rayon swabs are recommended. Calcium-alginate and cotton swabs may inhibit the PCR and are therefore not recommended [42]. However, it is important to note that room temperature may be too high in very hot countries/climates, particularly during summer, and therefore in these settings the use of cooler bags is recommended for the shipment.

2.3 Culture and storage

B. pertussis is a fastidious bacterium and the NPS/NPA should therefore be transported to the microbiology laboratory for culture at ambient temperature within four hours of collection. Transport times of up to 48 hours are acceptable when a suitable transport medium is employed (i.e. RL medium). If it is not possible to perform the culture within 48 hours of sampling, an aliquot can be stored at -80 °C and cultured later. It has been shown that children, especially infants, have high *B. pertussis* DNA loads in their NPS compared to adults [43]. Therefore, diagnosis of pertussis by culture is particularly useful in infants and young children. Positive cultures can usually only be obtained two to three weeks after the onset of cough.

After transport, the NPS or NSA are streaked onto fresh RL medium or Bordet-Gengou medium (BG) supplemented with 15% defibrinated sheep or horse blood. *B. pertussis* has a slow growth rate, and it is therefore necessary to use a selective medium to inhibit the growth of normal flora. RL medium contains cephalexin for this purpose, and cephalexin (40 µg/ml) can also be added to BG medium.

B. pertussis is a strictly aerobic bacterium. Plates should be incubated for seven days aerobically at 35–36°C, preferably with 60% relative moisture to avoid plate drying, and they should be inspected daily. Plates showing no growth after seven days of incubation can be discarded as negative. *B. pertussis* grows more slowly on BG, but isolation rates on RL and BG plates are similar after seven days of incubation, and a major advantage of BG is the possibility to visualise the haemolysis. Typical *B. pertussis* or *B. parapertussis* colonies on BG or RL plates are small, mercury-like droplets and glistening. *B. pertussis* or *B. parapertussis* will appear as typical small non-motile gram-negative coccobacilli. For confirmation, specific agglutination test (*B. pertussis* /*B. parapertussis*) or PCR are recommended [44,45]. Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) has also been shown to be reliable [46]. Biochemical tests can also be helpful to guide identification (oxydase and urease: +/- for *B. pertussis* and -/+ for *B. parapertussis*).

For storage, two different mediums can be used: Trypticase Soy Broth (TSB) – 10-20% Glycerol tubes or Bovine Serum Albumin (BSA) – Saccharose/phosphate/glutamate (SPG) buffer. More information can be found in the appendixes to WHO's 'Laboratory Manual for the diagnosis of whooping cough caused by bordetella pertussis/bordetella parapertussis' [40] and in Annex 1 of this manual.

It is important to perform culture in order to be able to monitor for vaccine antigen deficiency and evolution of the pathogen, as well as to evaluate its antibiotic resistance [18,47,48]. Since culture is less used in routine clinical practice and PCR is increasingly used for diagnosis of pertussis, one study has shown that culture from PCR-positive samples is a feasible approach to recover *B. pertussis* isolates, especially from samples with a high bacterial load (e.g. IS*481* or IS*1001* Ct value $\leq$25) [49]. During the 2024 outbreak of pertussis in Finland, 2 820 cases were laboratory-confirmed. In total, 40% of them were diagnosed using PCR, 37.2% by culture and 22.8% by serology. Most of the culture-positive samples were primarily PCR-positive, because the majority of cultures were made from PCR positive samples [22]. Detailed protocols regarding NPSs and culture can be found in WHO's laboratory manual for the diagnosis of pertussis [40].

2.4 Polymerase Chain Reaction (PCR) for nucleic acid detection of *Bordetella*

Diagnosis of pertussis by PCR is proven to be effective in patients who have been coughing for less than four weeks. However, the sensitivity is inversely related to time between onset of disease and specimen collection. PCR-based diagnosis can confirm an infection by different species of *Bordetella*. The key criteria for PCR-based diagnosis of a *Bordetella* infection are:

- a correctly taken nasopharyngeal swab or nasopharyngeal aspirate;
- an appropriate amplification target; and
- correct interpretation of results.

B. pertussis will mainly attach to ciliated epithelia in the airways and it is therefore preferred that the diagnostic sample is taken from the posterior nasopharynx (not from nose or throat). Swabs, as well as aspirates, can be used, and swabs made of rayon or dacron are suitable, whereas cotton or calcium alginate are not recommended [42]. Examples of suitable transport media for the swabs are Amies medium with charcoal or Stuart's medium, and the swabs can be transported at ambient temperature [50]. Prior to the PCR analysis, DNA should be extracted from the sample, for which there are several commercial kits available [51].

A number of genetic targets for detection of *Bordetella* species are possible. However, since several of these targets are present in the genome of more than one *Bordetella* species, choice of targets and interpretation of results are crucial. Insertion elements (IS) are most often used, in particular the IS*481* and IS*1001*. IS*481* is present in *B. pertussis*, *B. holmesii* and in some isolates of *B. bronchiseptica* [52], while IS*1001* is present in *B. parapertussis* and some isolates of *B. bronchiseptica* [53]. The IS elements are present in the genomes in a high number of copies (approx. 250 for IS*481* in *B. pertussis*), thereby increasing the sensitivity of detection.

Many laboratories and kits have interpreted an IS*481*-positive result as a confirmation of *B. pertussis* and an IS*1001*-positive result as a confirmation of *B. parapertussis*. This is, however, incorrect and for definitive confirmation of *B. pertussis*, specific *B. pertussis* assays are recommended, targeting the pertussis toxin promoter *(ptxP*) for *B. pertussis* or the IS*1001*-like element for *B. holmesii* [54] (named h-IS*1001*) as additional targets. To ensure optimal sensitivity and specificity, the use of controls is highly encouraged – i.e. controls for extraction, internal amplification, and positive and negative controls for the PCR step.

Diagnosis of pertussis by PCR is particularly useful in infants and young children, as they are more likely to be diagnosed in the early stages of infection, whilst adults tend to endure the symptoms for a longer period before seeking medical advice. If the patient has been coughing for two to three weeks or longer, diagnosis by serology (anti-PT IgG) is recommended, or a PCR on a secondary case since the disease is highly contagious.

Real-time quantitative PCR (qPCR) has several advantages compared to block-based PCR, as qPCR is faster and provides a quantitative result. The results can be monitored online, and agarose gel-based detection of PCR amplified products is not required. To avoid possible false-positive results due to the high number of PCR cycles, a maximum of 40 cycles of block-based PCR or qPCR is usually used [40]. ECDC published detailed guidance and a protocol in 2012 [45] and WHO in 2014 [40].

At present, multi-targeted qPCR combination analyses are widely used for pertussis diagnostics among clinical microbiology laboratories as a number of respiratory pathogens can be detected simultaneously. Although this approach is useful for detection, identification and differentiation of respiratory pathogens and increases the number of pertussis diagnostics performed, the kits are not as sensitive as PCRs specifically designed for pertussis diagnostics. According to studies and information from the kit producers, up to 22 different pathogens can be measured with these multi-target PCRs. However, the studies also show that multiplex PCRs show only 56–67% positivity rate for confirmation of pertussis compared to those identified by single target specific pertussis PCR among clinically confirmed pertussis cases [33,34,55]. Recently, a group of experts at the US Centers for Disease Control and Prevention(CDC) evaluated the trends in commercial laboratory testing and positivity for *Bordetella* species in the United States between 2019 and 2023 [56]. Among the 527 206 tests performed, they identified 316 428 (60.1%) PCR tests, 204 480 (38.8%) serological tests, and 5 840 (1.1%) cultures. While most PCR tests were ordered as part of a respiratory panel (83.5%), only 215 (0.08%) were positive for *B. pertussis*. During the study period, non-panel PCR positivity for *B. pertussis* was substantially higher but variable, ranging from 3% to 16%. This is possibly due to the use of *ptxP* as a target as only a single copy of *ptxP* is present in the genome. It could also be due to the collection of swab samples (it should be NP swab not nasal swab for specific detection of *B. pertussis*). Therefore, multi-target PCRs show a lower sensitivity for confirmation of pertussis among samples, especially with low content DNA/bacterial load. This aspect should be carefully considered when implementing a new PCR targeting multiple pathogens at a diagnostic laboratory. The IS elements have a high sensitivity but low specificity, and the *ptxP* PCR has a low sensitivity but high specificity. The optimal analysis for confirmation of pertussis by PCR is a combination of IS elements (IS*481* and IS*1001*) and *ptxP* and/or hIS*1001* as targets.

2.5 Serology

2.5.1 Blood sampling for detection of anti-PT IgG antibodies

Serological assays are mainly validated to test serum and therefore use of serum tubes without additives is recommended. Serum tubes are available from many manufacturers. Heparinised plasma or plasma collected in ethylenediaminetetraacetic acid (EDTA) tubes can also be used if the chosen serological assay has been validated for this. Serum or plasma should be kept at room temperature and separated quickly after blood sampling, preferably within 4 hours, although up to 24 hours is possible. If samples will be tested later, separated sera can be stored in the refrigerator (+2-8°C) for up to seven days. However, for long-term storage, serum samples should be frozen at −20°C or below [57,58].

2.5.2 Oral fluid sampling for detection of anti-PT IgG antibodies

Oral fluid swabs are considered more comfortable for the patients than blood sampling. The fluid is collected by rubbing the swab along the gum line for 1–2 minutes, which is especially attractive for children as they can even self-collect the sample under supervision. Then the swab is placed in a transport tube and sent to the laboratory where the oral fluid is eluted from the swab. The eluted fluid is tested for the presence of anti-pertussis toxin (PT) IgG antibodies. The results obtained are reliable, and there are commercially available kits for oral fluid sampling [59]. At present, testing for anti-PT IgG using oral fluid is not widely used apart from in England and Wales where it is specifically used for notified cases in those aged 2–17-years for the convenience of diagnosis and surveillance in this age group and for outbreak investigation [60,61].

2.5.3 Recommendations and methods for serological diagnosis

Serological diagnosis of pertussis is most effective in patients who have been coughing for at least two to three weeks, or longer. Antibodies against PT are the target of serology and PT is only produced by *B. pertussis*. To date, there are no serological tests available for *B. parapertussis* or other *Bordetella* infections.

Serology is primarily useful for diagnosis of children from two years of age, adolescents and adults. Infants and young children are more likely to be diagnosed in the early phases of pertussis, where PCR and/or culture are the methods of choice. Furthermore, as young children are likely to have been recently vaccinated against pertussis (and therefore have elevated levels of anti-PT antibodies), results from such individuals may be confounded.

Pertussis serology is performed by detecting specific *B. pertussis* antibodies to PT and can be performed with ELISAs or multiplex immunoassays (MIA). Key criteria for serological methods are that:

- only non-detoxified purified native PT is used as the coating antigen;
- results are expressed as international units (IU/ml) using calibrated standards such as the WHO Standard pertussis antisera 06/140 or 06/142 (NIBSC, Potters Bar, UK);
- IgG antibodies to PT are measured (anti-PT IgG);
- values from dilution series of the standard in use and of the diagnostic samples are parallel over a broad linear range [62]. However, it is possible to use fewer dilutions of the samples.

Several commercial kits with correct antigen and standards are currently available [63]. Kits that use antigens other than PT or mixed antigens and/or express results in arbitrary values are not recommended [64,65]. Therefore, validated in-house methods and commercial kits with only purified native PT as coating antigen and with reference or calibrants used in IU/mL are recommended [57].

Interpretation of results is critical. No universally recommended single cut-off exists, but those that are widely used are very similar. In general, a cut-off of 100 or 125 IU/ml serves as an indication of a recent infection within a year, and between 50/62.5 IU/mL to < 100 IU/mL as an indication of a recent infection within the past few years [62,65]. Comparison of data from Denmark, the Netherlands, and the United Kingdom showed that the single cut-off with optimal sensitivity and specificity for anti-PT IgG may be in the range between 60 IU/ml and 75 IU/ml [6,66].

In clinical practice, diagnosis is mostly based on a single sample of serum. If diagnosis cannot be confirmed from a single sample and is deemed necessary according to the clinical symptoms, a second (convalescent) serum sample should be taken two to four weeks after the first sample. If the second sample results in an anti-PT IgG concentration above the cut-off, or if a ≥100% increase or a ≥ 50% decrease of values above or below the level of diagnostic quantification in anti-PT IgG between the first and second sample is observed, the result can be interpreted as a confirmed diagnosis.

Since recent pertussis vaccination will result in elevated concentrations of anti-PT IgG antibodies, diagnostic pertussis serology is not recommended for at least one year after vaccination [3, 67, 68]. Detailed guidance and protocols for serological diagnosis have been published by ECDC [65] and by WHO [40].

2.6 Point-of-care (POC) assays

POC assays are generally more suitable for investigation in outbreaks as results are ready within a shorter period than is commonly the case for PCRs, and treatment of patients or prophylactic treatment of exposed subjects can be started sooner. Therefore, these tests are clinically relevant, however POC tests are still a rarity in pertussis diagnostics.

Development of POC tests for pertussis diagnosis is ongoing. A few options are described by von Koenig, of which Loop-mediated isothermal amplification (LAMP) has been commercialised [36,69,30]. This test is an alternative for PCR and is based on isothermal amplification. It does not require a stationary thermal device, and a mobile heater is enough to reach the elevated temperature needed. Urea can be used for DNA purification and the end product can be visualised (e.g. with a mobile device producing UV-light) [36]. Previous problems with specificity and sensitivity of this assay have been overcome and new innovative and low-cost platforms have been developed [69,70].

Recently, a CRISPR (clustered regularly interspaced short palindromic repeats)-based detection system was developed for rapid diagnosis of pertussis [71]. By combining this detection technique with a nucleic acid extraction-free method, the result can be ready within 30 minutes. When 82 clinical samples with confirmed pertussis were tested, a sensitivity and specificity of 97.3% and 100% were achieved compared with PCR. It seems that the CRISPR-based tests could offer a fast and accurate method to detect *B. pertussis* from clinical samples. However, further studies with a large number of samples are needed to compare with current diagnostic methods.

Another POC option is lateral flow (LF) assays, which target either antibodies from blood or *B. pertussis* antigens from the nasopharynx. The antibody-based tests have shown decent values for both sensitivity and specificity against PT [35,72]. Briefly, the sample is added on an absorbent sample pad and the test uses liquid chromatography. Visualisation is based on latex particles moving along with the sample, and results can be interpreted in a similar manner to COVID-19 LF tests, where a positive test will show as double lines (control and test line) on the device. The time from sampling to actual results is still relatively long, and further development is needed. This LF assay has not yet been commercialised.

2.7 Biosafety and biosecurity

B. pertussis is classified under Biosafety level 2 (BSL-2). Therefore, all work/research on this pathogen should be done in BSL-2 laboratories, which include proper safety cabinets (laminar hoods) to prevent the spread of aerosols and transmission of the bacteria to potent carriers of the disease (e.g. laboratory workers) [39]. In addition, laboratory coats, safety goggles and gloves should be used for protection.

Storage of *B. pertussis* isolates should be organised so that only certified persons can access them as a preventive measure to avoid possible abuse of the bacteria, although *B. pertussis* is not considered as a biological weapon due to existing vaccines and prophylaxis.

Individuals who contract pertussis should either stay in isolation (at home) or, if hospitalised, should be isolated from other patients/vulnerable persons to prevent the transmission of the disease. Early treatment with macrolides prevents transmission but will probably not alter the symptoms.

3. Molecular surveillance of *B. pertussis*

3.1 Serotyping

B. pertussis expresses two phase-variable major subunits of fimbriae: Fimbriae 2 (Fim2) and Fimbriae 3 (Fim3), which determine the serotype of isolates. Isolates can therefore be either Fim2, Fim3 or Fim2+Fim3 (Fim2,3). The fim2 and fim3 genes are regulated by single base insertions in their promoter regions, which result in serotype switching [73]. Serotyping of freshly isolated *B. pertussis* isolates was first described in 1953 [74], and has been an essential part of the characterisation of *B. pertussis* clinical isolates for a long time [75]. Studies on isolate variation have shown that *B. pertussis* populations fluctuate with respect to their serotypes, which may have important bearings on their recognition by the immune system [7,76–79]. Therefore, it is recommended that the serotype of circulating *B. pertussis* isolates be determined and this can be done using various methods:

- Slide agglutination: traditionally, serotyping is done using the slide agglutination method with bacteria from 24 to 48 hour cultures of *B. pertussis* mixed with specific serotyping antisera or monoclonal antibodies on glass slides [80]. The slide is rocked gently and observed for clumping. Specific agglutination occurs within 30 seconds.
- Micro-agglutination: this method [81] is based on the same principle as the slide method, except that instead of using glass slides, the assay is performed using V-bottom microtiter plates in which equal volumes of the bacterial suspension and a dilution in saline of the respective antiserum are mixed [82].
- ELISA: an indirect ELISA method can be used for serotyping [79,83]. ELISA plates are coated with heat inactivated bacterial suspensions and the serotype is determined by the use of Fim2/Fim3 specific monoclonal antibodies.

The bacterial agglutination method is comprehensive for a small number of isolates, whereas the ELISA protocol is more appropriate for processing larger numbers simultaneously [79].

For all serotyping methods, it is recommended that monoclonal antibodies (e.g. antibodies obtained from the National Institute for Biological Standards and Control (NIBSC), mAbs 06/124 (anti-Fim2) and 06/128 (anti-FIM-3) are used for serotyping of *B. pertussis* [84] and that Fim2- and Fim3-expressing isolates are included as controls [85–87]. No cross reactions have been found between monoclonal antibodies and reference isolates used as coating antigens [79]. Polyclonal antibodies have also been previously used for serotyping [88].

The recommended protocols for serotyping are presented in Annex 2.

3.2 Pulsed-field gel electrophoresis (PFGE)

The use of PFGE has become rare for *B. pertussis* typing. This method is laborious and requires a lot of time and cultured isolates, and cross-laboratory comparisons of PFGE profiles and interpretation of profiles are challenging. Furthermore, whole genome sequencing (WGS) provides high resolution, and sequence data can be directly compared. However, we still include a short description of PFGE in this handbook as a guideline for those laboratories still interested in using this method.

PFGE is based on electrophoretic separation of fragments obtained after digestion of genomic DNA with a restriction enzyme. This technique was first applied for epidemiological typing of *B. pertussis* in 1992 [89]. PFGE typing of *B. pertussis* has been successfully used in epidemiological investigations to identify outbreak-associated isolates and to monitor bacterial transmission [90–92].

In 2000, a group of experts working in the field of pertussis in Europe (EUpertstrain) established a detailed reference PFGE method based on two enzymes *Xba*I and *Spe*I [81]. The enzyme *Xba*I is recommended for PFGE analysis of *B. pertussis* isolates. Two nomenclatures are used when assigning isolates to different PFGE profiles. The French nomenclature is based on a cluster analysis, performed with the unweighted pair group method using the arithmetic average algorithm with 2% band tolerance and 1.5% optimisation settings. Based on this approach, seven clusters (I to VII) have been identified. The cluster IV also includes three subgroups named IVα, IVβ and IVγ [93]. The Swedish nomenclature is based on a cluster analysis with 1% band tolerance and 1% optimisation settings [94].

In general, the PFGE method analyses the whole genome, and results have proven to be stable and of a high resolution compared to Multi-locus Antigen Typing (MAST) and Multi Locus Variable Number of Tandem Repeat (VNTR) Analysis (MLVA) for epidemiological typing of *B. pertussis* [95,96]. To summarise, the PFGE technique and data can still be used to determine *B. pertussis* strains, although this is rare nowadays. For more details on PFGE, information can be found in the previous version of this laboratory handbook [97].

3.3 Multi-locus Antigen Sequence Typing (MAST)

Multi-locus Antigen Sequence Typing (MAST) was originally developed to study shifts in *B. pertussis* populations and to identify isolates producing protein variants which were distinct from the variants incorporated into pertussis vaccines [11,96,98,99]. MAST focuses on the gene coding for the five proteins used in current ACVs: PT, FHA, PRN, Fim2 and Fim3. The PT promoter (*ptxP*) is also included for isolate typing as the variant *ptxP3* is an important landmark mutation [8,25,100]. The allele numbers for the genes encoding all these different antigens can be found on the BIGSdb-Pasteur database (https://bigsdb.pasteur.fr/bordetella/). However, not all single nucleotide polymorphisms (SNPs) lead to an amino acid change in the sequence and therefore the conformation of the expressed protein is not affected. Protocols for gene amplification and sequencing are presented in Annex 3. Note that antigen gene sequences can also be derived from WGS, so nowadays the Sanger sequencing method is not often used.

3.3.1 Pertussis toxin (PT)

PT is comprised of five subunits, PtxA-PtxE (or Ptx1-Ptx5), with PtxA containing the toxic, catalytic activity. Of the genes encoding the five subunits, most sequence variation has been reported for *ptxA* [8]. The *ptxA* gene comprises 810 bases. In BIGSdb-Pasteur database, 46 alleles have been found for *ptxA* locus and 20 of them relate to *B. pertussis*. *ptxA* alleles 1, 2, 4, and 5 are the most frequent. The most common alleles are presented in Figure 2. While *ptxA*4 and *ptxA*5 were found in the pre-vaccine era, at present, the protein variant encoded by *ptxA1* is highly dominant among isolates across the globe [8,9,11,76,96,99,101–103].

Figure 2. Variation in the *ptxA* gene coding for the pertussis toxin A subunit*

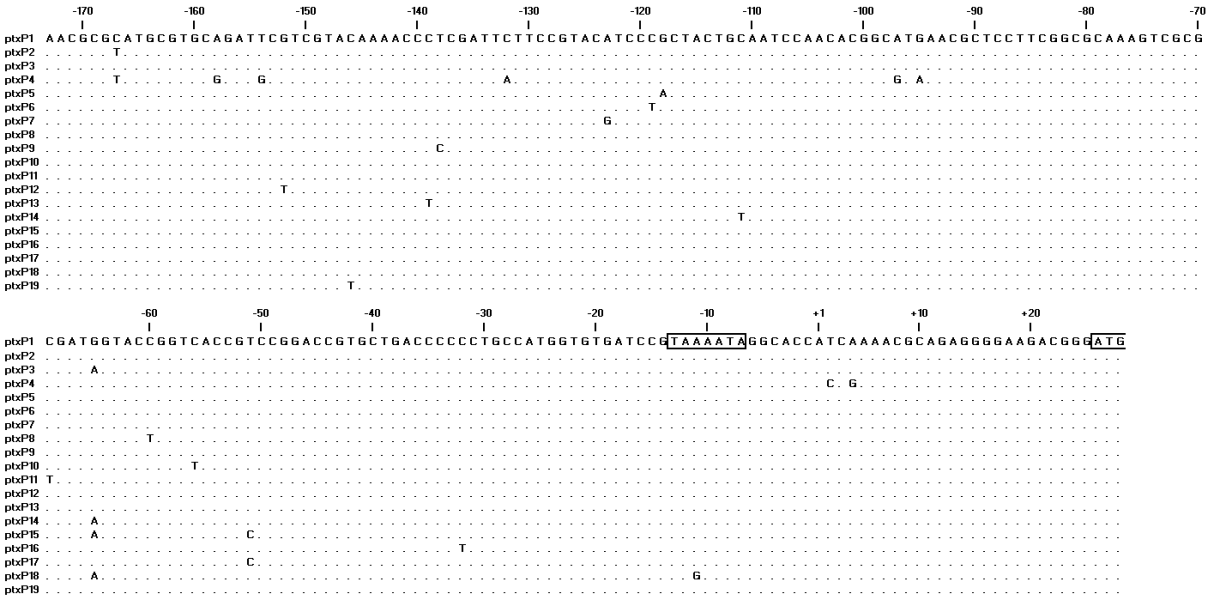
nt numbering	202	203	204	586	587	588	682	683	684	694	695	696
ptxA1	G	A	C	T	C	C	A	T	A	A	T	A
PtxA1		D			S			I			I	
ptxA2	.	.	.	.	.	.	.	.	G	.	.	.
PtxA2								M				
ptxA4	.	.	A	.	.	.	.	.	G	G	.	G
PtxA4		E						M			V	
ptxA5	.	.	A	C	.	.	.	.	G	.	.	G
PtxA5		E			P			M			M	
AA numbering		68			196			228			232	

** Dots indicate identity with the ptxA1 allele. Mutation sites are highlighted in ochre. Bases and amino acids are in black and red, respectively. Numbering (black for bases and red for amino acids), is relative to the start codon and first amino acid, respectively. For all ptxA alleles, the nucleic acid sequence can be found in the BIGSdb-Pasteur Bordetella database (https://bigsdb.pasteur.fr/bordetella/).*

3.3.2 The pertussis toxin promoter (*ptxP*)

Although *ptxP* does not code for a vaccine component, it is generally included in isolate typing. In the BIGSdb database, 42 alleles can be found for *ptxP* locus, with 20 of them relating to *B. pertussis*. *ptxP* is indeed considered as a marker for an emerged lineage, which has spread worldwide [7]. This lineage is marked by the *ptxP3* allele, which has been suggested to be associated with increased production of PT in vitro conditions [8,104]. In the pre-vaccination era, isolates with the *ptxP1* and *ptxP2 or ptxP4* alleles were predominant. After the introduction of vaccination, mainly *ptxP1* isolates were found, whereas nowadays *ptxP3* is predominant in Europe and many other countries [9–11,76,105]. Nowadays, *ptxP3* and *ptxP1* are the main alleles observed among the currently circulating isolates [22,106]. These two alleles differ by a single mutation in position -55 (G for *ptxP1* and A for *ptxP3*). Figure 3 shows some allelic differences.

Figure 3. Alleles for the pertussis toxin promoter (*ptxP*)



Bases are numbered –173 to +27 relative to the start of transcription (+1). The –10 sequence motif and initiation codon are highlighted with blocks. Alleles 20 to 49 are not presented, and can be found in the BIGSdb-Pasteur Bordetella database.

3.3.3 Filamentous hemagglutinin (FHA)

The FHA gene (*fhaB*) comprises around 12 000 bases and due to its large size, routine sequencing of this gene has not been performed, and the *fhaB* gene is rarely typed.

Nevertheless, when long-read sequencing is performed (using Oxford Nanopore (ONT) or PacBio technologies), the whole *fhaB* gene can be genotyped. This corresponds to the locus *fhaB* on the Bacterial Isolate Genome Sequence Database of Institut Pasteur (BIGSdb-Pasteur) displaying 60 different alleles for *B. pertussis*. The most frequent alleles are *fhaB2* and *fhaB39*. Most *B. pertussis* isolates are *fhaB2* but *fhaB39* allele has been reported in Shanghai, China as associated with macrolide-resistant *B. pertussis* (MRBP) isolates [107]. When *fhaB* alleles 2 and 39 are compared, a C5330T mutation leads to A1777C amino acid change.

When short-read sequencing is performed (as is usually routine with Illumina technology), only a part of *fhaB* gene can be sequenced and therefore genotyped. A locus corresponding to only a part of *fhaB* gene, fhaB-2400_5550, has been created in BIGSdb-Pasteur in order to catch the mutation associated with macrolide resistance in position 5330 (allele 39 of *fhaB* is corresponding to allele 3 for the locus fhaB-2400_5550).

A recent study has shown that the FhaB protein carries a C-terminal microtubule-binding domain (FhaB-CT), enhancing the bacterial colonisation [108]. A steady increase in FHA production of circulating isolates has been observed after the introduction of ACVs in Finland [109]. This may indicate that the gene should probably be monitored in the future for possible changes in response to ACV-induced selection pressure.

3.3.4 Pertactin (PRN)

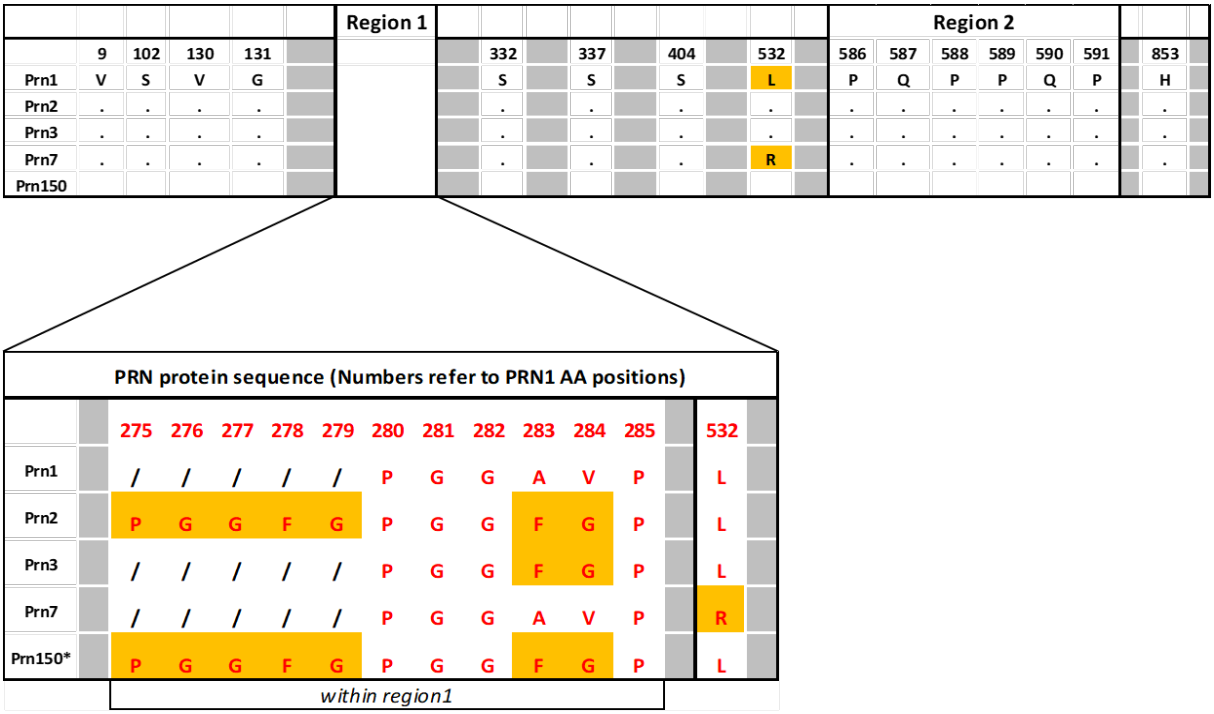
The *prn* gene comprises around 2 800 bases which encode for a large precursor protein of the autotransporter. The precursor molecule is proteolytically processed to produce P.69 and P.30 proteins located at the cell surface and in the outer membrane, respectively [110]. The integral outer membrane protein shows little variation and is not included in ACVs but the exported PRN P.69 is a vaccine antigen. P.69 protein is characterised by two polymorphic regions designed region 1 and region 2 that contain variable numbers of (GGXXP) and (PQP) repeats respectively. In *B. pertussis*, most variation is found in region 1 (Figure 3). Hence, typing is focused on region 1 for *B. pertussis*.

In BIGSdb-Pasteur database, the *prn* locus presents 170 alleles among the different *Bordetella* spp. With regard to *B. pertussis*, the *prn1* and *prn7* alleles were predominant in the pre-vaccine period and are also produced by many isolates used for production of vaccines (vaccine strains). In Europe, *prn3* was a transient allele observed in whole cell vaccine era and *prn2* is currently the predominant type found in approximately 95% of the circulating isolates [76,111]. Compared to *prn1*, *prn2* and *prn150* alleles lead to a longer PRN protein with an additional (GGFGP) repeat (Figure 3). In addition, *prn150* shows a synonymous mutation to *prn2* (C531T) [107]. In Asia, this new allele *prn150* has been found to be associated with MRBP strains [107].

In addition, the emergence and increase of clinical isolates which do not produce PRN has been observed in many countries. Some of the *prn* genotypes are therefore associated with PRN deficiency (e.g. with SNPs leading to stop codons) [112]. The issue of PRN-deficient isolates will be further described in chapter 3.5.

The *prn* allele identifiers are those available through the BIGSdb-Pasteur *Bordetella* database: https://bigsdb.pasteur.fr/bordetella/.

Figure 4. Variation in pertactin gene, regions 1 and 2*



	Region 1											Region 2							
	9	102	130	131		332		337		404		532	586	587	588	589	590	591	853
Prn1	V	S	V	G		S		S		S		L	P	Q	P	P	Q	P	H
Prn2	.	.	.	.		.		.		.		.	.	.	.	.	.	.	.
Prn3	.	.	.	.		.		.		.		.	.	.	.	.	.	.	.
Prn7	.	.	.	.		.		.		.		R	.	.	.	.	.	.	.
Prn150																			

PRN protein sequence (Numbers refer to PRN1 AA positions)													
	275	276	277	278	279	280	281	282	283	284	285		532
Prn1	/	/	/	/	/	P	G	G	A	V	P		L
Prn2	P	G	G	F	G	P	G	G	F	G	P		L
Prn3	/	/	/	/	/	P	G	G	F	G	P		L
Prn7	/	/	/	/	/	P	G	G	A	V	P		R
Prn150*	P	G	G	F	G	P	G	G	F	G	P		L
	within region1												

**Synonymous mutation to Prn2 (C531T), no amino acid change.*

***Region 1 (shown separately) and region 2 contain repeats. Only the amino acid sequences are shown. Dots indicate identity with prn1 and x-es indicate missing amino acids. Ochre colour refers to mutation site. Repeats have been blocked. Numbering is in amino acid (AA) sequence and relative to the initiation codon of prn1. prn14 and prn15 have been sequenced partially.*

3.3.5 Fimbriae (Fim)

B. pertussis produces two serologically different fimbriae major proteins FIM2 and FIM3 but also a minor subunit FIMD. The major *fim2* and *fim3* and minor *fimD* fimbrial subunit genes comprise around 600 and 1 098 bases, respectively. The *fimD* gene shows very little variation and to date only two alleles have been found [98]. The remaining two fimbrial major subunit genes, *fim2* (624 bp) and *fim3* (615 bp), are rarely expressed simultaneously by *B. pertussis* isolates due to a phase-variable promoter [73]. All the alleles identified for both *fim2* and *fim3* can be found in BIGSdb-Pasteur database. Isolates currently used for ACVs are characterised by *fim2-1* and *fim3-1* alleles.

With regard to *fim2* locus, the main alleles are *fim2-1* and *fim2-2.* They differ by a non-synonymous mutation in position 521 leading to an amino acid change in position 174 of FIM2 protein (Figure 5).

For *fim3* locus, the main alleles are *fim3-1*, *fim3-2*, *fim3-4* and *fim3-26* (Figure 4). In pre-vaccination isolates, only the *fim3-1* allele had been identified, nowadays both *fim3-1* and *fim3-2* alleles represent more than 95% of the circulating isolates. Compared to *fim3-1*, *fim3-2* allele is characterised by a non-synonymous mutation in position 260, leading to an amino acid change in position 87 of FIM3 protein.

Figure 5. Variation in the *fim2* and *fim3* genes coding for the serotype 2 and 3 fimbrial subunits

nt numbering	85	86	87	259	260	261	493	494	495
fim3-1	A	C	C	G	C	G	G	T	C
Fim3-1		T			A			V	
fim3-2	.	.	.	.	A	.	.	.	.
Fim3-2					E				
fim3-4	.	.	T	.	.	.	.	.	.
Fim3-1									
fim3-26	.	.	T	.	.	.	.	A	.
Fm3-26								D	
AA numbering		29			87			165	

nt numbering	520	521	522
fim2-1	A	G	A
Fim2-1		R	
fim2-2	.	A	.
Fim2-2		K	
AA numbering		174	

**Dots indicate identity with the fim2-1 and fim3-1 alleles. Mutation sites are highlighted in ochre. Bases and amino acids and their numbering are in black and red, respectively. Numbering is in nucleotides and relative to the start codon and the first amino acid. Note that Fim3-1 is encoded by two different alleles.*

***Fim3-3 is an artefact due to sequencing error.*

3.3.6 Bp- vaccine antigen genotyping scheme (Bp-agST)

In order to facilitate the genotyping of *B. pertussis*, a genotyping scheme has been developed in the BIGSdb-Pasteur *Bordetella* database based on all the main loci of interest: *ptxP*, *ptxA-E*, *fhaB2400-5550*, *fim2* and *fim3*. The main Bp-agST found in circulating isolates are Bp-agST 4 and 9. Other Bp-agST descriptions can be found on the BIGSdb-Pasteur platform [113].

Table 2. Genotyping scheme developed in BIGSdb-Pasteur *Bordetella* database based on loci *ptxP*, *ptxA-E*, *fhaB2400-5550*, *fim2* and *fim3*

BPagST	ptxP	ptxA	ptxB	ptxC	ptxD	ptxE	fhaB-2400_5550	fim2	fim3
4	3	1	1	4	1	4	1	1	1
9	3	1	1	4	1	4	1	1	2

3.4 Multi Locus Variable Number of Tandem Repeat (VNTR) Analysis (MLVA)

A bacterial variable-number tandem repeat (VNTR) locus is a genomic site containing tandem (direct) repeats of a DNA sequence. If the number of tandem repeats at an individual VNTR locus varies across a bacterial population, the repeat number can be used to differentiate isolates. Multi-locus VNTR analysis (MLVA) quantifies the numbers of repeats at several VNTR loci in each isolate and has been used for epidemiological typing of many bacterial species [114]. However, WGS has largely replaced the use of this method, which has lower resolution than whole-genome SNPs. Furthermore, the *B. pertussis* MLVA reference database (http://www.mlva.net/) is no longer maintained. For more details on MLVA, information can be found in the previous version of this laboratory handbook [97].

3.5 *B. pertussis* isolates deficient for the acellular pertussis vaccine antigen pertactin (PRN)

3.5.1 Background on PRN deficiency

The introduction of WCVs in the 1940s and 1950s changed the environment of *B. pertussis*. It appeared that vaccination was associated with emergence of new *B. pertussis* populations [7,25,78,79]. The hypothesis was that the later introduction of ACVs would further affect the remaining *B. pertussis* populations [115]. While WCV vaccination induces a broad immune response against hundreds of bacterial proteins, ACV only targets a few bacterial proteins.

Several years after the introduction of ACVs in many countries, isolates lacking the production of the vaccine antigen PRN emerged and spread in regions with high vaccination coverage [12, 26–30,67,116–119. A study conducted in nine European countries (Belgium, Denmark, Finland, France, Italy, the Netherlands, Norway, Sweden and UK) showed a significant increase in PRN deficient isolates from 1.0% in 1998–2001 to 25% in 2012–2015 [120]. In general, the earlier the introduction of ACVs, the higher the proportion of PRN deficient *B. pertussis* isolates [120]. Similar increases have been observed in Australia, Japan and the USA [28,31,121]. In contrast, in Argentina where primary vaccination with whole-cell vaccine was used during the period 2000–2017, PRN deficient isolates were rarely detected (only two out of 350 isolates were PRN deficient) [122]. Furthermore, a study in Japan showed a decrease in the frequency of PRN deficient isolates after a PRN containing ACV was replaced with an ACV without PRN, further suggesting a role of PRN-containing ACV vaccination on the evolution of circulating isolates [123]. By combining global isolates spanning more than 80 years across multiple countries, together with the specific ACV implementation dates in each country, mathematical models were used and allowed to quantify the increased fitness of PRN-deficient strains following the implementation of ACV vaccines, in a lineage-dependent manner [25]. However, during the COVID-19 pandemic, with common mitigation measures pertussis incidence sharply declined in many countries. After the pandemic period, in 2023–2024, pertussis outbreaks were reported in many European countries [2,22,124]. It is interesting to note that during this reemergence of pertussis the PRN deficient strains represented a minority of isolates [2,22,124]. This may have been due to the reimportation of PRN positive isolates from countries where they are still prevalent. Moreover, PRN-positive isolates may also have increased fitness compared to PRN deficient ones, as the population immunity after the COVID-19 pandemic could have been less directed towards PRN, since the previously-circulating isolates were predominantly PRN-negative, and some ACVs do not contain PRN. However, PRN-deficient isolates have an increased ability to colonise ACV-immunised mice than PRN-positive isolates [125,126]. The few PRN-deficient isolates that were detected were associated with an expanding macrolide-resistant lineage observed in China since 2016 and in Europe since 2024, which is of serious concern [22, 127].

Multiple mutations in the *B. pertussis* genome can result in PRN deficiency in *B. pertussis*, and such isolates have originated from different lineages [31,117,120,126]. The common mechanism observed was either an insertion of an IS*481* element in the *prn* gene or a partial deletion of the *prn* gene [27]. In Japan and Finland, a new deletion in the signal sequence of the *prn* gene was identified [28,29]. Since then, among other isolates, single nucleotide polymorphisms leading to a stop codon, deletion of the whole *prn* gene or inversions in the *prn* gene promoter area have been identified [120]. Recently, the *prn150* allele, which is prevalent in China, has also been mutated by the insertion of IS*481* insertion [107, 128].

Using a mouse model and human cell lines, no difference in virulence was observed between isolates not producing PRN and isolates producing PRN [12]. In addition, early studies failed to show that isolates deficient in PRN production differed in virulence from those producing PRN [129].

3.5.2 Methods to detect PRN production

The methods used for detection of PRN production by *B. pertussis* include Western blotting [12,27,28,30,116] and indirect ELISA [29]. Recently, new mAbs specific to PRN have been found (3-5, 69k/16, https://nibsc.org/). Both have been tested at the University of Turku and at the Institut Pasteur and show comparable specificity to PeM4 mAbs previously described [29].

Sanger sequencing of the whole *prn* gene was used to analyse the molecular basis for the loss of PRN expression. Further, WGS provides direct data on disruptions in the PRN gene and makes it possible to directly predict deficient isolates. However, a few isolates do not produce PRN, even though no disruptions have been identified in the *prn* gene or in the promoter area [120]. Therefore, phenotypic testing remains important to define PRN production status in isolates without apparent disruptions.

3.5.3 Other ACV antigens

In addition to PRN, other vaccine antigens were shown to be absent in some isolates, even though these have only been found very sporadically, with no evidence for transmission. The first PT deficient isolate was reported from France in 1996, and five such isolates were identified in France between 1996 and 2018 (0.35%, five out of 2 280) [27]. The cause for deficiency was deletion of the whole *ptx* operon and an unknown mechanism [12,27,77]. In addition, one PT and PRN negative isolate has been found in the US with the same deletion of the whole *ptx* operon [130]. Two FHA and PRN negative isolates have been reported in Australia and Slovenia [131,132]. FHA deficient isolates have been found from France (N=4), Slovenia (N=1), Sweden (N=2) and the US (N=5) [12,77,133–135]. Several mechanisms, including *IS481* insertion and single-base insertions/deletions in the *fhaB* gene, were found to cause the deficiency [133,135,136]. Furthermore, changes within the homopolymeric G tract (site: 1078-1087) from 10 Gs to 11 Gs in *fhaB*, result in a downstream stop codon leading to a truncated protein (97). In addition, sporadic isolates deficient in both Fim2 and Fim3 have been found [77,120]. Although not included in the ACVs, one isolate deficient in tracheal colonisation factor A has also been found [105,116,120,131,137,138].

So far, there is no evidence of isolates deficient in adenylate cyclase toxin (ACT), an important antigen of *B. pertussis* that is not included in ACVs. However, studies indicate that the currently circulating isolates seem to have shown an increase in the level of production of this antigen in Finland, where a large number of circulating isolates from the period 1991–2020 were studied [109].

The updated ELISA protocol for detection of vaccine antigen expression and sequencing primers of the *prn* gene and its promoter region are presented in Annex 4.

3.6 Antimicrobial susceptibility testing (AST)

Macrolides [azithromycin (AZT), clarithromycin (CLR) and erythromycin (ERY)] are the first-line drugs to treat *B. pertussis* infection [139,140]. Macrolide-resistant *B. pertussis* (MRBP) isolates are mainly found in China, and only sporadically in Europe, the Middle East and North and South America [15–19,141,142]. The frequency of MRBP isolates has increased to almost reach population fixation in different regions of China. According to Chinese studies performed in Beijing (2013–2014), Zhejiang province (2016) and Shanghai (2016–2017), frequencies of MRBP varied from 60% to 92% [143,144]. These isolates are widely found in different areas of China [145,146]. At present, MRBP isolates are increasing in Japan and Vietnam [24,147,148]. According to the latest study from Japan, it seems that these strains are highly related to the *ptxP3* cluster in China [148]. However, these strains also emerged in Europe in 2024 [21,22,149]. Furthermore, a study from France indicated that these strains are closely related to the same *ptxP3* cluster from China [127]. The expansion of these strains is alarming, and it is essential to develop the ability of laboratories to identify macrolide resistance. During 2024, three MRBP isolates were identified in Belgium and one strain was also found to be resistant to trimethoprim-sulfamethoxazole – an alternative drug for treatment of pertussis among patients over two months of age if the isolate is found to be macrolide resistant [unpublished data by Martini et al. UZB].

In 2025, there was a nationwide epidemic of pertussis in Japan. According to Japan's Institute for Health Security (JIHS), as of 22 October 2025 (epidemiological week 42), Japan had recorded 82 795 cumulative pertussis cases, representing the highest annual total since nationwide recordkeeping began in 2018. Moreover, it is very alarming that widespread macrolide resistance was reported in Okinawa where 227 pertussis cases have occurred since November 2024 [148]. Of 31 tested isolates, 18 were confirmed to be MRBP, carrying the A2047G mutation in the 23S rRNA gene. Genomic analysis showed all Okinawa MRBP belong to the *ptxP3* lineage, but split into three distinct clusters (≥8 SNPs apart), suggestive of multiple introductions.

The only mechanism identified to date to cause the macrolide resistance is a point mutation changing nucleotide A to G at position 2047 (A2047G, position according to Barktus et al 2003; A2037G in Tohama NC_02929.2) in the domain V of the *23S rRNA* gene (3 copies) of *B. pertussis* [14,15,18,140]. Culture-based AST is the golden standard for the determination of macrolide resistance. So far, all resistant *B. pertussis* isolates detected have been highly resistant (>64 µg/mL) to macrolides (ERY, CLR and AZT) when measured by the minimum inhibition concentration (MIC) test, whereas susceptible isolates show no tolerance against the macrolides (both ERY, CLR and AZT <0.250 µg/mL) [18,140,144,150]. There are no defined cut-offs from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for macrolides and *B. pertussis*.

Methods to detect macrolide resistance have been described in detail. Wang et al. developed an allele specific PCR for rapid detection of the A2047G mutation in the 23S rRNA gene, the primers used are presented in Table 3 and the complete protocol is also available [16]. A French group has recently developed an RT-PCR based approach for rapid identification of macrolide resistant *B. pertussis* directly from DNA extracted from the clinical samples [127]. PCR-based (partial) Sanger sequencing of *23S rRNA* gene or WGS (genotyping of the *23s rRNA* allele available at BIGSdb-Pasteur database) are other options to detect these isolates, although WGS may take longer and can only be applied after culture.

Table 3. Primers used for allele-specific PCR to detect macrolide resistance

Primer	Sequence (5´to 3´)	target	Length and details
FP	GTGATGGGGTGCAAGCTCTT	23S rRNA	286 bp, forward, resistance
RP	TCTGGCGACTCGAGTTCTGC	23S rRNA	286 bp, reverse, resistance
MP	ATCTACCCGCGGCTAGACAGG	23S rRNA	121 bp, forward, susceptible
WP	ATCTACCCGCGGCTAGACAGA	23S rRNA	121 bp, reverse, susceptible

MIC testing has also been described previously [140]. *B. pertussis* isolates are first cultured on Regan-Lowe or Bordet-Gengou agar. After 48 hours of incubation, new plates are prepared with 0.5 McFarland suspension (or DO650nm =1). Strips containing gradients of increasing concentrations of AZY, CLR and ERY are added on the plates (MH supplemented with 5% blood or Regan-Lowe). MIC can be determined after 48–72 hours.

Complete protocols for AST MIC, block-based PCR and RT-PCR testing are presented in Annexes 5 and 6.

3.7 Whole genome sequencing (WGS)

WGS is evolving from its use as a research tool to become a routine surveillance approach, providing new insights for investigating infectious disease emergence and transmission, expediting pathogen characterization, and promoting data sharing. *B. pertussis* is highly monomorphic and the traditional typing methods, MLVA and MAST, have limited discriminatory power [151].

For WGS, high-quality bacterial DNA is needed, which can often be achieved with commercial extraction kits. The DNA template purity should be ensured by measuring the 260/280 and 260/230 absorbance ratios by UV spectrophotometry. A 260/280 ratio around 1.8 and a 260/230 ratio of 2.0-2.2 are generally considered indicative of a pure DNA sample. In addition, DNA concentration and integrity should be verified through fluorometry and electrophoresis-based, respectively. Several high-throughput sequencing instruments are available for WGS [152,153]. The platforms include both short-read and long-read sequencers. Short-read sequencers generate millions of raw reads in parallel, ranging between 50 and 400 base pairs [152. Short-read sequencing technologies are known to provide highly accurate results, however, large repeats and regions with extreme G+C contents are problematic, especially for WGS reconstruction of *B. pertussis* [154]. Long-read sequencers, such as those from Oxford Nanopore Technologies (ONT), overcome these problems by generating reads of more than 10 kb [152,155], which allow for complete *B. pertussis* genome reconstruction despite its repeated regions (e.g. IS*481*) that are responsible for assembly fragmentation when using short-read sequencing [154]. Long-read sequencing with ONT initially had higher error rates than short-read sequencing [154,156]. However, accuracy has steadily increased over the years [157], and ONT has gained popularity in the field of infectious diseases since 2023, thanks to recent developments that are reported to have made its raw-read accuracy comparable to that of short-read sequencers. This technology has recently been shown to be suitable for high-resolution genomic typing of *B. pertussis* [158,159] and can be expected to be increasingly part of routine genomic surveillance in the future. Nonetheless, draft genomes assembled from short reads are still very suitable for molecular typing, virulence-associated gene detection, outbreak investigation and phenotype prediction of *B. pertussis*. It remains to be seen whether WGS gives comparable results to phenotyping (e.g. for vaccine antigen expression of the circulating *B. pertussis* isolates) as the mechanisms causing expression disruptions are not all identified.

The most popular means of exploring bacterial population diversity are gene-by-gene approaches, which consider alleles as the units of analysis [160]. The allele calling process is facilitated by the existence of international public databases, such as the BIGSdb-Pasteur Bordetella Database (available at https://bigsdb.pasteur.fr/bordetella/) [113,161]. User sequences can be scanned against key target genes for *B. pertussis,* such as vaccine antigens and 23S rRNA gene sequences. Analysis can cover single genes to complex classification schemes, such as the core genome multilocus sequence typing (cgMLST_pertussis) scheme, which is currently a popular approach for high-resolution subtyping [162]. Gene-by-gene approaches are easily scalable to large groups of isolates. Genomes can also be compared through the Genome Comparator tool directly within BIGSdb, to determine the differences among isolates for all protein-coding sequences in an annotated reference [25]Click or tap here to enter text..

Another widely used method for isolate subtyping by WGS is based on single nucleotide polymorphisms (SNPs). This approach relies on reference-based mapping of either reads or assembled contigs. The intrinsic high discriminatory power of this analysis makes the generated data particularly suitable to perform phylogenetic inferences among closely related isolates [25,163]. An issue which should be addressed for SNP typing is the lack of inter-laboratory comparability. There is a need to standardise the SNP-calling parameters and the choice of regions to be analysed (or excluded – e.g. repetitive regions) for detecting SNPs against a reference genome, as European-wide comparisons are only possible if compatible SNP sets are used.

Thanks to long-read sequencing technologies, it was demonstrated that isolate diversity is not only the result of small genetic changes including SNPs, but also of large rearrangements [164], facilitated by the high number of repetitive genetic elements present in the *B. pertussis* genome (such as IS*481* of which there are approximately 250 copies in the *B. pertussis* genome). However, monitoring these large changes is not currently part of the routine characterisation of *B. pertussis* isolates.

Several issues can hamper application of WGS to routine surveillance. The most obvious are workforce transformation, costs and the lack of standardised analytical procedures that could make data easily interpretable and comparable. However, one of the major problems is the continued decline of diagnostic culture and thereby a decline in available isolates. This could be avoided by optimising culture-independent sequencing directly from nasopharyngeal samples used for qPCR. However, this method requires bacterial loads to be high enough to successfully generate sufficient sequence data to be assembled and used for genomic typing, which is an important limiting factor for WGS to be directly used in clinical settings for diagnosis. Another limiting factor for metagenomic sequencing is residual host DNA (human), which represents most of the DNA present in clinical samples. This should be degraded for better results. The human eukaryotic cells are first lysed with hypotonic buffer and the released DNA is then digested with endonuclease enzyme [165]. Recently, an Australian group evaluated the use of targeted multiplex PCR (mPCR) amplicon sequencing and shotgun metagenomic sequencing for culture-independent typing of *B. pertussis* directly from respiratory swabs. The authors developed a nine-target mPCR amplicon assay that could accurately type major lineages [ptxP3/non-ptxpP3, fim3-1/2, fhaB3/non-fhaB3, and epidemic lineages (ELs) 1–5] circulating in Australia and showed that mPCR amplicon sequencing was highly sensitive, with a limit of detection of 4.6 copies [IS*481* cycle threshold (Ct) 27.3]. A shotgun metagenomic sequencing was also successful in genotyping *B. pertussis* in 84% of clinical specimens with PCR Ct < 24 and was concordant with mPCR typing results [166]. A similar study conducted by the US CDC also showed that direct WGS of residual pertussis positive specimens is possible after the targeted enrichment of *B. pertussis* DNA. The optimised protocol is suitable for nasopharyngeal specimens with diagnostic IS*481* Ct < 35 and >10 ng DNA [167].

4. Summary

Diagnosis of pertussis or whooping cough is mainly based on direct detection of nucleic acids of *B. pertussis* (PCR), culture from nasopharyngeal samples, and specific antibody recognition (serology) from serum samples of patients with suspected pertussis. Although the use of culture has been decreasing over time, it is still highly recommended as the isolates can be used for surveillance of the changes in bacterial populations. Monitoring of the circulating isolates is important for evaluation of vaccine effectiveness and for future vaccine development. In addition, cultured *B. pertussis* isolates will be important for the epidemiological surveillance of macrolide resistance and effective treatment of patients.

PCR (conventional or qPCR) is highly recommended for the diagnosis of pertussis, but the methods used vary and the wide implementation of multi-targeted PCRs may compromise sensitivity when compared to those targeting only *B. pertussis* [31,32,51]. This should be borne in mind when choosing a correct PCR method for diagnostic use. However, since the COVID-19 pandemic and during the global epidemic of pertussis in 2024, the use of PCR-based assays has clearly increased as a primary diagnostic method. Furthermore, it is essential to promote appropriate sampling and the use of correct swabs, which will not inhibit the PCR reaction.

Serological diagnosis is mainly used among adolescents and adults. For this, either in-house methods or commercial kits are used in the diagnostic laboratories. However, for reliable results it is crucial to determine only anti-PT IgG antibodies, to include validated international standards and to use proper cut-offs in the interpretation of results [65]. A seroepidemiology study is also important to estimate of the circulation of *B. pertussis* in the population, to provide relevant information on the burden of pertussis and immunological status, and act as a tool to evaluate the risk of infection for infants not yet vaccinated. It can also provide an opportunity to study waning immunity based on antibody decay in the population. All the information is valuable for surveillance of the vaccine-preventable disease, provided that the conditions set out above for serological assays are followed.

In addition to these common methods, new point-of-care (POC) based methods are constantly under development. CRISPR, Loop-mediated isothermal amplification (LAMP) and lateral flow (LF) assays have been shown to be specific, sensitive and easy to perform, and these methods would be practical in outbreak investigations. The importance of developing reliable POC assays is high, as it ensures rapid identification of pertussis, which in turn allows treatment and prophylactic action to start quickly in order to prevent transmission of the disease [35,36,71,72].

Bordetella pertussis isolates which do not express PRN have expanded globally, especially since the introduction of ACVs [31,78,120]. However, the frequency of PRN-deficient isolates has decreased considerably since the COVID-19 pandemic, and it remains to be seen whether these strains will increase again in the future. This highlights the importance of continuing to monitor the evolution of *B. pertussis*, in order to inform vaccine strategies.

Two approaches to isolate surveillance can be distinguished: a targeted or a generic approach. With the targeted approach, one focuses on particular genes assumed to be important for evasion of host immunity [98]. This approach is especially useful now that ACVs are widely used, limiting the number of genes to be targeted. In the generic approach, isolate typing is based on (highly) variable regions (whether or not they play a role in evasion of host immunity) as in the case of MLVA, PFGE or SNP-based typing [93, 168–170]. Such approaches should be sensitive enough to identify newly emerged lineages. Of these, WGS-based approaches have become very common as their discriminatory power is significantly higher than that of other typing methods [151].

Targeted approaches include serotyping (Chapter 3.1) and MAST or other WGS-based antigen typing approaches (Chapter 3.3). The simplest, and oldest approach to typing of *B. pertussis* is serotyping. However, the usefulness of serotyping for interpreting increases in pertussis is very limited, as only three serotypes can be distinguished. Serotype fluctuation among the circulating isolates is observed over time due to general evolution, and it has been suggested that in populations where WCVs are used, increases in serotype 2 isolates reflect a fall in population immunity [79,86,87]. However, in ACV-vaccinated populations shifts between Fim2 and Fim3 have also been observed [171]. Whether this could be used as an indicator to predict upcoming outbreaks on a national level remains to be seen. Moreover, the fimbrial expression cannot be detected with genome-based approaches.

Generic approaches involve MLVA (Chapter 3.3.5), PFGE (Chapter 3.2) and SNP typing (Chapter 3.3.7). However, the use of MLVA and PFGE are now mostly outdated. Whole-genome SNPs provide much higher resolution but need to be standardised for inter-laboratory comparisons. In contrast, an important advantage of cgMLST-based/gene-by-gene based approaches is that the results are unequivocal and reproducible, allowing European-wide comparisons [113,172].

It is important that PRN expression is monitored continuously, as it is one of the key components in the current ACVs. The probability that PRN deficient strains will become dominant again is high, given their high fitness in ACV-immunised populations [25,173].

The frequency of macrolide resistant *B. pertussis* isolates in China is currently extremely high, reaching almost 100% in several settings. Furthermore, these isolates have expanded in neighbouring countries, and also emerged in Europe in 2024. It is therefore important to monitor these strains. This is significant not only for bacterial surveillance, but also for effective treatment of pertussis patients and contacts, especially for infants who are at the

highest risk of hospitalisation and mortality. Ideally, rapid identification of these strains should be established not only in national reference but also in clinical microbiology laboratories performing pertussis diagnostics.

To summarise, the purpose of this guidance document for diagnostics and molecular surveillance of *B. pertussis* was to provide scientific context on the different approaches, and to help laboratories to correctly identify cases of pertussis. The correct use and interpretation of culture, PCR, serology and WGS will be crucial for making country-based comparisons of pertussis data. Weighing utility and feasibility, the minimum useful isolate surveillance package implemented in a reference laboratory should consist of serotyping, PRN vaccine antigen expression, and AST. WGS is currently the most powerful method to monitor population changes and vaccine antigen variants, and needs to be implemented more broadly.

5. References

1. World Health Organization (WHO). Pertussis reported cases and incidence 2025. WHO: 2025. Available at: https://immunizationdata.who.int/global/wiise-detail-page/pertussis-reported-cases-and-incidence
2. European Centre for Disease Prevention and Control (ECDC). ECDC Surveillance Atlas (2018). Available at: https://www.ecdc.europa.eu/en/surveillance-atlas-infectious-diseases
3. Berbers GAM, de Greeff SC, Mooi FR. Improving pertussis vaccination. Hum Vaccin 2009;5:497–503. Available at: https://doi.org/10.4161/hv.8112
4. Wendelboe AM, van Rie A, Salmaso S, Englund JA. Duration of immunity against pertussis after natural infection or vaccination. Pediatr Infect Dis J 2005;24:58. Available at: https://pubmed.ncbi.nlm.nih.gov/15876927/
5 Guiso N, Njamkepo E, Vié le Sage F, Zepp F, Meyer CU, Abitbol V, et al. Long-term humoral and cell-mediated immunity after acellular pertussis vaccination compares favourably with whole-cell vaccines 6 years after booster vaccination in the second year of life. Vaccine 2007;25:1390–7. https://doi.org/10.1016/j.vaccine.2006.10.048.
6. de Greeff SC, de Melker HE, van Gageldonk PG, Schellekens JF, van der Klis FR, Mollema L, et al. Seroprevalence of pertussis in The Netherlands: evidence for increased circulation of *Bordetella pertussis*. PLoS One 2010;1;5(12):e14183. Available at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0014183
7. Mooi FR, van der Maas NA, de Melker HE. Pertussis resurgence: waning immunity and pathogen adaptation - two sides of the same coin. Epidemiol Infect 2013:1–10. Available at: https://doi.org/10.1017/S0950268813000071
8. Mooi FR, van Loo IH, van Gent M, He Q, Bart MJ, Heuvelman KJ, et al. *Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis 2009;15:1206–13. Available at: https://pmc.ncbi.nlm.nih.gov/articles/PMC2815961/
9. Advani A, Gustafsson L, Åhrén C, Mooi FR, Hallander HO. Appearance of Fim3 and ptxP3-*Bordetella pertussis* strains, in two regions of Sweden with different vaccination programs. Vaccine 2011;29:3438–42. Available at: https://www.sciencedirect.com/science/article/pii/S0264410X11003057?via%3Dihub
10. Kallonen T, Mertsola J, Mooi FR, He Q. Rapid detection of the recently emerged *Bordetella pertussis* strains with the ptxP3 pertussis toxin promoter allele by real-time PCR. Clin Microbiol Infect 2012;18:377. https://doi.org/10.1111/j.1469-0691.2012.04000.x
11. Petersen RF, Dalby T, Dragsted DM, Mooi F, Lambertsen L. Temporal trends in *Bordetella pertussis* populations, Denmark, 1949-2010. Emerg Infect Dis 2012;18:767–74. Available at: https://pubmed.ncbi.nlm.nih.gov/22515990/
12. Hegerle N, Paris AS, Brun D, Dore G, Njamkepo E, Guillot S, et al. Evolution of French *Bordetella pertussis* and *Bordetella parapertussis* isolates: increase of *Bordetellae* not expressing pertactin. Clin Microbiol Infect 2012;18(9):E340-6. Available at: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1469-0691.2012.03925.x
13. Schmidtke AJ, Boney KO, Martin SW, Skoff TH, Tondella ML, Tatti KM. Population diversity among *Bordetella pertussis* isolates, United States, 1935-2009. Emerg Infect Dis 2012;18:1248–55. Available at: https://wwwnc.cdc.gov/eid/article/18/8/12-0082_article
14. Bartkus JM, Juni BA, Ehresmann K, Miller CA, Sanden GN, Cassiday PK, et al. Identification of a mutation associated with erythromycin resistance in *Bordetella pertussis*: implications for surveillance of antimicrobial resistance. J Clin Microbiol 2003;41:1167–72.
15. Wang Z, Han R, Liu Y, Du Q, Liu J, Ma C, et al. Direct Detection of Erythromycin-Resistant *Bordetella pertussis* in Clinical Specimens by PCR. J Clin Microbiol 2015;53:3418–22. Available at: https://journals.asm.org/doi/10.1128/jcm.01499-15
16. Wang Z, Cui Z, Li Y, Hou T, Liu X, Xi Y, et al. High prevalence of erythromycin-resistant *Bordetella pertussis* in Xi'an, China. Clin Microbiol Infect 2014;20:825. Available at: https://www.sciencedirect.com/science/article/pii/S1198743X14653349?via%3Dihub
17. US Centers for Disease Control and Prevention (US CDC). Erythromycin-resistant *Bordetella pertussis*-Yuma County, Arizona, May-October 1994. MMWR Morb Mortal Wkly Rep 1994;43:807–10.
18. Guillot S, Descours G, Gillet Y, Etienne J, Floret D, Guiso N. Macrolide-resistant *Bordetella pertussis* infection in newborn girl, France. Emerg Infect Dis 2012;18:966–8. Available at: https://wwwnc.cdc.gov/eid/article/18/6/12-0091_article
19. Shahcheraghi F, Nakhost Lotfi M, Nikbin VS, Shooraj F, Azizian R, Parzadeh M, et al. The First Macrolide-Resistant *Bordetella pertussis* Strains Isolated From Iranian Patients. Jundishapur J Microbiol 2014;7:e10880. Available at: https://brieflands.com/journals/jjm/articles/18730
20. Li Y, Liu X, Zhang B, He Q, Wang Z. Where macrolide resistance is prevalent. APMIS 2015;123:361–3. Available at: https://onlinelibrary.wiley.com/doi/10.1111/apm.12357
21. Rodrigues C, Bouchez V, Soares A, Trombert-Paolantoni S, Aït El Belghiti F, Cohen JF, et al. Resurgence of *Bordetella pertussis*, including one macrolide-resistant isolate, France, 2024. Euro Surveill 2024;29. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2024.29.31.2400459
22. Miettinen M, Barkoff A-M, Nyqvist A, Savolainen-Kopra C, Antikainen J, Mertsola J, et al. Macrolide-resistant *Bordetella pertussis* strain identified during an ongoing epidemic, Finland, January to October 2024. Euro Surveill 2024;29. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2024.29.49.2400765
23. Feng Y, Chiu C-H, Heininger U, Hozbor DF, Quanbee Tan T, Wirsing C-H, et al. Emerging macrolide resistance in *Bordetella pertussis* in mainland China: Findings and warning from the global pertussis initiative. Lancet Reg Health West Pac 2021;8:100098. Available at: https://pmc.ncbi.nlm.nih.gov/articles/PMC8315362/

24. Yamaguchi T, Kawasaki Y, Katsukawa C, Kawahara R, Kawatsu K. The First Report of Macrolide-Resistant *Bordetella pertussis* Isolation in Japan. Jpn J Infect Dis 2020;73:361–2. Available at: https://www.jstage.jst.go.jp/article/yoken/73/5/73_JJID.2019.421/_article
25. Lefrancq N, Bouchez V, Fernandes N, Barkoff A-M, Bosch T, Dalby T, et al. Global spatial dynamics and vaccine-induced fitness changes of *Bordetella pertussis*. Sci Transl Med 2022;14. Available at: https://www.science.org/doi/10.1126/scitranslmed.abn3253
26. Stefanelli P, Fazio C, Fedele G, Spensieri F, Ausiello CM, Mastrantonio P. A natural pertactin deficient strain of *Bordetella pertussis* shows improved entry in human monocyte-derived dendritic cells. New Microbiol 2009;32:159–66.
27. Bouchez V, Brun D, Cantinelli T, Dore G, Njamkepo E, Guiso N. First report and detailed characterization of *B. pertussis* isolates not expressing Pertussis Toxin or Pertactin. Vaccine 2009;27:6034–41.
28. Otsuka N, Han HJ, Toyoizumi-Ajisaka H, Nakamura Y, Arakawa Y, Shibayama K, et al. Prevalence and genetic characterization of pertactin-deficient *Bordetella pertussis* in Japan. PLoS One 2012;7:e31985. Available at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0031985
29. Barkoff AM, Mertsola J, Guillot S, Guiso N, Berbers G, He Q. Appearance of *Bordetella pertussis* strains not expressing the vaccine antigen pertactin in Finland. Clin Vaccine Immunol 2012;19:1703–4. Available at: https://journals.asm.org/doi/10.1128/cvi.00367-12
30. Queenan AM, Cassiday PK, Evangelista A. Pertactin-negative variants of *Bordetella pertussis* in the United States. N Engl J Med 2013;368:583–4. Available at: https://pubmed.ncbi.nlm.nih.gov/23388024/
31. Lam C, Octavia S, Ricafort L, Sintchenko V, Gilbert GL, Wood N, et al. Rapid Increase in Pertactin-deficient *Bordetella pertussis* Isolates, Australia. Emerg Infect Dis 2014;20:626–33. Available at: https://pubmed.ncbi.nlm.nih.gov/24655754/
32. He Q, Barkoff AM, Mertsola J, Glismann S, Bacci S, He Q, et al. High heterogeneity in methods used for the laboratory confirmation of pertussis diagnosis among European countries, 2010: Integration of epidemiological and laboratory surveillance must include standardisation of methodologies and quality assurance. Eurosurveillance 2012;17. Available at: https://www.eurosurveillance.org/content/10.2807/ese.17.32.20239-en
33. Guillot S, Mizrahi A, Armatys N, Chat L, le Monnier A, Brisse S, et al. Low detection rate of *Bordetella pertussis* using the BioFire filmarray respiratory panel 2plus. Open Forum Infect Dis 2020;7. Available at: https://pubmed.ncbi.nlm.nih.gov/32793765/
34. Jerris RC, Williams SR, MacDonald HJ, Ingebrigtsen DR, Westblade LF, Rogers BB. Testing implications of varying targets for *Bordetella pertussis*: Comparison of the Film Array Respiratory Panel and the Focus B. pertussis PCR assay. J Clin Pathol 2015;68:394–6.
35. Salminen T, Knuutila A, Barkoff AM, Mertsola J, He Q. A rapid lateral flow immunoassay for serological diagnosis of pertussis. Vaccine 2018;36:1429–34. Available at: https://www.researchgate.net/publication/322959255_A_rapid_lateral_flow_immunoassay_for_serological_diagnosis_of_pertussis
36. Wirsing von Koenig CH. Pertussis: Point-of-Care Testing in the Making. EClinicalMedicine 2019;8:4–5. Available at: https://www.sciencedirect.com/science/article/pii/S2589537019300367?via%3Dihub
37. Guiso N. Isolation, identification and characterization of *Bordetella pertussis*. Dev Biol Stand 1997;89:233–8.
38. Institut Pasteur. [Swabbing technique for collecting clinical specimens to diagnose *Bordetella pertussis*] 2010. (Educational video): prelevcoqueluche-desktop.m4v
39. Fry NK, Campbell H, Amirthalingam G. JMM Profile: *Bordetella pertussis* and whooping cough (pertussis): still a significant cause of infant morbidity and mortality, but vaccine-preventable. J Med Microbiol 2021;70. Available at: https://pubmed.ncbi.nlm.nih.gov/34668853/
40. World Health Organization (WHO). Laboratory Manual for the diagnosis of whooping cough caused by *Bordetella pertussis/Bordetella parapertussis*: update 2014. WHO. Available at: https://www.who.int/publications/i/item/WHO-IVB-14.03
41. Kenicer J, Hardie A, Hamilton F, Gadsby N, Templeton K. Comparative evaluation of the Diagenode multiplex PCR assay on the BD Max system versus a routine in-house assay for detection of *Bordetella pertussis*. J Clin Microbiol 2014;52:2668–70.
42. Cloud JL, Hymas W, Carroll KC. Impact of nasopharyngeal swab types on detection of *Bordetella pertussis* by PCR and culture. J Clin Microbiol 2002;40:3838–40.
43. Nakamura Y, Kamachi K, Toyoizumi-Ajisaka H, Otsuka N, Saito R, Tsuruoka J, et al. Marked difference between adults and children in *Bordetella pertussis* DNA load in nasopharyngeal swabs. Clinical Microbiology and Infection 2011;17:365–70.
44. World Health Organization (WHO). Pertussis: Vaccine Preventable Diseases Surveillance Standards. 2018. WHO. Available at: https://cdn.who.int/media/docs/default-source/immunization/vpd_surveillance/vpd-surveillance-standards-publication/who-surveillancevaccinepreventable-16-pertussis-r2.pdf?sfvrsn=a0157ae7_10&download=true
45. European Centre for Disease Prevention and Control (ECDC). Guidance and protocol for the use of real-time PCR in laboratory diagnosis of human infection with *Bordetella pertussis* or *Bordetella parapertussis.* 2012. Stockholm: ECDC; 2012. Available at: https://www.ecdc.europa.eu/en/publications-data/guidance-and-protocol-use-real-time-pcr-laboratory-diagnosis-human-infection
46. Zintgraff J, Irazu L, Lara CS, Rodriguez M, Santos M. The classical *Bordetella* species and MALDI-TOF technology: A brief experience. J Med Microbiol 2018;67:1737–42.

47. He Q, Mertsola J. Factors contributing to pertussis resurgence. Future Microbiol 2008;3:329–39. Available at: https://www.tandfonline.com/doi/full/10.2217/17460913.3.3.329
48. Zhang Q, Li M, Wang L, Xin T, He Q. High-resolution melting analysis for the detection of two erythromycin-resistant *Bordetella pertussis* strains carried by healthy schoolchildren in China. Clin Microbiol Infect 2013;19:260. Available at: https://www.sciencedirect.com/science/article/pii/S1198743X14615214?via%3Dihub
49. Vestrheim DF, Steinbakk M, Bjørnstad ML, Moghaddam A, Reinton N, Dahl ML, et al. Recovery of *Bordetella pertussis* from PCR-positive nasopharyngeal samples is dependent on bacterial load. J Clin Microbiol 2012;50:4114–5.
50. Riffelmann M, Wirsing von Konig CH, Caro V, Guiso N, Group PPCRC. Nucleic Acid amplification tests for diagnosis of *Bordetella* infections. J Clin Microbiol 2005;43:4925–9.
51. Riffelmann M, Schmetz J, Bock S, Wirsing von Koenig CH. Preparation of *Bordetella pertussis* DNA from respiratory samples for real-time PCR by commercial kits. Eur J Clin Microbiol Infect Dis 2008;27:145–8.
52. Diavatopoulos DA, Cummings CA, Schouls LM, Brinig MM, Relman DA, Mooi FR. *Bordetella pertussis*, the causative agent of whooping cough, evolved from a distinct, human-associated lineage of B. bronchiseptica. PLoS Pathog 2005;1:e45. Available at: https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.0010045
53. van der Zee A, Agterberg C, van Agterveld M, Peeters M, Mooi FR. Characterization of IS1001, an Insertion Sequence Element of *Bordetella parapertussis*. J Bacteriol 1993;175(1):141-7. Available at: https://journals.asm.org/doi/10.1128/jb.175.1.141-147.1993
54. Antila M, He Q, de Jong C, Aarts I, Verbakel H, Bruisten S, et al. *Bordetella holmesii* DNA is not detected in nasopharyngeal swabs from Finnish and Dutch patients with suspected pertussis. J Med Microbiol 2006;55:1043–51.
55. Leber AL, Everhart K, Daly JA, Hopper A, Harrington A, Schreckenberger P, et al. Multicenter Evaluation of BioFire FilmArray Respiratory Panel 2 for Detection of Viruses and Bacteria in Nasopharyngeal Swab Samples. J Clin Microbiol 2018;25;56(6):e01945-17.
56. Isenhour CJ, Pawloski L, Hariri S, Skoff TH. Trends in Commercial Laboratory Testing and Positivity for *Bordetella* Species in the United States, 2019 Through 2023. J Infect Dis 2025;232:e601–8. Available at: https://academic.oup.com/jid/article/232/4/e601/8090059
57. Guiso N, Berbers G, Fry NK, He Q, Riffelmann M, Wirsing von Konig CH, 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;30:307–12. https://doi.org/10.1007/s10096-010-1104-y.
58. Centers for Disease Control and Prevention (CDC). *Bordetella pertussis* Serology CDC-10166. Available at: https://www.cdc.gov/laboratory/specimen-submission/list.html.
59. Campbell H, Amirthalingam G, Fry NK, Litt D, Harrison TG, Wagner K, et al. Oral fluid testing for pertussis, England and Wales, June 2007-August 2009. Emerg Infect Dis 2014;20:968–75.
60. Edmunds M, Mearkle R, Folliard J, Anderson C, Balasegaram S, Chandra N, et al. Retrospective cohort study investigating extent of pertussis transmission during a boarding school outbreak, England, December 2017 to June 2018. Eurosurveillance 2021;5:1–9. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2021.26.26.1900736
61. Tessier E, Campbell H, Ribeiro S, Andrews N, Stowe J, Nicholls M, et al. Investigation of a pertussis outbreak and comparison of two acellular booster pertussis vaccines in a junior school in South East England, 2019. Eurosurveillance 2021;26. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2021.26.12.2000244
62. Markey K, Douglas-Bardsley A, Asokanathan C, Fry NK, Barkoff AM, Bacci S, et al. Improvement in serological diagnosis of pertussis by external quality assessment. J Med Microbiol 2019;68:741–7.
63. Fedele G, Leone P, Bellino S, Schiavoni I, Pavia C, Lazzarotto T, et al. Diagnostic performance of commercial serological assays measuring *Bordetella pertussis* IgG antibodies. Diagn Microbiol Infect Dis 2018;90:157–62.
64. Xing D, Markey K, Newland P, Rigsby P, Hockley J, He Q. EUVAC.NET collaborative study: evaluation and standardisation of serology for diagnosis of pertussis. J Immunol Methods 2011;372:137–45.
65. European Centre for Disease Prevention and Control (ECDC). Guidance and protocol for the serological diagnosis of human infection with *Bordetella pertussis*. Stockholm: ECDC; 2012. Available at: https://www.ecdc.europa.eu/sites/default/files/media/en/publications/Publications/bordetella-pertussis-guidance-protocol-serological-diagnosis.pdf
66. Dalby T, Petersen JW, Harboe ZB, Krogfelt KA. Antibody responses to pertussis toxin display different kinetics after clinical *Bordetella pertussis* infection than after vaccination with an acellular pertussis vaccine. J Med Microbiol 2010;59:1029–36.
67. Rodgers L, Martin SW, Cohn A, Budd J, Marcon M, Terranella A, et al. Epidemiologic and laboratory features of a large outbreak of pertussis-like illnesses associated with co-circulating *Bordetella holmesii* and *Bordetella pertussis*--Ohio, 2010-2011. Clin Infect Dis 2013;56:322–31.
68. Weston W, Messier M, Friedland LR, Wu X, Howe B. Persistence of antibodies 3 years after booster vaccination of adults with combined acellular pertussis, diphtheria and tetanus toxoids vaccine. Vaccine 2011;29:8483–6. Available at: https://pubmed.ncbi.nlm.nih.gov/21945698/
69. Dou M, Sanchez J, Tavakoli H, Gonzalez JE, Sun J, Dien Bard J, et al. A low-cost microfluidic platform for rapid and instrument-free detection of whooping cough. Anal Chim Acta 2019;1065:71–8. https://doi.org/10.1016/j.aca.2019.03.001.
70. Sun C, Xiao F, Fu J, Huang X, Jia N, Xu Z, et al. Loop-Mediated Isothermal Amplification Coupled With Nanoparticle-Based Lateral Biosensor for Rapid, Sensitive, and Specific Detection of *Bordetella pertussis*. Front Bioeng Biotechnol 2022;9.

71. Lin K, Guo J, Guo X, Li Q, Li X, Sun Z, et al. Fast and visual detection of nucleic acids using a one-step RPA-CRISPR detection (ORCD) system unrestricted by the PAM. Anal Chim Acta 2023;1248:340938.
72. Knuutila A, Rautanen C, Mertsola J, He Q. Multiplex point-of-care tests for the determination of antibodies after acellular pertussis vaccination. Diagnostics 2020;10. Available at: https://www.mdpi.com/2075-4418/10/4/187
73. Willems R, Paul A, van der Heide HG, ter Avest AR, Mooi FR. Fimbrial phase variation in *Bordetella pertussis*: a novel mechanism for transcriptional regulation. EMBO J 1990;9(9):2803-9. Available at: https://pubmed.ncbi.nlm.nih.gov/1975238/
74. Andersen EK. Serological studies on *H. pertussis*, *H. parapertussis* and *H. bronchisepticus*. Acta Pathol. Microbiol. Scand 1953;33:202–24. Available at: https://pubmed.ncbi.nlm.nih.gov/13123910/
75. Eldering G, Hornbeck C, Baker J. Serological study of *Bordetella pertussis* and related species'. J Bacteriol 1957;74(2):133-6. Available at: https://journals.asm.org/doi/10.1128/jb.74.2.133-136.1957
76. Barkoff A-M, Mertsola J, Pierard D, Dalby T, Hoegh SV, Guillot S, et al. Surveillance of circulating *Bordetella pertussis* strains in Europe during 1998 to 2015. J Clin Microbiol 2018;56.
77. Bouchez V, Guillot S, Landier A, Armatys N, Matczak S, French pertussis microbiology study group, et al. Evolution of *Bordetella pertussis* over a 23-year period in France, 1996 to 2018. Euro Surveill 2021;26. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2021.26.37.2001213
78. Hegerle N, Guiso N. Epidemiology of whooping cough & typing of *Bordetella pertussis*. Future Microbiol 2013;8(11):1391-403. Available at: https://pubmed.ncbi.nlm.nih.gov/24199799/
79. Heikkinen E, Xing DK, Olander RM, Hytonen J, Viljanen MK, Mertsola J, et al. *Bordetella pertussis* isolates in Finland: serotype and fimbrial expression. BMC Microbiol 2008;8:162.
80. Preston NW. Technical problems in the laboratory diagnosis and prevention of whooping-cough. Lab Pract 1970;19:482–6.
81. Mooi FR, Hallander H, Wirsing von Konig CH, Hoet B, Guiso N. Epidemiological typing of *Bordetella pertussis* isolates: recommendations for a standard methodology. Eur J Clin Microbiol Infect Dis 2000;19:174–81.
82. Guiso N, von Konig CH, Becker C, Hallander H. Fimbrial typing of *Bordetella pertussis* isolates: agglutination with polyclonal and monoclonal antisera. J Clin Microbiol 2001;39:1684–5.
83. Tsang RS, Sill ML, Advani A, Xing D, Newland P, Hallander H. Use of monoclonal antibodies to serotype *Bordetella pertussis* isolates: comparison of results obtained by indirect whole-cell enzyme-linked immunosorbent assay and bacterial microagglutination methods. J Clin Microbiol 2005;43:2449–51.
84. Li ZM, Brennan MJ, David JL, Carter PH, Cowell JL, Manclark CR. Comparison of type 2 and type 6 fimbriae of *Bordetella pertussis* by using agglutinating monoclonal antibodies. Infect Immun 1988;56:3184–8. Available at: https://pubmed.ncbi.nlm.nih.gov/2903125/
85. Borisova O, Kombarova SY, Zakharova NS, van Gent M, Aleshkin VA, Mazurova I, et al. Antigenic divergence between *Bordetella pertussis* clinical isolates from Moscow, Russia, and vaccine strains. Clinical and Vaccine Immunology 2007;14:234–8.
86. Hallander HO, Advani A, Donnelly D, Gustafsson L, Carlsson RM. Shifts of *Bordetella pertussis* variants in Sweden from 1970 to 2003, during three periods marked by different vaccination programs. J Clin Microbiol 2005;43:2856–65.
87. Preston NW, Carter EJ. Serotype specificity of vaccine-induced immunity to pertussis. Commun Dis Rep CDR Rev 1992;2:R155-6.
88. Xing, D, World Health Organization (WHO), Newland P, Corbel M, WHO Expert Committee on Biological Standardization. International collaborative study: evaluation of proposed international standard monoclonal antibodies for serotyping *Bordetella pertussis* fimbrial antigen 2 and fimbrial antigen 3. Geneva: WHO; 2009. Available at: https://iris.who.int/handle/10665/70158
89. Khattak MN, Matthews RC, Burnie JP. Is *Bordetella pertussis* clonal? BMJ 1992;304:813–5. Available at: https://pubmed.ncbi.nlm.nih.gov/1392709/
90. Beall B, Cassiday PK, Sanden GN. Analysis of *Bordetella pertussis* isolates from an epidemic by pulsed-field gel electrophoresis. J Clin Microbiol 1995;33:3083–6.
91. de Moissac YR, Ronald SL, Pepplerl MS. Use of Pulsed-Field Gel Electrophoresis for Epidemiological Study of *Bordetella pertussis* in a Whooping Cough Outbreak. J Clin Microbiol 1994;32(2):398-402.
92. Syedabubakar SN, Matthews RC, Preston NW, Owen D, Hillier V. Application of pulsed field gel electrophoresis to the 1993 epidemic of whooping cough in the UK. Epidemiol Infect 1995;115(1):101-13.
93. Caro V, Njamkepo E, van Amersfoorth SC, Mooi FR, Advani A, Hallander HO, et al. Pulsed-field gel electrophoresis analysis of *Bordetella pertussis* populations in various European countries with different vaccine policies. Microbes Infect 2005;7:976–82.
94. Advani A, Donnelly D, Hallander H. Reference system for characterization of *Bordetella pertussis* pulsed-field gel electrophoresis profiles. J Clin Microbiol 2004;42:2890–7.
95. Advani A, Van der Heide HG, Hallander HO, Mooi FR. Analysis of Swedish *Bordetella pertussis* isolates with three typing methods: characterization of an epidemic lineage. J Microbiol Methods 2009;78:297–301. Available at: https://pubmed.ncbi.nlm.nih.gov/19577594/
96. Litt DJ, Neal SE, Fry NK. Changes in genetic diversity of the *Bordetella pertussis* population in the United Kingdom between 1920 and 2006 reflect vaccination coverage and emergence of a single dominant clonal type. J Clin Microbiol 2009;47:680–8.

97. European Centre for Disease Prevention and Control (ECDC). Laboratory diagnosis and molecular surveillance of *Bordetella pertussis*: Recommendations from ECDC. Stockholm: ECDC; 2022. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/bordetella-pertussis-antimicrobial-susceptibility-testing-2022_0.pdf
98. van Loo IH, Heuvelman KJ, King AJ, Mooi FR. Multilocus sequence typing of *Bordetella pertussis* based on surface protein genes. J Clin Microbiol 2002;40:1994–2001.
99. Elomaa A, Advani A, Donnelly D, Antila M, Mertsola J, He Q, et al. Population dynamics of *Bordetella pertussis* in Finland and Sweden, neighbouring countries with different vaccination histories. Vaccine 2007;25:918–26.
100. Bart MJ, van Gent M, van der Heide HG, Boekhorst J, Hermans P, Parkhill J, et al. Comparative genomics of prevaccination and modern *Bordetella pertussis* strains. BMC Genomics 2010;11:627.
101. Mosiej E, Zawadka M, Krysztopa-Grzybowska K, Polak M, Augustynowicz E, Piekarska K, et al. Sequence variation in virulence-related genes of *Bordetella pertussis* isolates from Poland in the period 1959-2013. Eur J Clin Microbiol Infect Dis 2015;34:147–52.
102. Fu P, Zhou J, Meng J, Liu Z, Nijiati Y, He L, et al. Emergence and spread of MT28 ptxP3 allele macrolide-resistant *Bordetella pertussis* from 2021 to 2022 in China. Int J Infect Dis 2023;128:205–11.
103. Moosa F, du Plessis M, Weigand MR, Peng Y, Mogale D, de Gouveia L, et al. Genomic characterization of *Bordetella pertussis* in South Africa, 2015-2019. Microb Genom 2023;9.
104. de Gouw D, Hermans PWM, Bootsma HJ, Zomer A, Heuvelman K, Diavatopoulos DA, et al. Differentially expressed genes in *Bordetella pertussis* strains belonging to a lineage which recently spread globally. PLoS One 2014;9. Available at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0084523
105. Polak M, Zasada AA, Mosiej E, Krysztopa-Grzybowska K, Witkowski L, Rzeczkowska M, et al. Pertactin-deficient *Bordetella pertussis* isolates in Poland-a country with whole-cell pertussis primary vaccination. Microbes Infect 2019;21(3-4):170-175.
106. Fu P, Zhou J, Yang C, Nijiati Y, Zhou L, Yan G, et al. Molecular Evolution and Increasing Macrolide Resistance of *Bordetella pertussis*, Shanghai, China, 2016-2022. Emerg Infect Dis 2023;30:29–38.
107. Cai J, Chen M, Liu Q, Luo J, Yuan L, Chen Y, et al. Domination of an emerging erythromycin-resistant ptxP3 *Bordetella pertussis* clone in Shanghai, China. Int J Antimicrob Agents 2023;62:106835.
108. Costello MS, Neumann BC, Cuthbert BJ, Holubová J, Raimondi MW, Garza-Sánchez F, et al. Bacteria deliver a microtubule-binding protein into mammalian cells to promote colonization. BioRxiv 2025. https://doi.org/10.1101/2025.06.17.660209.
109. Niinikoski V, Barkoff A-M, Mertsola J, Holubova J, Masin J, Sebo P, et al. Alterations in the expression of *Bordetella pertussis* antigens in relation to the use of acellular pertussis vaccine in Finland. Vaccine 2025;58:127279.
110. Mooi FR, van Oirschot H, Heuvelman K, van der Heide HG, Gaastra W, Willems RJ. Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. Infect Immun 1998;66:670–5.
111. Mooi FR. *Bordetella pertussis* and vaccination: the persistence of a genetically monomorphic pathogen. Infect Genet Evol 2010;10:36–49. Available at: https://pubmed.ncbi.nlm.nih.gov/19879977/
112. Tsang RSW, Shuel M, Cronin K, Deng S, Whyte K, Marchand-Austin A, et al. The evolving nature of *Bordetella pertussis* in Ontario, Canada, 2009-2017: strains with shifting genotypes and pertactin deficiency. Can J Microbiol 2019;65:823–30.
113. Bridel S, Bouchez V, Brancotte B, Hauck S, Armatys N, Landier A, et al. A comprehensive resource for Bordetella genomic epidemiology and biodiversity studies. Nat Commun 2022;13:3807.
114. van Belkum A. Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA). FEMS Immunol Med Microbiol 2007;49:22–7.
115. Guiso N. *Bordetella pertussis* and pertussis vaccines. Clin Infect Dis 2009;49:1565–9.
116. Miyaji Y, Otsuka N, Toyoizumi-Ajisaka H, Shibayama K, Kamachi K. Genetic Analysis of *Bordetella pertussis* Isolates from the 2008-2010 Pertussis Epidemic in Japan. PLoS One 2013;4;8(10):e77165. Available at: https://pubmed.ncbi.nlm.nih.gov/24124606/
117. Quinlan T, Musser KA, Currenti SA, Zansky SM, Halse TA. Pertactin-negative variants of *Bordetella pertussis* in New York State: A retrospective analysis, 2004-2013. Mol Cell Probes 2014;28(4):138-40.
118. Lam C, Octavia S, Ricafort L, Sintchneko V, Gilbert L, Wood N, et al. Emergence of pertactin deficient *Bordetella pertussis* in Australia is due to independent events. 10th International Symposium on Bordetella, Dublin, Ireland 2013 [poster].
119. Pawloski LC, Queenan AM, Cassiday PK, Lynch AS, Harrison MJ, Shang W, et al. Prevalence and Molecular Characterization of Pertactin-Deficient *Bordetella pertussis* in the United States. Clin Vaccine Immunol 2014;21:119–25.
120. Barkoff A-M, Mertsola J, Pierard D, Dalby T, Hoegh SV, Guillot S, et al. Pertactin-deficient *Bordetella pertussis* isolates: Evidence of increased circulation in Europe, 1998 to 2015. Eurosurveillance 2019;24. Available at: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2019.24.7.1700832
121. Martin SW, Pawloski L, Williams M, Weening K, DeBolt C, Qin X, et al. Pertactin-negative *Bordetella pertussis* strains: evidence for a possible selective advantage. Clin Infect Dis 2015;60:223–7.
122. Carriquiriborde F, Regidor V, Aispuro PM, Magali G, Bartel E, Bottero D, et al. Rare Detection of *Bordetella pertussis* Pertactin-Deficient Strains in Argentina. Emerg Infect Dis 2019;25:2048–54.
123. Hiramatsu Y, Miyaji Y, Otsuka N, Arakawa Y, Shibayama K, Kamachi K. Significant Decrease in Pertactin-Deficient *Bordetella pertussis* Isolates, Japan. Emerg Infect Dis 2017;23:699–701.

124. Kastrin T, Barkoff A-M, Seme K, Kosec L, Triglav T, Zorko NŠ, et al. Pertussis epidemic in Slovenia in 2024-no pertactin-deficient or macrolide-resistant *Bordetella pertussis* isolates identified. Clin Microbiol Infect 2025;31:1398–9.
125. Safarchi A, Octavia S, Luu LD, Tay CY, Sintchenko V, Wood N, et al. Pertactin negative *Bordetella pertussis* demonstrates higher fitness under vaccine selection pressure in a mixed infection model. Vaccine 2015;33:6277–81.
126. Hegerle N, Dore G, Guiso N. Pertactin deficient *Bordetella pertussis* present a better fitness in mice immunized with an acellular pertussis vaccine. Vaccine 2014;32:6597–600.
127. Bouchez V, Lefrancq N, Toubiana J, Rodrigues C, Brisse S. Microbes know no borders: importation of macrolide-resistant *Bordetella pertussis* into France in 2024. Clin Microbiol Infect 2025. Available at: https://pubmed.ncbi.nlm.nih.gov/40602497/
128. Qin S, Wang Z, Wu Y, Hu R, Cao L, Shan M, et al. High Prevalence of Macrolide-resistant ptxP3-prn150 *Bordetella pertussis* in Jiangsu Province, China, 2024. Pediatr Infect Dis J 2026;1;45(1):24-28.
129. Bodilis H, Guiso N. Virulence of Pertactin-Negative *Bordetella pertussis* Isolates from Infants, France. Emerg Infect Dis 2013;19(3):471-4.
130. Williams MM, Sen K, Weigand MR, Skoff TH, Cunningham VA, Halse TA, et al. *Bordetella pertussis* Strain Lacking Pertactin and Pertussis Toxin. Emerg Infect Dis 2016;22:319–22.
131. Xu Z, Octavia S, Luu LDW, Payne M, Timms V, Tay CY, et al. Pertactin-Negative and Filamentous Hemagglutinin-Negative *Bordetella pertussis* , Australia, 2013–2017. Emerg Infect Dis 2019;25:1196–9.
132. Barkoff A-M, Kastrin T, Seme K, Vitek MG, Mertsola J, He Q. Prevalence of Pertactin-Deficient *Bordetella pertussis* Isolates, Slovenia. Emerg Infect Dis 2024;30:2429–32.
133. Bart MJ, van der Heide HG, Zeddeman A, Heuvelman K, van Gent M, Mooi FR. Complete Genome Sequences of 11 *Bordetella pertussis* Strains Representing the Pandemic ptxP3 Lineage. Genome Announc 2015; Nov 25;3(6):e01394-15. Available at: https://pubmed.ncbi.nlm.nih.gov/26607899/
134. Kastrin T, Barkoff A-M, Paragi M, Vitek MG, Mertsola J, He Q. High prevalence of currently circulating *Bordetella pertussis* isolates not producing vaccine antigen pertactin in Slovenia. Clinical Microbiology and Infection 2019;25(2):258-260.
135. Weigand MR, Pawloski LC, Peng Y, Ju H, Burroughs M, Cassiday PK, et al. Screening and Genomic Characterization of Filamentous Hemagglutinin-Deficient *Bordetella pertussis*. Infect Immun 2018 Mar 22;86(4):e00869-17.
136. Bouchez V, Hegerle N, Strati F, Njamkepo E, Guiso N. New Data on Vaccine Antigen Deficient *Bordetella pertussis* Isolates. Vaccines (Basel) 2015;3:751–70.
137. Shuel M, Jamieson FB, Tang P, Brown S, Farrell D, Martin I, et al. Genetic analysis of *Bordetella pertussis* in Ontario, Canada reveals one predominant clone. Int J Infect Dis 2013;17:413.
138. Wakimoto Y, Otsuka N, Yanagawa Y, Koide K, Kamachi K, Shibayama K, et al. The First Reported Case of *Bordetella pertussis* Bacteremia in a Patient With Human Immunodeficiency Virus Infection. Open Forum Infect Dis 2022;9:ofac020. Available at: https://academic.oup.com/ofid/article/9/3/ofac020/6523701
139. Kilgore PE, Salim AM, Zervos MJ, Schmitt HJ. Pertussis: Microbiology, Disease, Treatment, and Prevention. Clin Microbiol Rev 2016;29:449–86.
140. Lönnqvist E, Barkoff A-M, Mertsola J, He Q. Antimicrobial susceptibility testing of Finnish *Bordetella pertussis* isolates collected during 2006–2017. J Glob Antimicrob Resist 2018;14.
141. Torres R, Moraes P, Oliveira K. *Bordetella pertussis* Antibiotic Resistance in Southern Brazil: A 3-Year Surveillance Study. Brasilin society of Microbiology. 2015 [poster].
142. Wilson KE, Cassiday PK, Popovic T, Sanden GN. *Bordetella pertussis* isolates with a heterogeneous phenotype for erythromycin resistance. J Clin Microbiol 2002;40:2942–4.
143. Yang Y, Yao K, Ma X, Shi W, Yuan L, Yang Y. Variation in *Bordetella pertussis* Susceptibility to Erythromycin and Virulence-Related Genotype Changes in China (1970-2014). PLoS One 2015;10:e0138941.
144. Fu P, Wang C, Tian H, Kang Z, Zeng M. *Bordetella pertussis* Infection in Infants and Young Children in Shanghai, China, 2016-2017: Clinical Features, Genotype Variations of Antigenic Genes and Macrolides Resistance. Pediatr Infect Dis J 2018.
145. Cai J, Liu Q, Chen B, Jiang Y, Zeng X, Huang J, et al. Waning immunity, prevailing non-vaccine type ptxP3 and macrolide-resistant strains in the 2024 pertussis outbreak in China: a multicentre cross-sectional descriptive study. Lancet Reg Health West Pac 2025;60:101628.
146. Mai Q, Wen J, Luo Y, Guo J, Qin Y, Lai W, et al. Molecular epidemiology and increasing macrolide resistance of *Bordetella pertussis* isolates in Guangzhou, China. BMC Infect Dis 2025;25:1152.
147. Kamachi K, Duong HT, Dang AD, Hai T, Do D, Koide K, et al. Macrolide-Resistant *Bordetella pertussis*, Vietnam, 2016-2017. Emerg Infect Dis 2020;26:2511–3.
148. Kakita T, Taira H, Kudeken T, Kuniyoshi M, Takara T, Teruya M, et al. Multiclonal dissemination of ptxP3 allele macrolide-resistant *Bordetella pertussis* in Okinawa, Japan. Jpn J Infect Dis 2026;21;79(3):125-128.
149. Bouchez V, Lefrancq N, Toubiana J, Rodrigues C, Brisse S. Microbes know no borders: importation of macrolide-resistant *Bordetella pertussis* into France in 2024: author's reply. Clin Microbiol Infect 2025;31(12):2090-2091.
150. Stefanelli P, Buttinelli G, Vacca P, Tozzi AE, Midulla F, Carsetti R, et al. Severe pertussis infection in infants less than 6 months of age: Clinical manifestations and molecular characterization. Hum Vaccin Immunother 2017;13:1073–7.

151. Belcher T, Preston A. *Bordetella pertussis* evolution in the (functional) genomics era. Pathog Dis 2015;73:ftv064. Available at: https://pubmed.ncbi.nlm.nih.gov/26297914/
152. Goodwin S, McPherson JD, McCombie WR. Coming of age: Ten years of next-generation sequencing technologies. Nat Rev Genet 2016;17:333–51.
153. Besser J, Carleton HA, Gerner-Smidt P, Lindsey RL, Trees E. Next-generation sequencing technologies and their application to the study and control of bacterial infections. Clinical Microbiology and Infection 2018;24:335–41.
154. Ring N, Abrahams JS, Jain M, Olsen H, Preston A, Bagby S. Resolving the complex *Bordetella pertussis* genome using barcoded nanopore sequencing. Microb Genom 2018;4.
155. Wang Y, Zhao Y, Bollas A, Wang Y, Au KF. Nanopore sequencing technology, bioinformatics and applications. Nat Biotechnol 2021;39:1348–65.
156. Oehler JB, Wright H, Stark Z, Mallett AJ, Schmitz U. The application of long-read sequencing in clinical settings. Hum Genomics 2023;17:73.
157. Prior K, Becker K, Brandt C, Cabal Rosel A, Dabernig-Heinz J, Kohler C, et al. Accurate and reproducible whole-genome genotyping for bacterial genomic surveillance with Nanopore sequencing data. J Clin Microbiol 2025;63:e0036925.
158. Wagner GE, Dabernig-Heinz J, Lipp M, Cabal A, Simantzik J, Kohl M, et al. Real-Time Nanopore Q20+ Sequencing Enables Extremely Fast and Accurate Core Genome MLST Typing and Democratizes Access to High-Resolution Bacterial Pathogen Surveillance. J Clin Microbiol 2023;61:e0163122.
159. Zidane N, Rodrigues C, Bouchez V, Rethoret-Pasty M, Passet V, Brisse S, et al. Accurate genotyping of three major respiratory bacterial pathogens with ONT R10.4.1 long-read sequencing. Genome Res 2025;35:1758–66.
160. Maiden MCJ, van Rensburg MJJ, Bray JE, Earle SG, Ford SA, Jolley KA, et al. MLST revisited: The gene-by-gene approach to bacterial genomics. Nat Rev Microbiol 2013;11:728–36.
161. Jolley KA, Maiden MCJ. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 2010;11.
162. Uelze L, Grützke J, Borowiak M, Hammerl JA, Juraschek K, Deneke C, et al. Typing methods based on whole genome sequencing data. One Health Outlook 2020;18:2:3.
163. Schürch AC, Arredondo-Alonso S, Willems RJL, Goering R v. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene–based approaches. Clinical Microbiology and Infection 2018;24:350–4.
164. Weigand MR, Peng Y, Loparev V, Batra D, Bowden KE, Burroughs M, et al. The History of *Bordetella pertussis* Genome Evolution Includes Structural Rearrangement. J Bacteriol 2017;199:10.1128/JB.00806-16.
165. Nelson MT, Pope CE, Marsh RL, Wolter DJ, Weiss EJ, Hager KR, et al. Human and Extracellular DNA Depletion for Metagenomic Analysis of Complex Clinical Infection Samples Yields Optimized Viable Microbiome Profiles. Cell Rep 2019;26:2227-2240.e5.
166. Luu LDW, Rafique R, Payne M, Octavia S, Robson J, Sintchenko V, et al. Deciphering *Bordetella pertussis* epidemiology through culture-independent multiplex amplicon and metagenomic sequencing. J Clin Microbiol 2024;62:e0117824.
167. Peng Y, Williams MM, Xiaoli L, Simon A, Fueston H, Tondella ML, et al. Strengthening *Bordetella pertussis* genomic surveillance by direct sequencing of residual positive specimens. J Clin Microbiol 2024;62:e0165323.
168. Schouls LM, van der Heide HG, Vauterin L, Vauterin P, Mooi FR. Multiple-locus variable-number tandem repeat analysis of Dutch *Bordetella pertussis* strains reveals rapid genetic changes with clonal expansion during the late 1990s. J Bacteriol 2004;186:5496–505.
169. Advani A, Hallander HO, Dalby T, Krogfelt KA, Guiso N, Njamkepo E, et al. Pulsed-field gel electrophoresis analysis of *Bordetella pertussis* isolates circulating in Europe from 1998 to 2009. J Clin Microbiol 2013;51:422–8.
170. van Gent M, Bart MJ, van der Heide HGJ, Heuvelman KJ, Kallonen T, He Q, et al. SNP-based typing: A useful tool to study *Bordetella pertussis* populations. PLoS One 2011;6. Available at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0020340
171. Barkoff AM, He Q. Molecular Epidemiology of *Bordetella pertussis*. Adv. Exp. Med. Biol., vol. 1183, Springer; 2019, p. 19–33.
172. Bouchez V, Guglielmini J, Dazas M, Landier A, Toubiana J, Guillot S, et al. Genomic sequencing of *Bordetella pertussis* for epidemiology and global surveillance of whooping cough. Emerg Infect Dis 2018;24:988–94.
173. Ma L, Caulfield A, Dewan KK, Harvill ET. Pertactin-Deficient *Bordetella pertussis*, Vaccine-Driven Evolution, and Reemergence of Pertussis. Emerg Infect Dis 2021;27:1561–6.
174. Barkoff AM, Guiso N, Guillot S, Xing D, Markey K, Berbers G, et al. A rapid ELISA-based method for screening *Bordetella pertussis* strain production of antigens included in current acellular pertussis vaccines. J Immunol Methods 2014;408:142–8.

Annex 1. Storage medium used to preserve *Bordetella pertussis* isolates

Preparation of TSB – Glycerol tubes

Tryptone Soy Broth	3 g
Glycerol	20 mL (amount of glycerol may vary between 10–20%)
Water	80 mL
Final volume	100 mL.

- Autoclave the mixture in +121°C for 15 minutes
- Cool down to room temperature
- Divide into cryotubes (1mL/tube).

The tubes can be stored at +4°C for up to six months.

Preparation of Bovine Serum Albumine (BSA) – Saccharose/phosphate/glutamate (SPG) tubes

The instructions are described in the publicly available WHO 'Laboratory Manual for the Diagnosis of Whooping Cough caused by *Bordetella pertussis*/*Bordetella parapertussis*'. The instructions are presented in Annex 5 [40].

Annex 2. Serotyping of *B. pertussis*.

Reagents

International standards for monoclonal antibodies are used for serotyping *Bordetella pertussis* Fimbrial Antigen 2 (NIBSC code 06/124) and Fimbrial Antigen 3 (NIBSC code 06/128). Each ampoule of 06/124 or 06/128 contains the freeze-dried powder from 1 ml of concentrated cell culture supernatant, adjusted with phosphate-buffered saline to give a concentration of 10mg/ml IgG, as determined by UV at 480 nm.

The ampoule contents should be reconstituted with 1 ml of saline, dispensed into suitable aliquots (normally 100 µl) and stored at -20°C until needed. Repeat freeze-thawing should be avoided. Data from an in-use stability study suggests that the reconstituted material can be used if it has been suitably stored, but this should be validated under individual laboratory conditions.

From an international collaborative study [88], the following working dilutions were suggested, although these may vary under different laboratory conditions:

- For slide agglutination: dilutions in the range 1/10 to 1/50
- For microplate assay: dilutions in the range 1/100 to 1/800
- For whole cell ELISA: dilutions in the range 1/100 to 1/1000.

A. Protocol for microtiter plate method for serotyping of *B. pertussis* (Swedish Institute for Infectious Disease Control (SIIDC)) – adapted from Institut Pasteur

Materials

- 96 well microtiter plate (V-bottom, Nunc products, Denmark)
- Spectrophotometer (Pharmacia Biotech, Novaspec II)
- Plate sealers 8.3x13.3cm (Nunc products, Fasson S695, Denmark)
- Cuvettes (Plastibrand, GMBH, Germany)
- 2.0 mL Tubes (Sarstedt yellow top tubes)
- Sterile loops.

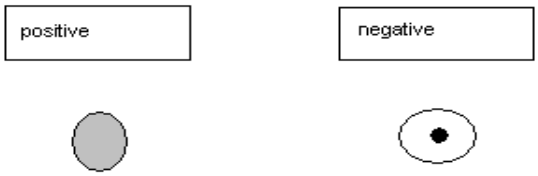
- **Bacterial isolates**
  All determinations are performed twice on the same plate.
  50µl of bacterial isolate of isolate FDA460 (Fim2,3) with an OD_{650nm} = 1.0* is placed in wells A1 to A6.
  50µl of bacterial isolate of isolate FDA460 (Fim2,3) with an OD_{650nm} = 0.5* is placed in wells A7 to A12.
  Unknown samples are allocated to the wells of rows B to H, and wells 1 to 6 are used for the bacterial isolate sample with an OD_{650nm} = 1.0*, and wells 7 to 12 are used for the bacterial isolate sample with an OD_{650nm} = 0.5*.
- **06/124 antibodies (anti-Fim2)**
  50µl anti-Fim 2 at the right concentration are distributed in wells of columns 1, 2, 7 & 8 of rows A to H depending on the number of samples.
- **06/128 antibodies (anti-Fim3)**
  50µl anti-Fim 3 at the right concentration are distributed in wells of columns 3, 4, 9 & 10 of rows A to H depending on the number of samples.
- **PBS 1X (negative control)**
  50µl of PBS 1X are distributed in wells 5, 6, 11 & 12 of rows A to H depending on number of samples.

Cover the plate with a plate sealer and incubate for 12–36 hours at 37°C.

Results

- Positive results are observed as the formation of a diffuse layer of antigen-antibody complex in the bottom of the well. The control should be read first!
- Repeat the entire assay if the control failed.
- Negative results are observed when bacteria sediment forms as a clump at the bottom of the well without forming any antibody-antigen complex.
- The plate should be read by two independent readers in order to validate a correct result.
- If the result is uncertain: document as untypeable or repeat the test.
- Agglutinations are easier to read after incubation at 35–36°C.

Figure 6. Appearance of results on the plate



**: It is important that the inoculum should be locally adjusted and standardised. Results are much easier to determine, using one optical density of 0.8 (650nm) for all samples and controls.*

B. Protocol of slide agglutination for serotyping of *B. pertussis* isolates

Materials

- Charcoal (Regan-Lowe) agar plates
- International standards 06/124 and 06/128 antibodies
- Microscope slides e.g. BDH Superfrost
- Sterile saline and distilled water
- Sterile loops (plastic)
- Pipette P10 or P20 with tips
- Reference *B. pertussis* isolates: isolates of known serotype 2,3 (e.g. FDA 460).

Method

1. Grow reference and test isolates at 37°C for 24–48 hours on charcoal agar plates.
2. Make a dilution of the monoclonal or polyclonal antibodies:
   Thaw the frozen aliquots of 06/124 or 06/128 (if using monoclonal antibodies, see section A above). Make a 1/50 dilution of this material in saline for immediate use.
3. Slide agglutination: use prewashed clean microscope slides e.g. BDH Superfrost.
   3.1. Label the slide (on frosted end with pencil or with permanent marker)
   3.2. Using a pipette place one 10 µl drop of saline and one drop each of anti-fim 2 antibody and anti-fim 3 antibody on the slide (i.e. three drops in a line across the slide).
   3.3. Using a sterile loop remove a tiny portion of bacteria from the surface of the charcoal agar plate and mix this directly into the drop on the microscope slide. Use three separate mixings into the three separate drops with fresh loops on each slide.
   3.4. Mix the bacteria and antibody with the loop in each drop and, when all three drops contain bacteria, gently rock the slide to continue mixing.
   3.5. Observe the drops for agglutination against a black background, in good light using a suitable lens and record the results.
   3.6. Discard the slides after recording the result. The result should be read rapidly before the drops start to dry out.

Results

- Agglutination is observed as 'clumping' of the bacteria within the drop.
- The saline control drop should remain a clear smooth suspension, if this shows signs of agglutination the test must be repeated since auto-agglutination can occur.

C. Protocol of ELISA serotyping for *B. pertussis* isolates

Materials

- 96-well plate, plate is sealed during the incubation
- Plate cover
- Isolate S1 (Fim2) (can be purchased from NIBSC) or Tohama (can be purchased from CIP, CIP81.32)
- Isolate S3 (Fim3) (can be purchased from NIBSC) or FR4624 (can be purchased from CIP, CIP 112614**)**
- Normal sheep sera (NSS) or skimmed milk (SM)
- Phosphate-buffered saline (PBS)
- 06/128 monoclonal mouse antibodies (anti-Fim3), NIBSC
- 06/124 monoclonal mouse antibodies (anti-Fim2), NIBSC
- Anti-mouse conjugate (e.g. Anti-Mouse IgG (γ-chain specific), A3673 Sigma)
- Substrate (phosphatase or other, depending on the conjugate of choice, e.g. Biolegend TMB Substrate Set 421101)
- Optical density (OD) reader
- Charcoal agar plates.

Preparation of bacterial suspension

Bacterial suspension for coating:
- Grow bacteria for 48–72 hours on a charcoal agar or Bordet-Gengou plate.
- Suspend bacterial growth in PBS.
- Dilute in PBS to give an OD of 0.1 or 0.2 at 620 nm for Fim2 and Fim3 isolates.
- Heat inactivate the suspension at 56°C for one hour.
- Store at +4°C until testing.

ELISA protocol

Day 1

1. Coating (two wells/sample) 100 µl/well. Overnight at room-temperature.
   - S1 and S3 isolates serve as both negative and positive control for Fim2 (S1=positive, S3=negative) and Fim3 (S1=negative and S3=positive), OD=0.1 (at 620nm) diluted in PBS.
   - Samples, OD = 0.1 (at 620mn) diluted in PBS for Fim2 and Fim3.
   - PBS as a reagent control (see plate layout at the end of the protocol).

Day 2

1. Start with three washes with NaCl-0,05 % Tween 200 µl/well.
   (NaCl-0,05% Tween: 0,5 ml Tween 20 + 1000 ml 0,9 % NaCl).

2. Blocking 150 µl/well one hour + 37°C.
   - Add 1% NSS-PBS (or 5% SM-PBS) to all wells
     (0.5 ml Lamb serum + 49.5 ml PBS).

3. After one hour, wash three times with NaCl-0,05% Tween 200 µl/well.

4. Monoclonal antibodies 100 µl/well – two hours + 37°C.
   - Monoclonal antibodies 06/124 (Fim2) and 06/128 (Fim3) diluted in 1% NSS-PBS (or 5% SM-PBS).
   - Dilution 1:1000.

5. After 2 hours, wash three times with NaCl-0,05% Tween 200 µl/well.

6. Anti-Mouse Conjugate 100 µl/well -two hours + 37°C.
   - Anti-mouse conjugate diluted in 1% NSS-PBS (or 5% SM-PBS) buffer.
   - Dilution 1:1000.

7. After two hours, wash three times with NaCl-0,05% Tween 200 µl/well.

8. Substrate 100 µl/well – 30 minutes/one hour at room temperature.
   - Phosphatase substrate or similar diluted in Diethanolamine-MgCl2-buffer (or the one recommended for your substrate).
   - 5 mg/5 ml buffer = one tablet/5ml buffer (sensitive to light, cover the plate with aluminium foil after addition of substrate).

9. STOP-solution 100 µl/well.
   - 3M NaOH (or the one recommended for your substrate) is added to wells with substrate to stop the reaction.
   - After 30min for Fim2.
   - After 60min for Fim3.
   - Measure absorbance at wavelength 405 nm.

10. Analysing the results
    - Compare sample values to Fim2/Fim3 controls to identify the correct serotype.

Figure 7. Plate layout First half is for Fim2 (MAbs to Fim2) and second half for Fim3 (MAbs to Fim3)

S1(Fim2)	S1(Fim2)	sample	sample	sample	sample	S3(Fim3)	S3(Fim3)	sample	sample	sample	sample
S3(Fim3)	S3(Fim3)	sample	sample	sample	sample	S1(Fim2)	S1(Fim2)	sample	sample	sample	sample
PBS	PBS	sample	sample	sample	sample	PBS	PBS	sample	sample	sample	sample
sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample
sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample
sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample
sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample
sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample

Annex 3. Protocol for Multi-locus Antigen Sequence Typing (MAST)/genotyping of single genes

The following genes are targeted: *prn* (pertactin), *ptxA* (pertussis toxin A subunit), *ptxP* (pertussis toxin promoter), *tcfA* (tracheal colonisation factor), *fim2* (fimbrial subunit 2), and *fim3* (fimbrial subunit 3). This protocol describes the PCR protocols for amplification and sequencing of these genes. All genes are sequenced completely, except *prn* and *tcfA* – due to their large size, in these genes only the regions which are known to be polymorphic are sequenced.

Chemicals, reagents and media

- HotStart Taq Mastermix.
- Betain 5M solution.
- DMSO 5%.
- Tris- Ethylenediaminetetraacetic acid (EDTA) buffer: Tris 10 mM + EDTA 1 mM, DNAse free pH 8.0.
- DNAse free PCR grade water.
- Primer sets as mentioned in Table 4, select standard purification as supplied by manufacturer.
- DNA marker: e.g. 1 Kb DNA Ladder
- Exo-sap it.
- Sequencing materials (buffers, BigDyeTerminator).
- 200 µl thin-walled PCR reaction tubes for small sample numbers.
- PCR tray 96 wells 200µl.

Table 4. PCR, Sequencing primers and PCR product size

Target	Primer	PCR product size (bp)	Sequence	PCR	Seq
prn-region 1	prn-AF	585	GCC AAT GTC ACG GTC CAA	X	X
	prn-AR		GCA AGG TGA TCG ACA GGG	X	X
prn-region 2	prn-BF	535	AGC TGG GCG GTT CAA GGT	X	X
	prn-BR		CCG GAT TCA GGC GCA ACT C	X	X
ptxP	ptxP-F	575	AAT CGT CCT GCT CAA CCG CC	X	X
	ptxP-R		GGT ATA CGG TGG CGG GAG GA	X	X
ptxA	PTS1F2	934	CCC CCT GCC ATG GTG TGA TC	X	
	ptxS1-seqF		GTG GGG AAA CAA CGA CAA		X
	PTS1R2		AGA GCG TCT TGC GGT CGA TC	X	
	ptxS1-seqR		GTG TAG GGG TTG GGA TTG		X
tcfAnew - F2	tcfA	548	CTT TCT CCT CCC TCG GCA TGG	X	X
tcfAnew - R2	tcfA		AGC GCC GTC CGG ATT CAA G	X	X
fim2	fim2-F	842	GCG CCG GGC CCT GCA TGC AC	X	
	fim2-seqF		TCA TCA CCG GCA CCA TCA		X
	fim2-R		GGG GGG TTG GCG ATT TCC AGT TTC TC	X	
	fim2-seqR		GTT CTT TTT GAC GTA GGA GG		X
fim3	fim3-F	713	GAC CTG ATA TTC TGA TGC CG	X	
	fim3-seqF		CAC CCT CAA CCA TAT CAA		X
	fim3-R		CGC AAG GCT TGC CGG TTT TTT TTG G	X	
	fim3-seqR		TCT TGC TGC CAT TGG TGA		X

Biological materials

Bordetella isolates are kept in 20% TSB-glycerol at -80°C. The frozen cells are grown on Regan-Lowe or Bordet Gengou agar plates and incubated for 2–7 days at 35°C. Cultured *B. pertussis* cells can be used for isolating chromosomal DNA or to prepare lysates by boiling. This DNA or lysate can be used as a source for sequence PCRs. The PCR will work well with both types of templates.

Preparing lysates

- Approximately 5–30µl freshly grown cells are suspended in 500µl TE, boiled for five minutes, and centrifuged for two minutes at maximum speed in a micro centrifuge.
- 1 µl of the lysate supernatant is then used for PCR.
- Lysates of *B. pertussis* can be stored and used for up to two years at 4°C.

Using DNA

- 5–10 ng of purified genomic DNA is used per reaction. The quality of DNA prepared from generally available rapid genomic DNA isolations kits is sufficient.
- DNA of *B. pertussis* can be stored and used for up to 5–10 years at 4°C.

Apparatus and disposables

- PCR apparatus
- DNA analyser
- 96-well PCR reaction plates
- 200 µl coloured thin-walled PCR reaction tubes.

Method

General PCR setup

- Use filter tips to minimise PCR product contamination. Further precautions are unnecessary because abundant target DNA (cells) are used in the PCR reaction.
- Dilute primer stocks to 10 pmol/µl.
- Prepare PCR mix for the amount of samples desired + 2 (See Table 5).
- Add 1 µl DNA or lysate dilution to the bottom of the reaction tubes.
- Add 19 µl of the corresponding PCR mix (see Table 5).
- Prepare extra sample mix for use as a negative control. Use at least 2 extra reactions without DNA or lysate.

Table 5. PCR reaction mix composition (per reaction)

Target		**Target**	
prn region 1, *prn* region 2, *ptxP, tcfA, fim2*		*ptxS1, fim3*	
Component	µl	**Component**	µl
DNA/lysate dilution	1	DNA/lysate dilution	1
Primer 1 10 pmol/ul	1	Primer 1 10 pmol/ul	1
Primer 2 10 pmol/ul	1	Primer 2 10 pmol/ul	1
Betain 5M	4	DMSO 5%	1
DNAse free PCR grade water	3	DNAse free PCR grade water	6
HotStar Taq Mix	10	HotStar Taq Mix	10
Reaction Volume	**20**	**Reaction Volume**	**20**

Incubate the PCR reactions according to Table 6 and Table 7.

Table 6. PCR programme

Cycles	**Time**	**Temperatures** *Prn, ptxP, tcfA*	**Temperatures** *Fim2*	**Temperatures** *Fim3*
1	15 minutes	95°C	95°C	95°C
30	15 seconds	95°C	95°C	95°C
	30 seconds	**55°C**	**65°C**	**56°C**
	1 minute	72°C	72°C	72°C
1	10 minutes	72°C	72°C	72°C
1	Hold	20°C	20°C	20°C

Table 7. *ptx*S1 touch down PCR

15 min 95ºC:1 cycle
15 sec 95ºC -> 30 sec 74ºC -> 60 sec 72ºC: 2 cycles
15 sec 95ºC -> 30 sec 72ºC -> 60 sec 72ºC: 2 cycles
15 sec 95ºC -> 30 sec 70ºC -> 60 sec 72ºC: 2 cycles
15 sec 95ºC -> 30 sec 68ºC -> 60 sec 72ºC: 2 cycles
15 sec 95ºC -> 30 sec 66ºC -> 60 sec 72ºC: 2 cycles
15 sec 95ºC -> 30 sec 64ºC -> 60 sec 72ºC: 25 cycles
10 min 72°C: 1 cycle

Sequencing

- Analyse 1 µl of each PCR product on a 1% standard quality agarose gel in Tris-borate-EDTA (TBE) buffer containing a 1 kb ladder to estimate the size of the PCR product.
- Run for 30 minutes (for example) on a I-MyRun or other electrophoresis apparatus.
- Check for yield and product size according to Table 4. The yield is satisfactory if the DNA band is clearly visible.
- Primers are removed enzymatically as follows: add to 5 µl PCR product 2µl Exo-sap solution and incubate these in a PCR incubator for 15 minutes at 37ºC, followed by 15 minutes at 80ºC.
- Sequence the forward as well as the reverse sequence.

Table 8. Sequence reaction mix per sample

Solution	µl
DNAse free PCR grade water	6.68
Primer 10 pmol/µl	0.32
Buffer	7
Q solution	4
Reaction Mix BigDye terminator	1
PCR prod µl	1
TOTAL	20

Table 9. PCR programme sequence reaction

Cycles	Time	Temperature
1	1 minute	96°C
25	10 seconds	96°C
	5 seconds	50°C
	4 minutes	60°C
1	Hold	20°C

Quality

- PCR products should have the sizes for gel as per Table 4.
- If negative PCR controls are positive, wash and dry the trays, replace all the buffers and redo all the PCR reactions done that day.

Identifications of alleles

- This can best be done by performing blast analysis of the sequence.

Annex 4. ELISA protocol for Antigen Expression and sequencing primers to detect mutations in the PRN gene

The ELISA protocol has been described previously by Barkoff et al. [174].

Materials*

- 96-well plate (polystyrene, high binding), plate is sealed during the incubation
- Plate cover
- PRN-negative isolate, OD = 0.1 (e.g. FR7302 (CIP 112615) or FR4624 (CIP 112614))
- FHA-negative isolate, OD = 0.1 (e.g. FR4624 (CIP 112614))
- PT-negative isolate, OD = 0.2 (e.g. FR3749 (CIP 112613))
- Monoclonal antibodies (see Table 10 below)
- Normal sheep sera (NSS) or skimmed milk (SM, better results at IP)
- Phosphate-buffered saline (PBS)
- Anti-mouse conjugate
- Substrate (phosphatase or other, depending on the conjugate of choice)
- Optical density (OD) reader
- Charcoal or Bordet-Gengou agar plates.

**For OD dilutions, instead of OD 0.1, you can use OD 0.2 throughout the protocol.*

Table 10. Monoclonal mouse antibodies

Antigen	mAb	Target	Source
PT	99/512* 99/542*	S1 subunit S3 subunit	NIBSC
FHA	99/572 JNIH-11	NK**	NIBSC
PRN	PeM4 3-5*** 69K/16***	Region I of *prn* gene NK** NK**	RIVM NIBSC NIBSC

** A mixture of the two monoclonal antibodies is used.*
*** Not known*
**** New mAbs from NIBSC. The authors have tested the functionality, which is comparable to or better than PeM4 mAbs. The use of Prn mAbs 97/558 is no longer recommended. Serotyping can be done simultaneously with other antigens.*

Preparation of bacterial suspension

Bacterial suspension for coating:

- Grow bacteria for 48–72 hours on a charcoal agar plate (For PT: optional grow in +5% CO_2).
- Suspend bacterial growth in PBS.
- Dilute in PBS to give an OD of 0.1 at 620 nm for FHA and PRN isolates.
- Dilute in PBS to give an OD of 0.2 at 620 nm for PT isolates.
- Heat inactivate the suspension at 56°C for one hour.
- Store at +4°C until testing.

ELISA PROTOCOL

(RT = Room Temperature, o/n = overnight)

Day 1

1. Coating (two wells/sample) 100 µl/well o/n RT

- Positive control: purified PRN, FHA and PT 1.0 µg/ml diluted in PBS.
- Negative controls: PRN isolate, FHA isolate OD = 0.1 (at 620nm) diluted in PBS. PT isolate OD = 0.2 (at 620 nm).
- Samples, OD = 0.1 (at 620mn) diluted in PBS for PRN and FHA.
- Samples, OD = 0.2 (at 620nm) diluted in PBS for PT.
- PBS as a reagent control (see plate layout at the end of protocol).

Day 2

1. Wash three times with NaCl-0,05% Tween 200 µl/well.
   (NaCl-0,05% Tween: 0,5ml Tween 20 + 1 000 ml 0.9% NaCl)
2. Blocking 150 µl/well one hour + 37°C.
   Add 1% NSS-PBS or 5% SM-PBS* to all wells.
   (0.5ml Lamb serum + 49.5ml PBS).

** If problems are notified with NSS (e.g. higher background), milk can be used as a replacement.*

3. Wash three times with NaCl-0,05 % Tween 200 µl/well.
4. Monoclonal antibodies 100 µl/well two hours + 37°C
   - Monoclonal antibodies 3-5 or 69K/16 (PRN), 99/572 (FHA) and 99/512 (anti-PT S1) & 99/542 (anti-PT S3) (If Fim serotyping performed simultaneously: 06/124 (Fim2) and 06/128 (Fim3)) diluted in 1% NSS-PBS or 5% SM-PBS.
   - Dilution 1:1000.
5. Wash three times with NaCl-0,05 % Tween 200 µl/well.
6. Anti-Mouse Conjugate 100 µl/well two hours + 37°C.
   - Anti-mouse conjugate diluted buffer 1% NSS-PBS or 5% SM-PBS.
   - Dilution 1:1000.
7. Wash three times with NaCl-0,05% Tween 200 µl/well.
8. Substrate 100 µl/well 30 minutes/one hour in RT.
   - Phosphatase substrate diluted in Diethanolamine-MgCl2-buffer (or according to conjugate recommendations).
   - 5 mg/5 ml buffer = one tablet/5 ml buffer (sensitive to light, cover the plate with aluminium foil after addition of substrate).
9. STOP-solution 100 µl/well.
   - 3M NaOH is added to wells with substrate to stop the reaction (or the one recommended for your substrate).
   - After 60 minutes for all antigens (PT, FHA, PRN).
   - Measure absorbance at wavelength 405 nm.
10. Analysing the results:
    - Compare sample values to negative control to identify negative isolates (confirm with sequencing).

Table 11. 96-well plate layout (example for multiple antigen measurement)

	1	2	3	4	5	6	7	8	9	10	11	12
A	PRN 1.0	PRN1.0	FHA 1.0	FHA 1.0	PT 1.0	PT 1.0						
B	PRNneg	PRNneg	FHA neg	FHA neg	PT neg	PT neg						
C	PBS	PBS	PBS	PBS	PBS	PBS						
D	sample1	sample1	sample1	sample1	sample1	sample1						
E	sample2	sample2	sample2	sample2	sample2	sample2						
F	sample3	sample3	sample3	sample3	sample3	sample3						
G	sample4	sample4	sample4	sample4	sample4	sample4						
H	sample5	sample5	sample5	sample5	sample5	sample5						

Sequencing primers for detection of mechanisms and direct typing of the *prn* gene

The sequencing primers for *prn* gene has been previously published by Barkoff et al. [120]. Here we present a table of primers (Table 12), which can be used for sequence purposes. Note that these are suggestions and other in-house primers can also be used.

Table 12. Primers for *prn* gene sequencing*

NAME OF PRIMER	SEQUENCE (5'-3')	TARGET (position in the genome)
PrnG_A2 FOR	CCC ATT CTT CCC TGT TCC AT	*prn* gene(+seq), first part (1098027-1098046)
PrnG_A REV	TGT TGG CAA GGG TAA AGG TC	*prn* gene, first part (1099698-1099717)
prn-BF_1322bp	AGC TGG GCG GTT CAA GGT	*prn* gene (+seq), second part (1099489–1099505)
PrnGeneR	GCC TGA GCC TGG AGA CTG G	*prn* gene, second part (1100877-1100895)
PrnProm FOR	GCT CAA AGC AGG AAA AAG CA	*prn* Promoter (+seq) (1097635-1097654)
PrnProm REV	CGC TTA CCT TGA TGG TGG TT	*prn* Promoter (1098287-1098306)
PrnSeq1F	GCC AAT GTC ACG GTC CAA	*prn* gene, sequencing (1098595-1098612)
PrnSeq2F	TGT CGA TCA CCT TGC AGG	*prn* gene, sequencing (1099166-1099183)
PrnSeq3F	AAC GGC AAT GGG CAG TG	*prn* gene, sequencing (1099765-1099781)
PrnSeq4F	GAC AGC GGT TTC TAC CTG GA	*prn* gene, sequencing (1100269-1100288)

** If WGS is not used, these primers will cover the whole Prn gene.*

Annex 5. Protocol to determine Minimum Inhibitory Concentration (MIC) of macrolides

The MIC protocol has been described previously by Lönnqvist et al. 2018 [140].

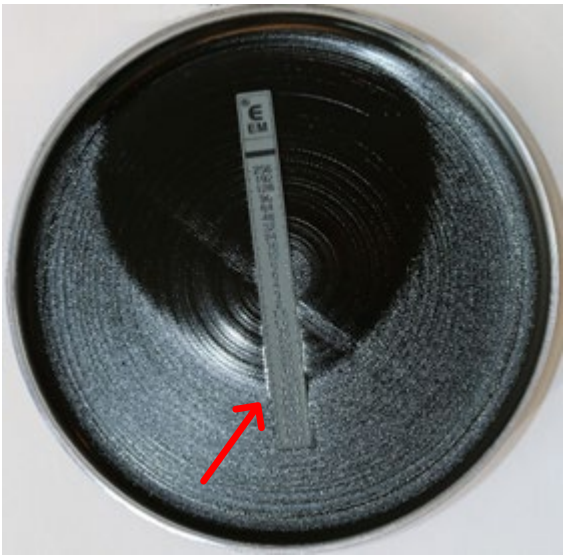
Materials

- Etest® Erythromycin (BioMérieux)
- Etest® Azithromycin (BioMérieux)
- Densitometer
- Petri Dish Rotator
- Regan-Lowe Charcoal Agar.

MIC protocol

1. Culture *B. pertussis* on Regan-Lowe agar (charcoal blood agar, without cephalexin) for 48 to 72 hours.
2. From the fresh culture, use a cotton swab to suspend a small amount of bacteria in 2ml of 0.9% NaCl solution to a density equivalent of 0.5 McFarland standard.
3. Inoculate a Regan-Lowe plate (without cephalexin) with the 0.5 McFarland bacterial suspension by dipping a cotton swab in the solution, and evenly distributing it onto the plate using an automatic rotating device. Gently push the side-top of the cotton swab into the centre of the plate and start moving the swab towards the edge of the plate in vertical direction (straight line) and back to the centre of the plate. You can spin the swab gently during the procedure to have more suspension on the plate.
4. Place the Etest on the plate. Take sterilised tweezers and open the Etest package. Grip the Etest from the top end. Place the bottom end of the Etest on the plate, so that the whole Etest will be approximately in the middle of the plate. Gently lay the Etest on the plate and remove any air bubbles by pressing the Etest gently with the tweezers. Let it rest for one minute before incubation.
5. Incubate upside-down at 35°C for 48 to 72 hours.
6. Record the MIC values where the inhibition zone line touches the strip (Figure 8a).
7. Read the plate after 48h and 72h. For pertussis, the growth is slow, so usually incubation times of 72h is needed.
8. Results from sensitive and resistant isolates are presented in Figures 8a and 8b, respectively. NB! There is currently no approved MIC from the EUCAST.

Figure 8a. Erythromycin Etest on macrolide sensitive control isolate ATCC 9797



Note: the arrow shows the edge of the inhibition zone that determines the MIC

Figure 8b Erythromycin Etest on macrolide resistant control isolate NAP-12-30 (China)

Note: no inhibition zone can be detected.

Annex 6. Protocols for *B. pertussis* DNA-based identification of macrolide resistance

1. Block-based macrolide PCR for *B. pertussis*

A SNP changing A to G at position 2047 in the 23S rRNA of *B.pertussis* has been associated with macrolide resistance[2]. An allele-specific PCR assay has been developed to detect this mutation[3]. However, this protocol is designed for detection of the mutation from heated water tubes or for isolated purified DNA from single strain. Tests for clinical samples have shown good results as well, but for clinical laboratories this method might not be feasible.

1. Prepare the DNA template by diluting 1 µl loopful of bacteria in 200 µl molecular biology grade water in an Eppendorf tube. Transfer 50 µl of this suspension to a new tube with 350 µl of MBG water. Alternatively, purified DNA can be used (e.g. commercial kits).
2. Heat the suspension at 95ºC for 30 minutes. Centrifuge at 13000 rpm for five minutes, and transfer the supernatant to a new Eppendorf tube.
3. Prepare the PCR reaction mixture in an Eppendorf tube according to Table 13.

Table 13. Sensitive PCR reaction mixture for detection of 23S rRNA without the A2047G mutation

Reagents	1	x mix	Final conc.
Molecular biology grade H_2O	25	ul	
10x buffer	5	ul	1x
dNTP's (1,25 mM each)	8	ul	200 uM each
FP_23S_rRNA (10 pmol/ul)	2	ul	20 pmol
WP_23S_rRNA (10 pmol/ul)	2	ul	20 pmol
RP_23S_rRNA (10 pmol/ul)	2	ul	20 pmol
Dynazyme (2U/ul)	1	ul	2.5 U
Total	45	ul	

4. Transfer 45 µl of the reaction mixture to a 0.2 ml PCR tube and add 5 µl of DNA template.
5. Include control strains ATCC 9797 (no mutation), and NAP-12-30 and 12072GH (mutation) in every assay.
6. Run the PCR amplification with the following programme:

   95°C 15 min

   95°C 1 min

   59°C 30 s x 39

   72°C 30 s

   72°C 10 min

   10°C.

7. Run the PCR product on a 1.5% agarose gel stained with Midori green at 110 V for 75 minutes.
8. Detect the bands with GelDoc XR+ Imaging System (BioRad, USA). Strains without the mutation will give two bands of 286 bp and 121 bp (Figure 9, lane 1). Strains with the A2047G mutation give only one band of 286 bp (Figure 9, lane 2).
9. Use MP_23S_rRNA primer instead of WP_23S_rRNA primer to detect resistant strains with A2047G mutation using the same PCR protocol. In this assay, strains with the mutation will give two bands of 286 bp and 121 bp. Strains without the A2047G mutation give only one band of 286 bp.

[2] Bartkus JM, Juni BA, Ehresmann K, et al. Identification of a mutation associated with erythromycin resistance in *Bordetella pertussis*: Implications for surveillance of antimicrobial resistance. Journal of Clinical Microbiology. 2003;41(3):1167-1172.
[3] Wang Z, Han R, Liu Y, et al. Direct detection of erythromycin-resistant bBordetella pertussis in clinical specimens by PCR. J Clin Microbiol. 2015;53(11):3418-3422.

Figure 9. Sensitive PCR assay using WP_23S_rRNA primer

Lane 1) ATCC 9797, Lane 2) NAP-12-30, Lane 3) H_2O, and Lanes 4-7) Samples without the A2047G mutation.

Table 14. Primers included in this protocol

FP_23S_rRNA	GTG ATG GGG TGC AAG CTC TT
RP_23S_rRNA	TCT GGC GAC TCG AGT TCT GC
WP_23S_rRNA	ATC TAC CCG CGG CTA GAC AGA
MP_23S_rRNA	ATC TAC CCG CGG CTA GAC AGG

References

1. .
2. Wang Z, Han R, Liu Y, et al. Direct detection of erythromycin-resistant *Bordetella pertussis* in clinical specimens by PCR. J Clin Microbiol. 2015;53(11):3418-3422.

2. Real-time PCR amplification and genotyping of the 23S RRNA from *Bordetella pertussis* using the Lightcycler 480

This method is based on the approach published by Institut Pasteur [127], which is itself based on the method published by Kamachi et al. [147].

Principle

The method is an adaptation for LC480 (version II, Roche) of the one described by Kamachi *et al.,* EID 2020 (PMID: 32946738 - Supplementary Materials 'Cycleave Real-Time PCR Assay for Detection of the A2047G Mutation'). qPCR targeting 23S rRNA differentiates two alleles of the ribosomal RNA of the 23S subunit of *Bordetella pertussis* (present in three copies per genome). The A2047G mutation distinguishes macrolide-susceptible isolates, with an A at position 2047, from macrolide-resistant *Bordetella pertussis* isolates (MRBP), with a G at position 2047.

Real-time amplification is performed from DNA extracted from a respiratory sample that has previously given a positive result for the detection of pertussis toxin (qPCR PTa, one copy per genome). Two distinct fluorescent probes are employed: one specific for the susceptible allele (FAM) and the other for the resistant allele (HEX). These probes specifically detect a 123 bp fragment of the 23S ribosomal RNA of *B. pertussis* and identify the A2047G mutation.

This qPCR is performed only for samples that have given a positive qPCR-PTa result (Ct <32), which has a similar analytical sensitivity.

Equipment and materials

- - Sterile 1.5 mL Eppendorf tubes
- - LightCycler 480 Multiwell plate 96 (Plates & Adhesives), Roche, ref. no. 04 729 692 001
- - Centrifuge for 1.5 mL Eppendorf tubes and centrifuge for microplates
- - PCR refrigerated racks
- - LightCycler 480 Thermal Cycler Version II, Roche.

Reagents

- DNAaway, MBP, Ref. 038188
- LightCycler 480 Probes Master Kit, Roche, ref. no. 04 707 494 001
- Water, molecular biology quality, supplied with kit Ref. 04 707 494 001 or by default Sterile Distilled H2O (10 mL ampoule, Braun Medical B, Ref. 3511810).

Positive or standard controls

Genomic DNA extracted from the reference strain Tohama (CIP 8132), either using the QIAGEN 'DNeasy Blood & Tissue Kit (A69504)' or via the MAXWELL automatic extractor with the 'Maxwell® CSC Blood DNA Kit (AS1321)'.

This DNA serves as a susceptible positive control and is used at the concentration of 100 pg/µL and 10 pg/µL. It is aliquoted to be stored between -15°C and -25°C. The tube is identified by the name of the strain and the date of aliquoting of the batch.

Genomic DNA extracted from the FR4991 strain (CIP 112499), either using the QIAGEN DNeasy Blood & Tissue Kit (A69504) or via the MAXWELL Automatic Extractor with the Maxwell® CSC Blood DNA Kit (AS1321). This DNA serves as a resistant positive control and is used at the concentration of 100 pg/µL and 10 pg/µL. It is aliquoted to be stored between -15°C and -25°C.

Primers and probes

(HPLC quality, supplier TibMolBiol, Berlin, Germany)

These are supplied freeze-dried for 'long-term' storage, protected from light, between +2°C and +8°C. Probes and primers are resuspended in H2O, molecular biology quality, and can be stored between -15°C and -25°C. Refer to the supplier's data sheets for quantities per tube.

o **Primers**

23S-rRNA-F and 23S-rRNA-R

23S-rRNA-F: 5'-GAATGGCGTAACGATG -3'

23S-rRNA-R: 5'-TGCAAAGCTACAGTAAAGG -3'

o **Probes**

23s_rRNA_SUS: 6FAM-CGGCTAGACGGAAAGACCCCA-BHQ2

23s_rRNA_RES: HEX-CGGCTAGACGGGAAGACCCCA-BHQ2

Sample processing

DNA from respiratory samples is extracted according to the protocol 'Genomic DNA extraction with the High Pure PCR Template Preparation Kit (ROCHE) for use with the Bordetella R-gene and Parapertussis R-gene kits'.

Instructions

- Follow the steps outlined in the forward circuit for preparing the reaction mixture (mix room, clean room) and the subsequent deposition of DNA samples.
- For the preparation of the reaction mix, the 96-well plates should be placed in a dedicated refrigerated rack, stored in the freezer of the PCR room. **Important:** This rack must never leave the clean room.
- A separate refrigerated rack is used for subsequent handling. **Important:** This second rack must never enter the clean room.
- Before each procedure, thoroughly clean the work surface and all equipment with DNAaway.

Experimental protocol

Plate plan

The plate design must include:

- a negative amplification control (H2O tube, Roche kit)
- a negative H2O extraction control, if extraction carried out in the lab
- positive amplification control for susceptible *B. pertussis*: CIP 8132 at 100 pg/µL and 10 pg/µL
- positive amplification control for resistant *B. pertussis*: CIP 112499 at 100 pg/µL and 10 pg/µL
- samples to test (Case 1).

Case 1

For a respiratory sample received already extracted or extracted with the procedure 'Genomic DNA extraction by the High Pure PCR Template Preparation Kit (ROCHE) for use with the *Bordetella* R-gene and parapertussis R-gene kits', add 5 µL of the extracted DNA to the dedicated well (according to the plate plan).

Preparation of the reaction mixture (volumes are given for one sample)

- In the reaction mix preparation room, prepare the stock of Assay Mix 20X.

Reagent (C final)	Volume
H2O	56 µL
Primer 23srRNA-F (18 µM)	18 µL
Primer 23srRNA-R (18 µM)	18µL
23s_rRNA_SUS (4 µM)	4 µL
23s_rRNA_RES (4 µM)	4 µL
Total volume	**100 µL**

- Mix gently by aspiration/discharge with a pipette.
- Storage between -15°C and -25°C (maximum 12 months).

Reaction Mix

- Prepare the reaction mixture (by multiplying the quantity in the 'Volume' column by the number of reactions to be performed, plus two additional reactions).
- Mix in a 1.5 mL Eppendorf tube:

Reagent	Volume	Final Concentration
H2O	4 µL	
Assay Mix 20X	1 µL	1X
Probes Master 2X	10 µL	1X
Total volume	**15 µL**	

- Mix gently by aspiration/discharge with a pipette.
- Place the 96-well plate in the refrigerated rack.
- Dispense 15 µL of the reaction mixture into the wells following the plate plan.
- Cover the plate with aluminum foil stored in the room.
- Do not forget to use the adhesive to seal the plate later.

Reminder

When the reaction mix preparation part comes out, place the plate in the dedicated refrigerated rack.

At a DNA sample repository:

- Add 5 µL of extracted and control DNA to the wells according to the plate plan.
- Immediately cover the plate with the appropriate adhesive.

In the PCR room where the thermal cycler is located:

- Centrifuge the plate for one minute at about 1000 g at room temperature.
- Put the plate in the LightCyclerÒ480 and start the 'qPCR 23s rRNA' programme.

Figure 10. Amplification programme

Program Name	pre-incubation						
Cycles	1	Analysis Mode	None				
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:10:00	4.40		0	0	0
Program Name	amplification						
Cycles	30	Analysis Mode	Quantification				
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:00:10	4.40		0	0	0
60	Single	00:01:00	2.20		0	0	0
72	None	00:00:01	4.40		0	0	0
Program Name	cooling						
Cycles	1	Analysis Mode	None				
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
40	None	00:00:30	2.20		0	0	0

Data analysis

- The analysis of the bacterial target is performed in **'Endpoint Genotyping'** mode, with FAM (465-510 nm filter) and HEX (533–580 nm filter) readout. In the **'Sample Editor'** tab, negative and extraction controls should be set as **'Negative Control'** and positive controls as **'Standards'** in the **'EndPt Sample Type'** column.
- For standards, add **'susceptible'** for CIP 8132 at 10 and 100 pg/µL to the **'EndPt Genotype**' column, and **'resistant'** for FR4991 at 10 and 100 pg/µL.
- The samples to be tested must be set as 'Unknown' in the **'Sample Type'** column.
- Finally, carry out the analysis.

Table 15. Results of analysis

Results

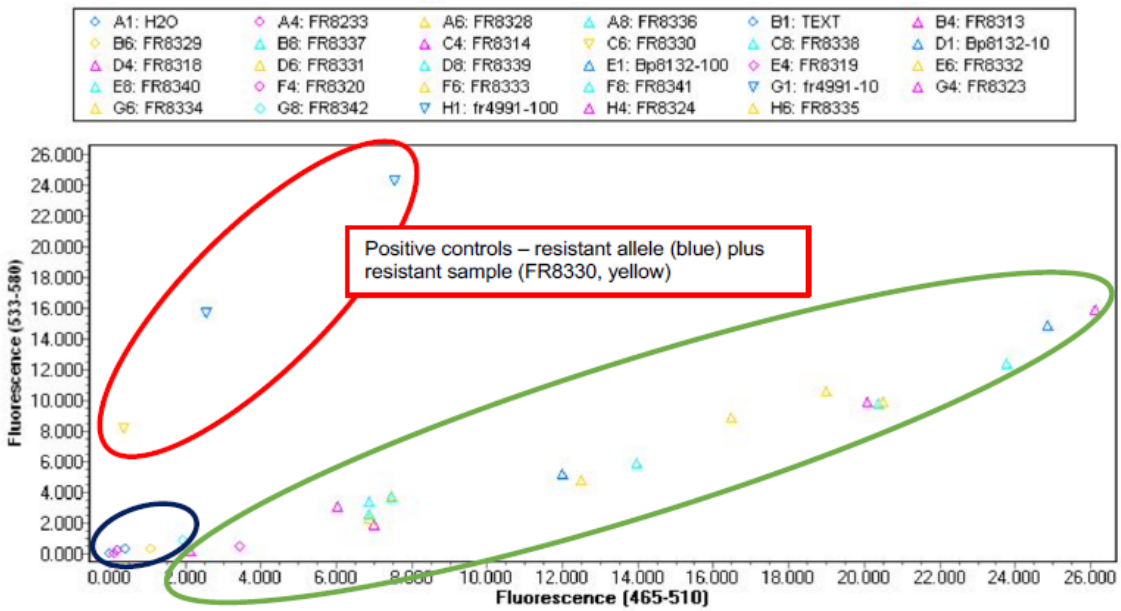
Inc	Pos.	Sample Name	Endpoint Fluorescence Allele X	Endpoint Fluorescence Allele Y	Call	Score	Status
☑	A1	H2O	0.41	0.35	Negative		
☑	A4	FR8233	0.22	0.16	Negative		
☑	A6	FR8328	7.47	3.79	SENSIBLE	0.90	
☑	A8	FR8336	6.89	3.42	SENSIBLE	0.91	
☑	B1	TEXT	0.00	0.00	Negative		
☑	B4	FR8313	2.12	0.23	SENSIBLE	0.76	
☑	B6	FR8329	1.07	0.33	Negative		
☑	B8	FR8337	13.95	5.88	SENSIBLE	1.00	
☑	C4	FR8314	6.99	1.94	SENSIBLE	0.83	
☑	C6	FR8330	0.38	8.18	RESISTANT	0.80	
☑	C8	FR8338	20.40	9.79	SENSIBLE	0.94	
☑	D1	Bp8132-10	11.99	5.24	SENSIBLE	0.98	
☑	D4	FR8318	20.08	9.95	SENSIBLE	0.92	
☑	D6	FR8331	20.49	9.93	SENSIBLE	0.93	
☑	D8	FR8339	6.88	2.63	SENSIBLE	0.96	
☑	E1	Bp8132-100	24.89	14.88	SENSIBLE	0.81	
☑	E4	FR8319	3.44	0.53	Unknown	0.44	
☑	E6	FR8332	12.51	4.84	SENSIBLE	0.97	
☑	E8	FR8340	7.47	3.69	SENSIBLE	0.92	
☑	F4	FR8320	0.12	0.04	Negative		
☑	F6	FR8333	16.49	8.88	SENSIBLE	0.87	
☑	F8	FR8341	23.79	12.37	SENSIBLE	0.89	
☑	G1	fr4991-10	2.57	15.70	RESISTANT	0.95	
☑	G4	FR8323	26.13	15.92	SENSIBLE	0.79	
☑	G6	FR8334	6.87	2.34	SENSIBLE	0.91	

Negative Controls

Positive controls – susceptible allele

Positive controls – resistant allele

Figure 11. Endpoint fluorescence scatter plot



The final conclusion for a sample will be determined based on the alleles called. In general, the 'endpoint fluorescence' for a susceptible sample is always higher for the susceptible allele (FAM) than for the resistant allele (HEX), and vice versa for a resistant sample. It is important to note that for samples with cycle thresholds (Ct) greater than 35 in the PTa qPCR, the majority will be categorised as negative in the qPCR 23S rRNA PCR. In all cases, the final decision on the interpretation of the results is taken by the NRC (deputy) head, who is also responsible for the diagnosis made.

Quality assurance

When the batch of the LightCyclerÒ480 Probes Master Kit (Roche, Art. No. 04 707 494 001) expires, it can still be used, as long as it is systematically revalidated with the positive amplification controls.

European Centre forDisease Prevention and Control (ECDC)

Gustav III:s Boulevard 40
169 73 Solna, Sweden

Tel. +46 858 60 10 00
ECDC.info@ecdc.europa.eu

www.ecdc.europa.eu

Publications Office
of the European Union