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**TECHNICAL** REPORT

# Laboratory diagnosis and molecular surveillance of *Bordetella pertussis*

Recommendations from ECDC

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# Laboratory diagnosis and molecular surveillance of *Bordetella pertussis*

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This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Dr Sabrina Bacci, Dr Marlena Kaczmarek and Christiana Carstairs MSc, and produced by Prof Qiushui He, Mr Alex-Mikael Barkoff, Prof Jussi Mertsola, University of Turku (UTU) (Turku, Finland), Dr Norman Fry, UK Health Security Agency (UKHSA) (London, UK), Dr Tine Dalby, Department of Bacteria, Parasites & Fungi, Statens Serum Institut (SSI) (Copenhagen, Denmark), Dr Paola Stefanelli, Department of Infectious Diseases, Istituto Superiore di Sanità (ISS) (Rome, Italy) and Dr Kevin Markey, Division of Bacteriology, National Institute for Biological Standards and Control (NIBSC) (Potters Bar, the UK) on behalf of the European Reference Laboratory Network for Pertussis (ERLNPert-Net) consortium, as part of the coordination of activities for laboratory surveillance of whooping cough in Member States/EEA countries (referring to Framework Contract ECDC/2019/023).

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# 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 | Centers for Disease Control and Prevention |
| 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 | Multi-Locus Variable-Number Tandem Repeat Analysis |
| 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 |
| TSB | Trypticase Soy Broth |
| VNTR | Variable-Number Tandem Repeat Analysis |
| WCV | Whole Cell Pertussis Vaccine |
| WGS | Whole Genome Sequencing |
| WHO | World Health Organization |

# 1. Introduction

Despite widely implemented childhood immunisation campaigns, pertussis remains one of the world's leading causes of vaccine-preventable deaths. In 2012, nationwide epidemics of pertussis occurred in several countries including the Netherlands, the United Kingdom (UK) and the United States [1]. According to the World Health Organization (WHO), 151 074 cases of pertussis were reported globally in 2018 [2]. In the EU/EEA, pertussis vaccines and vaccination schedules vary among countries. At present, all European countries (except Poland) switched from whole-cell pertussis vaccines (WCVs) to acellular pertussis vaccines (ACVs). ACVs consist of one to five antigens (in different formulations) of *Bordetella pertussis* [3]. Vaccine-induced immunity is reported to last only up to 10 years [4-5], and increasing numbers of pertussis cases are reported in adolescents and adults [6]. 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 isolates and circulating isolates [7]. Furthermore, *B. pertussis* isolates with the *ptxP3* allele for the pertussis toxin promoter have become prevalent in many countries since 2000, a genotype that is reported to confer enhanced virulence [8-13]. Since 1994, *B. pertussis* isolates resistant to macrolides have been identified in the US, France, Iran and China [14-19], and have especially increased in China during the past 10 years [15, 16, 20]. 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 [21-22], as shown by a recent study in which 3344 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 [23]. Finally, *B. pertussis* isolates which do not express some of the ACV antigens, such as PRN, have been reported globally, including Australia, Finland, France, Italy, Norway, Sweden, UK and the US [12, 24-29].

The current methods for laboratory diagnosis of pertussis includes 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 the EU/EEA[i] found that there is high heterogeneity in methods used for the laboratory confirmation of pertussis among national pertussis reference laboratories [30]. To evaluate the effects of different pertussis immunisation programmes in the EU/EEA, standardisation and harmonisation of laboratory methods are clearly needed. Additionally, commercial real-time quantitative PCR kits (qPCR) and respiratory panels are now widely used in diagnostics, allowing many respiratory pathogens to be tested 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 [31-32]. Recently, new rapid point-of-care (POC) tests for pertussis diagnosis have also been developed and other insights for better diagnostics have been studied [33-34].

For more effective national immunisation programs to prevent pertussis in the EU/EEA, it is important to apply standardised diagnostic methods which will make country-specific 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. This guidance 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 within the local technical and financial provisions. Some laboratory protocols have been previously published and are cited in this document in the appropriate sections.

[i] This study was conducted when the UK was part of the EU/EEA so all references to the EU/EEA in this report include the UK.

# 2. Diagnostics

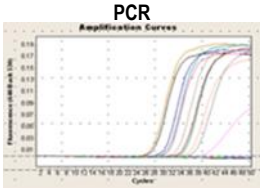
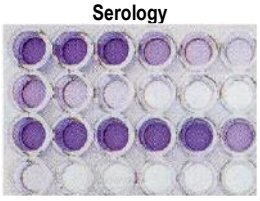
## 2.1 Nasopharyngeal sampling for culture and PCR

*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 in aliquots and saved for other investigations. However, for adolescents and adults, NPSs are usually preferred by medical staff. For older children, adolescents and adults, NPSs are mostly used, as NPAs are difficult to obtain in this population [35]. A video of the correct sampling technique can be found in a video produced by Nicole Guiso at the Institut Pasteur, Paris, France ("Prelevcoqueluche-desktop.m4v", YouTube) [36].

Since *B. pertussis* mainly grows in the human nasopharynx, throat swabs are considered suboptimal for culture and PCR testing and are therefore not recommended. However, they are sometimes used by general practitioners due to their wider availability.

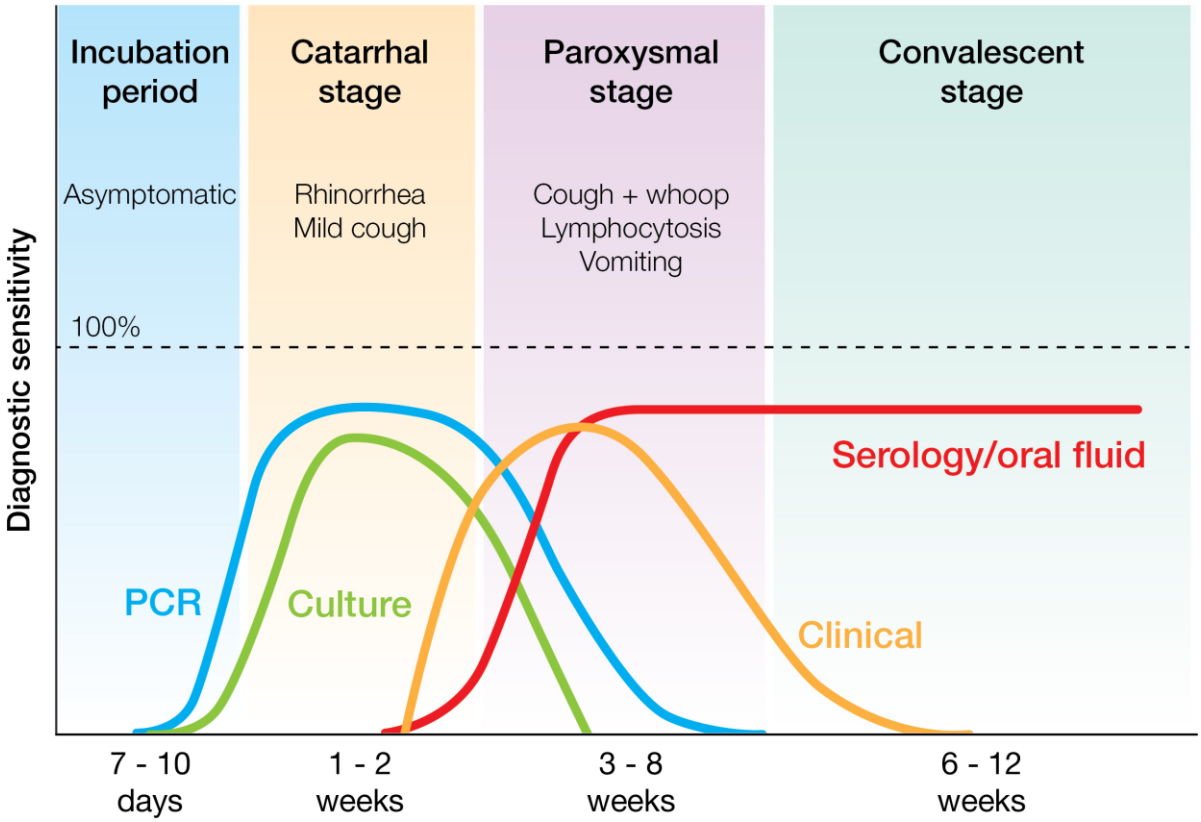
Sampling, isolation, culture and recommended diagnostics are summarised in Table 1 and Figure 1.

**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 last 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***




** adapted from Fry et al. 2021 [37].*

## 2.2 Transport of swabs for culture and PCR

After sampling, a NPS or NPA intended for detection of *B. pertussis* or other *Bordetellae* (e.g. *B. parapertussis*) should be transported quickly. Prior to transportation, the NPS/NPA should be stored at room temperature (RT) to avoid bacterial loss [38]. 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 [38-39]. Transport should also be at RT to avoid bacterial loss and arranged with minimal delay as a transportation time of more than 48h will affect negatively on viable bacteria [38]. Dry swabs are adequate for PCR testing and either nylon, dacron or rayon swabs are recommended, whereas calcium-alginate and cotton swabs may inhibit the PCR and are therefore not recommended [40]. However, it is important to note that RT may be too high in very hot countries/climates, particularly during summer months, and in these settings, the use of a cooler bag within the shipment is recommended.

## 2.3 Culture and storage

*B. pertussis* is a fastidious bacterium and the NPS/NPA should therefore be transported at an ambient temperature within four hours of collection to the microbiology laboratory for culture. Transport times of up to 48 hours are acceptable when a suitable transport medium is employed (i.e. Reagan Lowe medium). It has been shown that children, especially infants, have very high *B. pertussis* DNA loads in their NPS compared to adults [41]. Therefore, diagnosis of pertussis by culture is particularly useful in infants and young children. Positive cultures are usually only possible to obtain within two to three weeks of the onset of a cough.

After transport, the NPS or NSA are streaked onto fresh Regan-Lowe medium (RL) 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. Regan-Lowe medium contains cephalexin for this purpose, and cephalexin (40 µg/ml) can also be added to Bordet-Gengou medium.

*B. pertussis* is a strictly aerobic bacterium. Plates should be incubated for seven days, aerobically at 35–36°C, with preferably 60% relative moisture to avoid plate drying, and inspected daily. Plates showing no growth after seven days of incubation can be discarded as negative. *B. pertussis* grows slower 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 tests (*B. pertussis* / *B. parapertussis*) or PCR are recommended [38,42]. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has also shown to be reliable [43].

For storage, two different mediums can be used: 1) Trypticase Soy Broth (TSB) – 10-20% Glycerol tubes or 2) Bovine Serum Albumin (BSA) – Saccharose/phosphate/glutamate (SPG) buffer. More information can be found in Appendix I of the WHO pertussis laboratory manual [38].

It is important to perform culture to be able to monitor for vaccine antigen deficiency and evolution of the pathogen as well as to evaluate its antibiotic resistance [18,44-45]. Detailed protocols regarding NPSs and culture can be found from the WHO guidelines for laboratory diagnostics of pertussis [38].

## 2.4 PCR for Nucleic Acid Detection of *Bordetellae*

Diagnosis of pertussis by PCR is possible in patients who have been coughing for less than four weeks (<28 days), however the sensitivity is inversely related to time between onset of disease and specimen collection. Therefore specimen collection is recommended as soon as possible after symptom onset, and ideally <21 days from onset. 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;
- 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 nasopharynx (not from nose or throat). Swabs, as well as aspirates, can be used, and swabs made of rayon, nylon or dacron are suitable whereas cotton or calcium alginate are not recommended [40]. 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 [46]. Prior to the PCR analysis, DNA should be extracted from the sample, for which several commercial kits are available [47].

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 species of *Bordetella*, the choice of targets and interpretation of the 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* [48], while IS*1001* is present in *B. parapertussis* and some isolates of *B. bronchiseptica* [49]. The IS elements are present in the genomes in a high number of copies, 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 targeting the pertussis toxin promoter *(ptxP*) for *B. pertussis* or the IS*1001*-like element for *B. holmesii* [50] as additional targets are recommended. 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 an infection whilst adults tend to endure the symptoms for a longer period before seeking medical advice. If the patient has coughed 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.

Quantitative PCR has several advantages compared to block-based PCR, as qPCR is both 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 [38]. A detailed guidance and a protocol was published by ECDC in 2012 [42] and WHO in 2014 [38].

At present, multi-targeted PCR 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 designed 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 detected 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 [31-32, 51]. This is mostly due to the single use of *ptxP* as a target, as only a single copy of *ptxP* is present in the genome. Therefore, multi-target PCRs show a lower sensitivity for confirmation of pertussis among samples with low contents of DNA/bacterial load. This aspect should be carefully considered when a new PCR targeting multiple pathogens is implemented in 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* 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 the 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 in RT and separated quickly after blood sampling, preferably within four hours, but it is even possible up to 24 hours. 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 [52-53].

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

Oral fluid swabs are considered more comfortable for 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 (anti-PT) IgG antibodies. The results obtained are reliable, and there are commercially available kits for oral fluid sampling [54]. Currently, testing for anti-PT IgG in oral fluid is not widely used apart from in England and Wales where it is specifically used for notified cases for two to 17-year-olds to increase data in this age group and for outbreak investigation [55-56].

## 2.5.3 Recommendations and methods for serological diagnosis

Serologic 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 in serology and PT is only produced by *B. pertussis*. To date, there are no available serological tests for *B. parapertussis* or infections with other *Bordetellae*.

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. Also, 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 [57]. However, fewer dilutions of the samples may be used.

Several commercial kits with correct antigen and standards are currently available [58]. Kits that use other antigens than PT or mixed antigens, and/or express results in arbitrary values are not recommended [59-60]. Therefore, validated in-house methods and commercial kits with only purified native PT as a coating antigen and with reference or calibrants used in IU/mL are recommended [52].

Interpretation of results is critical. No universally recommended single cut-off exists, but those 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 [57, 60]. 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, 61].

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, 62-63]. Detailed guidance and protocols for serological diagnosis have been published by ECDC [60] and by WHO [38].

## 2.6 Point-of-care assays

Point-of-care assays are generally more suitable for investigation in outbreaks as results are ready in a shorter time than commonly used PCRs, and treatment of patients or prophylactic treatment of exposed subjects can be started soon. Therefore, these tests are clinically relevant. However, POC tests are still a rarity among pertussis diagnostics.

Development of POC tests for pertussis diagnosis is constantly ongoing. A few options were described by von Koenig, of which Loop-mediated isothermal amplification (LAMP) has been commercialised [34, 64-65]. 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 [34]. Previous problems with specificity and sensitivity of this assay have been overcome and new innovative and low-cost platforms have been developed [64-65].

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 [33, 66]. 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. Commercial approaches have not yet been done for this LF assay.

# 3. Molecular surveillance of circulating *B. pertussis*

## 3.1 Serotyping

*B. pertussis* expresses two phase-variable major subunits of fimbriae: Fimbriae2 (Fim2) and Fimbriae3 (Fim3), which determine the serotype of isolates. Isolates can therefore be either Fim2, Fim3 or Fim2,3. The *fim2* and *fim3* genes are regulated by single base insertions in their promoter regions, which result in serotype switching [67]. Serotyping of freshly isolated *B. pertussis* isolates was first described in 1953 [68], and has been an essential part of characterising *B. pertussis* clinical isolates for a long time [69]. Studies on isolate variation have shown that *B. pertussis* populations are dynamic and continuously evolving [7, 70-73]. Therefore, determining the serotype of circulating *B. pertussis* isolates is recommended and can be performed with various methods:

- Slide agglutination: traditionally, serotyping is done using the slide agglutination method with bacteria from 24 to 48 hours cultures of *B. pertussis* mixed with specific serotyping antisera or monoclonal antibodies on glass slides [74]. The slide is rocked gently and observed for clumping. Specific agglutination occurs within 30 seconds.
- Micro-agglutination: the micro-agglutination method [75] 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 [76].
- ELISA: an indirect ELISA method can be used for serotyping [73, 77]. 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 at one time [73].

For all serotyping methods, it is recommended that monoclonal antibodies (e.g., obtained from the National Institute for Biological Standards and Control (NIBSC)) are used for serotyping of *B. pertussis* [78] and that Fim2- and Fim3-expressing isolates are included as controls [79-81]. No cross reactions have been found between monoclonal antibodies and reference isolates used as coating antigens [73]. Previously, polyclonal antibodies have also been used for serotyping [82].

The recommended protocols for serotyping are presented in Annex II.

## 3.2 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (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 [83]. PFGE typing of *B. pertussis* has been successfully used in epidemiological investigations to identify outbreak-associated isolates and to monitor bacterial transmission [84-86].

In 2000, a group of experts working in the field of pertussis in the EU/EEA (EUpertstrain) established a detailed reference PFGE method based on two enzymes *Xba*I and *Spe*I [75]. The method analysed bands with a molecular size between 23 and 500 kb. Band patterns of the test isolates should be interpreted in relation to the patterns obtained from international reference isolates such as ATCC 18323, Tohama I, Bp134, FR287, FIN12, B902 and FR743. Using the reference method, Weber and co-workers classified isolates into five clusters or groups [87]. Groups I and II contained historical isolates (ATCC 18323 and Tohama I) and did not represent circulating isolates. Groups III to V represented the circulating isolates. Using only enzyme *Xba*I, Advani and co-workers analysed 1810 Swedish clinical isolates collected between 1970 and 2003 and identified 176 distinct PFGE profiles [88]. Thirty-five profiles, each represented by more than seven isolates, comprised 85% of all 1 810 isolates. The profiles were designated BpSR1, BpSR2, BpSR3, etc.

The European Bordetella expert group (EUPertStrain) has analysed a total of 661 *B. pertussis* clinical isolates collected between 1998 and 2015 [70]. These isolates were collected from nine European countries: Denmark, Finland, France, Germany, the Netherlands, Norway, Poland, Sweden and the UK. Altogether, the 661 isolates produced 104 different PFGE profiles, with five main profiles of BpSR3, BpSR5, BpSR10, BpSR11, and BpSR12. In regard to profiles, the US Centers for Disease Control and Prevention (CDC) use their own nomenclature. However, the above mentioned five main profiles can be traced and CDC profiles CDC002 (BpSR3), CDC046 (BpSR5), CDC010 (BpSR10), CDC013 (BpSR11) and CDC082 (BpSR12) represent these main profiles in the EU/EEA [70, 89].

The PFGE profiles or clusters are analysed by computer software, such as BioNumerics (Applied Maths). 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% optimization 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 [90]. The Swedish nomenclature is based on a cluster analysis with 1% band tolerance and 1% optimization settings [88].

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* [91]. However, performing PFGE is time consuming and its band patterns are relatively difficult to standardise and compare between laboratories. In addition, PFGE requires cultured isolates. The advantages of this method are the simplicity of the method and the low cost of the equipment used.

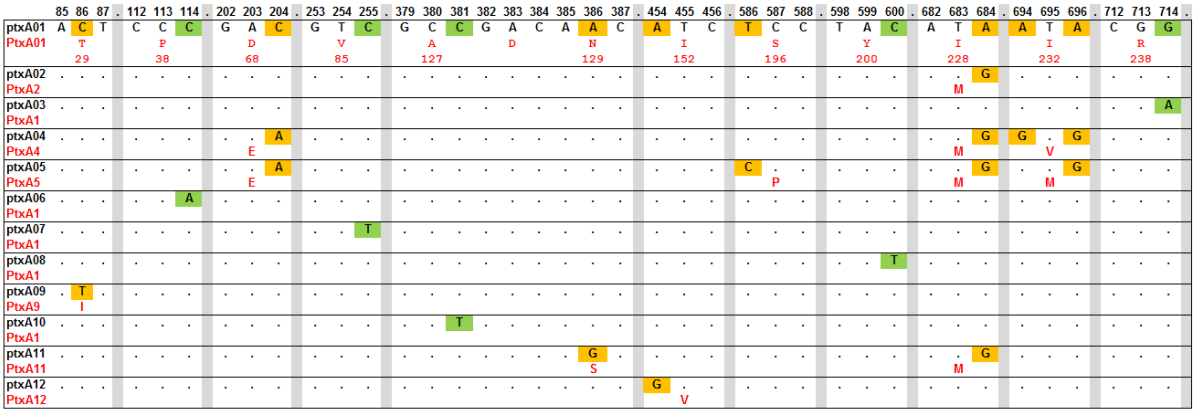
# 3.3 Multi-Locus Antigen Sequence Typing

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 in pertussis vaccines [11, 92-94]. MAST is focused on the genes 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 supposed to enhance the production of toxin [8]. All current ACVs contain PT, whereas the other components are used in varying combinations [3].

## 3.3.1 Pertussis toxin

Pertussis toxin (PT) is comprised of five subunits, PtxA-PtxE or Ptx1-Ptx5, of which PtxA contains the toxic, catalytic activity. Of the genes encoding the five subunits, most sequence variation by far has been reported for *ptxA* [8]. The *ptxA* gene comprises 810 bases, and 13 alleles have been found to date (2021) (*ptxA13* not presented, Figure 2). As a number of single nucleotide polymorphisms (SNPs) in *ptxA* are silent, this results in seven protein variants: Six of the 12 alleles encode variants of the PtxA1 protein, whereas the remaining six (*ptxA1*, *3*, *6*, *7*, *8*, *10*) contain silent mutations and encode the wild-type PtxA1 protein. In addition, the effect of the mutation (A185G) in the recently found *ptxA13* is not known [95]. Currently, the protein variant *ptxA1* is highly dominant among isolates, especially in the EU/EEA [8, 9, 11, 70, 93-95].

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




** Dots indicate identity with the ptxA1 allele. Silent and non-silent SNPs are highlighted in green and ochre, respectively. 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.*

## 3.3.2 Filamentous hemagglutinin

The filamentous hemagglutinin (FHA) gene (*fhaB*) comprises about 12 000 bases and due to its large size, routine sequencing of this gene has not been performed and the FHA gene is rarely typed among other vaccine antigens.

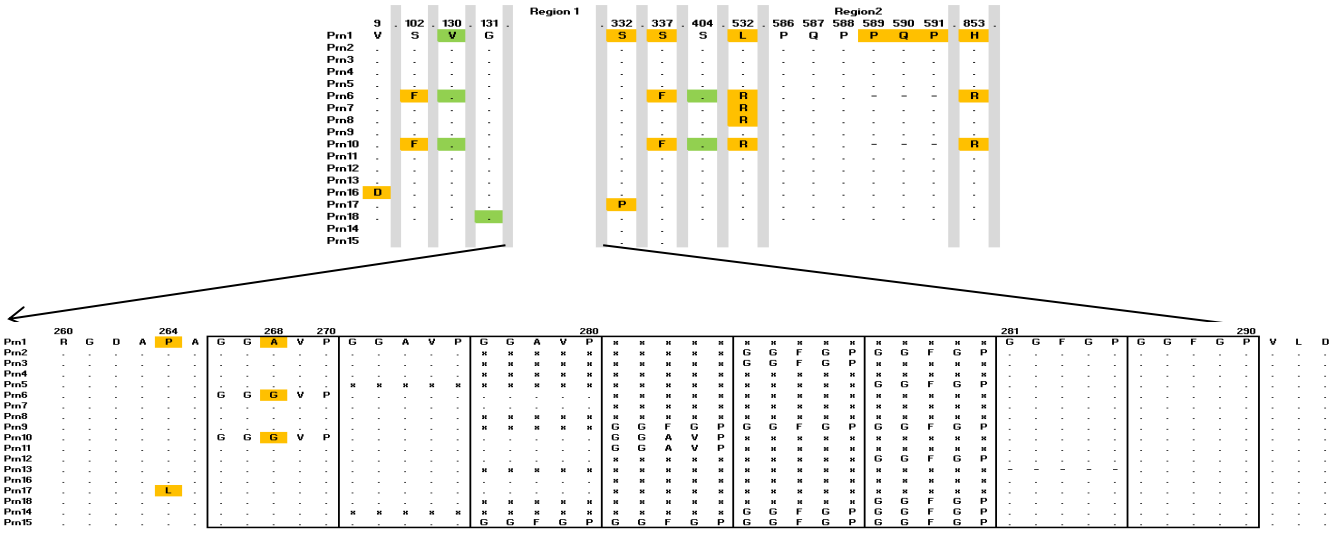
Only three alleles of *fhaB* (varying by one non-silent SNP) have been reported [92], although recent whole genome sequencing (WGS) revealed further polymorphisms, encoding several FHA variants, from which a novel *fhaB3* allele was described. The isolate carrying this allele had a five-fold higher mutation rate than other isolates in the study [96-97].

## 3.3.3 Pertactin

The gene for pertactin (PRN) comprises about 2 800 bases, which code for a large precursor protein. The 5'-end of the gene codes for the part of the PRN precursor, which is exported from the cell, while the 3'-end codes for an integral outer membrane protein required for this export. The integral outer membrane protein shows little variation and is not included in ACVs. Although the exported PRN contains SNPs, most variation is found in one of the two repeat regions (region 1) in PRN (Figure 3). Hence, gene typing is focused on region 1.

*Prn* is one of the most polymorphic genes found in *B. pertussis* and to date 18 alleles have been identified (*prn1-15* shown in the figure 3, *prn16-18* not presented). The *prn1* and *prn7* alleles predominated in the pre-vaccine period and are also produced by many isolates used for production of vaccines (vaccine isolates). The isolates used for vaccine production were later replaced, mainly by isolates carrying *prn2* and *prn3* genotypes. In the EU/EEA, *prn2* is currently the predominant type, with 95% of the circulating isolates having this genotype [70, 98]. Furthermore, the emergence and increase of clinical isolates which do not express PRN has been observed. This matter will be further described in chapter 3.3.6.

**Figure 3. Variation in pertactin (*prn)* gene, regions 1 and 2***




**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. Green and ochre colours refer to silent and non-silent mutations, respectively. Repeats have been blocked. Numbering is relative to the initiation codon of Prn1. Prn14 and Prn15 have been sequenced partially.*

## 3.3.3 Fimbriae

*B. pertussis* harbours four major fimbrial (fim) subunits (*fimA, fimX, fim2* and *fim3*) and one minor subunit (*fimD*) [99]. The major and minor fimbrial subunit genes comprise about 600 and 1 098 bases, respectively. The *fimD* gene shows very little variation and to date only two alleles have been found [92]. The *fimA* gene contains a large deletion and the protein is therefore not assembled into fimbriae. The *fimX* gene is intact, but it is presumed that its promoter is not active due to a deletion. The remaining two fimbrial major subunit genes, *fim2* and *fim3*, may be expressed simultaneously, or separately, by *B. pertussis* isolates due to a phase-variable promoter [67]. Two and seven alleles for *fim2* and *fim3* are known, respectively (Figure 4). Isolates currently used for ACVs produce *fim2-1* and/or *fim3-1*. Both the *fim2-1* and *fim2-2* alleles are found in isolates from the pre-vaccine era. However, *fim2-2* has now been found in modern isolates, suggesting that the mutation has arisen anew. In pre-vaccination isolates, only the *fim3-1* allele has been identified. The *fim3-2, fim3-4, fim3-5, fim3-6* and *fim3-7* alleles probably arose after the introduction of vaccination [100]. Interestingly, all the novel *fim3* alleles are associated with the *ptxP3* allele (see below).

**Figure 4. Variation in the *fim2* and *fim3* genes coding for the serotype 2 and 3 fimbrial subunits***

| allele | protein | 85 | 86 | 87 | 259 | 260 | 261 | 388 | 389 | 390 | 493 | 494 | 495 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| fim3-1 | Fim3-1 | A | C | C | G | C | G | A | C | C | G | T | C |
| | | | T | | | A | | | T | | | V | |
| | | | 29 | | | 87 | | | 130 | | | 165 | |
| fim3-2 | Fim3-2 | . | . | . | . | A | . | . | . | . | . | . | . |
| | | | | | | E | | | | | | | |
| fim3-3 | Fim3-3 | . | . | . | . | A | . | G | . | . | . | . | . |
| | | | | | | E | | | A | | | | |
| fim3-4 | Fim3-1 | . | . | T | . | . | . | . | . | . | . | . | . |
| fim3-5 | Fim3-5 | . | . | . | . | A | . | . | T | . | . | . | . |
| | | | | | | E | | | I | | | | |
| fim3-6 | Fim3-6 | . | . | . | . | A | . | . | . | . | . | C | . |
| | | | | | | E | | | | | | A | |
| fim3-7 | Fim3-7 | . | . | T | . | . | . | . | . | . | . | A | . |
| | | | | | | | | | | | | D | |

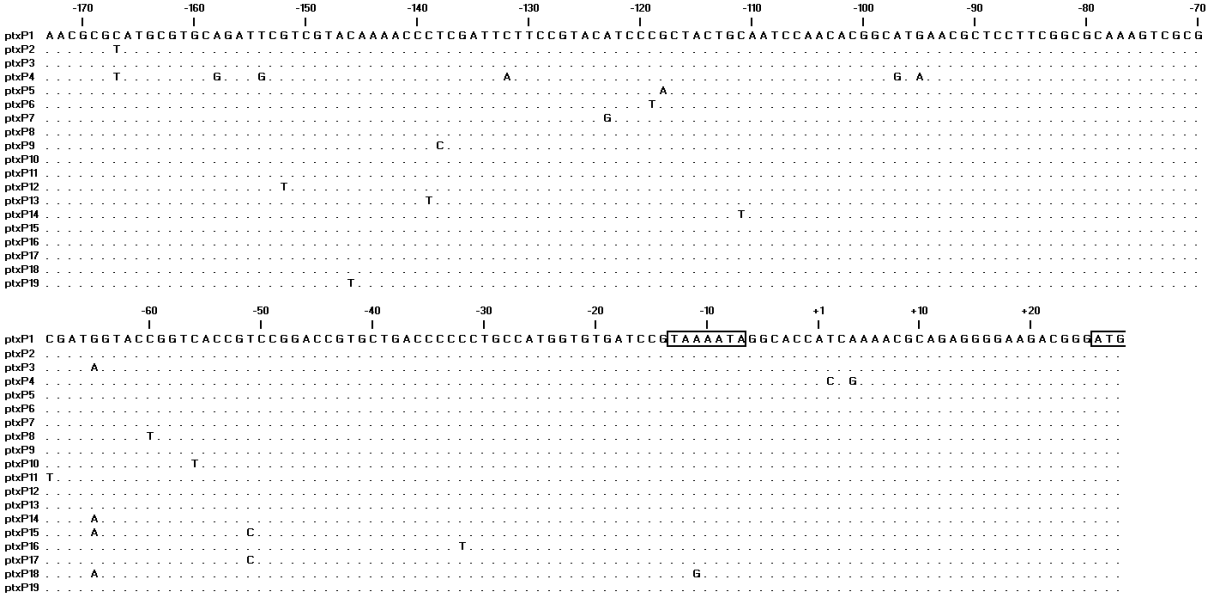
| allele | protein | 520 | 521 | 522 |
|---|---|---|---|---|
| fim2-1 | Fim2-1 | A | G | A |
| | | | R | |
| | | | 174 | |
| fim2-2 | Fim2-2 | . | A | . |
| | | | K | |

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

## 3.3.4 The pertussis toxin promoter

Although the pertussis toxin promoter (*ptxP)* does not code for a vaccine component, it is generally included in isolate typing as a marker for an emerged lineage, which has spread worldwide [7]. This lineage contains the *ptxP3* allele, which is associated with increased production of PT and other virulence factors under *in vitro* conditions [8, 101]. Nineteen *ptxP* alleles have been found (Figure 5). In the pre-vaccination era, isolates with the *ptxP1* and *ptxP2* alleles predominated. After the introduction of vaccination, mainly *ptxP1* isolates were found, whereas nowadays *ptxP3* predominates in the EU/EEA [9-11, 70, 102].

**Figure 5. 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 blocked.*

## 3.3.5 Multi-Locus Variable Number of Tandem Repeat Analysis

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 [103].

A MLVA typing scheme for *B. pertussis* was first described by Schouls et.al [104]. The scheme was designed using the whole genome sequences of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* [105]. Six VNTR loci were originally chosen (VNTR1 to 6), which contain DNA repeat units of between 5 and 15 bp (Table 2). The VNTR loci are located in the putative open reading frames of genes encoding for proteins with different functions (Table 2). VNTR2 was excluded from the final MLVA scheme due to its low variability and VNTR3 has been split into VNTR3a and VNTR3b [104].

**Table 2. Characteristics of VNTR loci in *B. pertussis*.***

| VNTR | Repeat | Locus** | Gene | Putative function ORF |
|---|---|---|---|---|
| VNTR1 | GAACCCGCCAAGCAG | BP2075 | | Inner membrane efflux protein |
| VNTR2 | CCGCCCATGCCG | BP2498 | *dnaJ* | Chaperone |
| VNTR3 | CTGGC | BP2450 | | Membrane protein (pseudogene) |
| VNTR4 | CAAGGACAAGGG | BP0184 | | Exported protein |
| VNTR5 | TGGTGC | BP0967 | *cysT* | Sulfate permease (pseudogene) |
| VNTR6 | CGAGCCGCC | BP1988 | | Membrane protein (pseudogene) |

**Adapted from Schouls 2004 [104].*
***Locus designation in Tohama I [105].*

The number of repeats at each VNTR locus is typically determined by amplifying across each locus using PCR and measuring the length of the amplified product [93, 104]. The results are reported either as a profile that describes the number of repeats at each locus in the order VNTR1, 3a, 3b, 4, 5 and 6 (e.g. 8,7,0,7,6,7) or as an MLVA type (e.g. MT-27). Unfortunately, the *B. pertussis* MLVA reference database (http://www.mlva.net/) is no longer maintained.

The six locus MLVA scheme has been widely adopted for typing *B. pertussis* isolates from various European countries, the US, Australia and Japan [11, 13, 91, 93, 104, 106-108]. Over 250 unique MLVA profiles have been reported. The results of these studies have shown that the diversity of MTs within circulating *B. pertussis* isolates has decreased since the introduction of mass vaccination. This is partly due to the emergence of MT-28 and especially MT-27 isolates, which have grown to become dominant in the majority of European countries (from 48% to 91%) as well as in US (76%) and Australia (47%) [70, 109-110].

## 3.3.6 *B. pertussis* isolates deficient to acellular pertussis vaccine antigens

Pertussis is known to be an endemic disease with epidemic episodes occurring every two to five years. Despite the introduction of vaccination, *B. pertussis* has managed to avoid eradication and still circulates in the human population. The introduction of WCVs in the 1940s and1950s changed the environment of this organism. It appeared that controlling circulating vaccine-type isolates allowed for new *B. pertussis* populations to emerge [7, 72-73]. The later introduction of ACVs also changed the vaccine-induced immunity and, along with booster vaccination for adolescents and adults in some countries, increased the population's herd-immunity, thus introducing a new challenge for circulating *B. pertussis* populations. It was hypothesised that ACVs impact on the remaining *B. pertussis* populations would be different than WCVs [111]. WCV vaccination induces a broad immune response against hundreds of bacterial proteins, leading to control of the circulation of isolates that are similar to the vaccine isolates. However, this vaccination strategy did not control the circulation of other isolates, nor did it control the virulence of the isolates.

ACVs were introduced in many developed countries in the late 1990s or early 2000s to replace WCVs, due to a high prevalence of adverse effects with WCVs. ACV-induced immunity only targets few bacterial proteins that directly interact with the host. Several years after the introduction of ACVs as the only vaccines in use in many countries, isolates lacking the production of one vaccine antigen (PRN) emerged and spread in regions of high vaccine coverage [12, 42-28, 62, 108, 112-114]. A recent study conducted in the nine European countries (Belgium, Denmark, Finland, France, Italy, the Netherlands, Norway, Sweden and UK) showed a significant increase of PRN deficient isolates from 1.0% in 1998-2001 to 25% in 2012-2015 [115]. Similar increases have also been observed in Australia, Japan and the US [26, 29, 115]. Of note, a recent study performed 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 stressing the effect of ACV vaccination on the expression of vaccine-antigens in circulating isolates [117].

Multiple mutations in the *B. pertussis* genome can result in isolates that are not producing PRN, and such isolates seem to have originated from different lineages [29, 112, 115, 118]. The first mechanism observed was either an insertion of an IS*481* element in the *prn* gene or a partial deletion of the *prn* gene [25]. In Japan and Finland, a new deletion in the signal sequence of the *prn* gene was identified [26-27]. Later, 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 among other isolates [115].

Using a mouse model and human cell lines, no difference in virulence was observed between isolates not producing PRN and isolates producing PRN [12]. However, PRN-deficient isolates have an increased ability to colonise ACV-immunised mice compared to PRN-positive isolates [119]. In addition, one study showed that these isolates are as virulent in infants less than six months of age and as transmissible as the isolates producing PRN [120]. The emergence and particularly the frequency of PRN-deficient isolates has been shown to increase as a function of time since the introduction of ACVs. In general, the longer time since introduction leads to higher frequency of PRN deficient *B. pertussis* isolates [115]. However, other evolutionary advantages could also be part of the explanation. As vaccine coverage increases in adolescents and adults, monitoring the changes in the *B. pertussis* populations should continue through molecular typing.

The methods used for detection of PRN production by *B. pertussis* include western blotting [12, 25-26, 108] and indirect ELISA [27]. Sequencing of the whole *prn* gene was used for analysing the molecular basis for the loss of PRN expression.

In addition to PRN deficient isolates, other vaccine antigen deficient isolates have sporadically been found. The first PT deficient isolate to be reported was from France in 1996, and five such isolates have been identified in France from 1996 to 2018 (0.35%, 5 out of 2280) [25]. The cause for deficiency was deletion of the whole *ptx* operon and an unknown mechanism [12, 25, 71]. In addition, one PT+PRN negative isolate has been found in the US with the same deletion of the whole *ptx* operon [121]. One FHA+PRN negative isolate was reported in Australia [97]. The FHA inactivation was probably resulting from changes within the homopolymeric G tract (site: 1078-1087) from 10 Gs to 11 Gs in *fhaB*, resulting in a downstream stop codon that produced a truncated protein.

FHA deficient isolates have been found from France (N=4), Slovenia (N=1), Sweden (N=2) and the US (N=5) [12, 71, 96, 122-123]. Several mechanisms, including *IS481* insertion and SNP insertions/deletions in the *fhaB* gene, were found to cause the deficiency [96, 122, 124]. In addition, three isolates deficient in Fim2/3 and one deficient in tracheal colonization factor A have been found [102, 108, 115 125].

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 show an increase in the production of this antigen in Finland, where a large number of circulating isolates from the period 1991-2020 were studied (Barkoff and He, unpublished data).

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

### 3.3.6 Antimicrobial susceptibility testing

Macrolides [erythromycin (ERY) and azithromycin (AZT)] are the first-line drugs to treat *B. pertussis* infection [126-127]. So far, macrolide-resistant isolates have mainly been found in China, but such isolates have also been found sporadically in the EU/EEA, the Middle East and in North and South America [15-19, 128-129]. The frequency of the isolates resistant to macrolides has increased, especially in China. According to Chinese studies performed in Beijing (2013–2014), Zhejiang province (2016) and Shanghai (2016–2017), frequencies of macrolide-resistant *B. pertussis* varied from 60% to 92% [130-131]. These isolates are found in different areas in China, which indicates that they are not clonally expanded in one location. Recently, macrolide resistant *B. pertussis* isolates have appeared in Japan and Vietnam, which are neighbouring countries to China [22, 132].

The only mechanism identified to date to cause the macrolide resistance has been a point mutation changing nucleotide A to G at position 2047 (A2047G) in the domain V of the 23S rRNA gene of *B. pertussis*, as described previously [14, 15, 18, 127]. It remains to be shown if other mutations or molecular changes in the genome confer macrolide resistance. Therefore, culture-based antimicrobial susceptibility testing (AST) is the golden standard for the determination of macrolide resistance. So far, all resistant *B. pertussis* isolates detected have been highly resistant (>256 µg/mL) to macrolides (both ERY and AZT) when measured by the minimum inhibition concentration (MIC) test, whereas sensitive isolates show no tolerance against the macrolides (both ERY and AZT <0.250 µg/mL) [18, 127, 131, 133]. Crucially, at the moment there are no defined cut-offs from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) regarding 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 available [16]. PCR-based (partial) Sanger sequencing of 23S rRNA gene or WGS are other options to detect these isolates, but WGS is not as practical for small-scale laboratories as PCR or PCR-based Sanger sequencing of the 23S rRNA. This method is further described in chapter 3.3.7.

**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 [127]. *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. Strips containing gradients of increasing concentrations of AZT and ERY are added on the plates. MIC can be determined after 48 hours. A complete protocol for AST MIC testing is presented in Annex 5.

## 3.3.7 Whole genome sequencing

Whole genome sequencing is evolving from a research tool to a routine surveillance instrument, providing new insights for investigating infectious disease emergence and transmission, expediting pathogen characterisation, and promoting data sharing. *B. pertussis* is highly monomorphic and the traditional typing methods, MLVA and MAST, have limited discriminatory power [134]. Thanks to long-read sequencing technologies, it was demonstrated that isolate diversity is the result of small genetic changes as well as large rearrangements [135] facilitated by the high number of long repetitive mobile genetic elements present in the *B. pertussis* genome, (such as IS*481* of which there are more than 200 copies found in the *B. pertussis* genome). 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. Nevertheless, this could be avoided by optimising culture-independent sequencing directly from nasopharyngeal samples used for qPCR.

For WGS, high-quality DNA is needed, which can often be achieved with commercial extraction kits. The template quality should be ensured by measuring the 260/280 and 260/230 absorbance ratios by UV spectrophotometry or fluorometry. 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 quantity and integrity should be verified with gel electrophoresis and fluorimetric methods, respectively. However, human DNA in the samples may sometimes cause problems and therefore it can 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 [136]. Several high-throughput sequencing instruments are available for WGS and are well described [137-138]. 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 [137]. Short-read sequencing technologies are cost-effective and provide highly accurate results. However, large repeats and regions with extreme GC contents are problematic especially for short-read WGS typing of *B. pertussis* [139]. Long-read sequencers overcome these problems by generating reads of more than 10 kb [137]. However, long-read platforms have higher error rates than short-read sequencers [139]. Draft genomes assembled from short reads are very suitable to speed-up molecular typing, virulence-associated gene detection, outbreak investigation and phenotype prediction of *B. pertussis*. However, it remains to be shown whether WGS gives comparable results to phenotyping e.g. on vaccine antigen expression of the circulating *B. pertussis* isolates as the mechanisms causing these are not all identified nor fully understood.

The most popular means of exploring genome diversity is the gene-by-gene approach, which considers the alleles as the units of analysis [140]. The allele calling process is facilitated by the existence of international databases, such as the Bacterial Isolate Genome Sequence Database (BIGSdb; (*B. pertussis* is available at https://bigsdb.pasteur.fr/bordetella/) [141]. The sequences uploaded into the BIGSdb can be scanned against all known alleles, which present key targets for *B. pertussis*. Analysis can cover single genes to complex schemes, such as the Core Genome Multi-Locus Sequence Typing (cgMLST), which is currently the most popular approach for high-resolution subtyping [142]. The gene-by-gene approach provided by the BIGSdb is easily scalable to large groups of isolates and the generated allelic profiles can be compared through the Genome Comparator tool to determine the correlation between different isolates.

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 [143]. The easiest way to visualise relationships from WGS data is to convert the multiple sequence alignment into a distance matrix showing the number of allelic or SNPs differences between all pairs of sequences in the dataset.

# 4. Summary

Diagnosis of 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 from year to year, it is still highly recommended as the isolates can be used for molecular surveillance of the changes in bacterial populations. Monitoring of the circulating isolate variants is important for evaluation of vaccine effectiveness and for future vaccine development. In addition, cultured *B. pertussis* isolates will be important for surveillance of macrolide resistance and epidemiology.

PCR is highly recommended for diagnosis of pertussis in infants and small children, but the methods used vary and the wide implementation of multi-targeted PCRs may have compromised sensitivity in comparison to those targeting only *B. pertussis* [31, 32, 51]. This should be kept in mind when choosing a PCR method for diagnostic use.

Serology is the most commonly used method among adolescents and adults. For this method, 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 [60].

In addition to these common methods, new point-of-care (POC) based methods are under development. 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 actions to be quickly started to stop transmission of the disease [33-34, 66].

*B. pertussis* has shown a surprising ability to adapt to immunised populations since the introduction of vaccination in the 1950s and 1960s [98]. Initial changes involved the expansion of (presumably) pre-existing isolates containing small mismatches in virulence factors compared to vaccine isolates. Later, new mutations arose in *fim3* and *ptxP* [7]. More recently, isolates which do not express PRN have expanded globally [29, 72, 115]. Understanding how *B. pertussis* adapts, facilitates the selection of the best currently available vaccine and the development of improved vaccines. Any outbreak of pertussis leads to the questioning of what has changed; the population immunity, the vaccination coverage, the vaccine or the pathogen? Often several factors are involved in pertussis epidemics and without isolate surveillance, it is difficult to establish their cause. Furthermore, immunity is not lifelong, and individuals may have several episodes of pertussis in a lifetime. Also, world-wide eradication has shown to be difficult to achieve despite widespread vaccination, and frequent epidemics cannot be easily avoided.

One can distinguish two approaches to isolate surveillance: a targeted or a generic approach. With a targeted approach, one focuses on particular genes assumed to be important for evasion of host immunity [92]. 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 [90, 104, 144-145]. Such approaches should be sensitive enough to identify newly emerged isolates. However, WGS based approaches have become more and more common and the discriminatory power has diminished the use of many other typing methods [134].

Targeted approaches include serotyping (Chapter 3.1) and MAST (Chapter 3.3). The simplest, and oldest, approach to typing of *B. pertussis* is serotyping. The usefulness of serotyping for interpreting increases in pertussis is very limited, however, as only three serotypes can be distinguished. Serotype switches among the circulating isolates are observed in 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 [73, 80-81]. However, in ACV vaccinated populations, shifts between Fim2 and Fim3 have been observed [146]. Whether this could be used as an indicator to predict upcoming outbreaks on a national level remains to be shown. MAST involves the sequencing of genes which are mainly used in ACVs. For laboratories which do not have a DNA sequencer, MAST can be outsourced by sending *B. pertussis* DNA to commercial companies. The costs are very reasonable when compared to buying (or leasing), maintaining and running a DNA sequencer. An important advantage of both serotyping and MAST is that the results are unequivocal, allowing European-wide or global comparisons.

Generic approaches involve MLVA (section 3.3.5), PFGE (section 3.2) and SNP typing (section 3.3.7). The disadvantage of MLVA is that it lacks discriminatory power in isolate comparison as the majority of currently circulating isolates in the EU/EEA are dominated by MLVA type MT27 [70, 147]. SNP typing is mostly carried out by WGS. An issue which should be addressed for SNP typing is the choice of regions to be analysed for occurrence of SNPs against a reference genome (like the reference strain Tohama I), as European-wide comparisons are only possible if the same SNP set is used. Further, it is anticipated that the SNP set will have to be continuously adapted as new SNPs in new isolates emerge.

An important advantage of both MAST and SNP typing is that the results are unequivocal, allowing European-wide comparisons. PFGE has been most widely used for *B. pertussis* isolate surveillance as it has the next most discriminative power after WGS. Unfortunately, PFGE does not accurately reflect genetic relationships, which may be important to assess epidemiological trends. A major drawback of PFGE is that it is difficult to standardise and compare between laboratories. Furthermore, one possibility is to use WGS data to build up a new PFGE-like identification. However, if WGS data are available, cgMLST exhibits the highest discriminatory power and reproducibility [141, 148]. Importantly, PRN expression should be tested phenotypically as it is one of the key components in the current ACVs. The rapid increase of PRN deficient isolates is alarming as such isolates have shown better fitness in ACV immunised mice [118].

Although macrolide resistant *B. pertussis* isolates are mostly circulating in China, it is important to monitor both phenotypically and genotypically whether, or when, resistant *B. pertussis* isolates appears in the EU/EEA. This is an important issue not only for bacterial surveillance but also for (prophylactic) effective treatment of pertussis patients, especially infants who are at the highest risk of hospitalisation and death.

In summary, the purpose of this guidance and protocols for diagnostic and typing methods is to enable laboratories to correctly identify cases of pertussis and to make country-based comparisons of pertussis data possible. The correct use and interpretation of culture, PCR, serology, WGS and POC tests is crucial for this. Furthermore, weighing utility and feasibility, the minimum useful isolate surveillance package implemented in a reference laboratory should consist of serotyping, vaccine antigen expression, MAST and AST. WGS is currently the most discriminative method, but requires both equipment/external utilities and standardisation, for which BIGSdb can be used.

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

Preparation of TSB - Glycerol tubes

| | |
|---|---|
| Tryptone Soy Broth | 3g |
| 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 in +4°C up to 6 months.

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

The instructions are well described in the publicly available WHO manual for "Laboratory Manual for the Diagnosis of Whooping Cough caused *by Bordetella pertussis/Bordetella parapertussis*". The instructions are presented in Annex 5 [38].

# Annex 2. Serotyping of *B. pertussis*

## Reagents

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

The ampoule contents should be reconstituted with 1ml 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 [82], working dilutions were suggested as following, however 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 with an $OD_{650nm}$ = 1.0* is placed in wells A1 to A6.
- 50µl of bacterial isolate of isolate FDA460 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)**

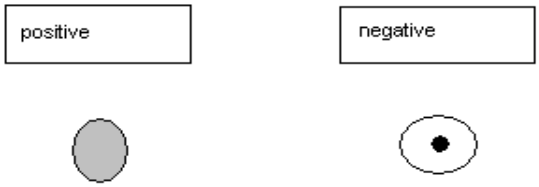
- 50µl anti-Fim 3 at the right concentration are distributed in wells of columns 3, 4, 9 & 10 of rows A to H depending on the number of samples.

**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 a 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 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 not typeable or repeat the test.
- Agglutinations are easier to read after incubation at 35-36°C.




** 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 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 and 2,3.

**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 10µl drop of saline and one drop each of anti-fim 2 antibody and anti-fim 3 antibody on the slide. (i.e. 3 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)
Isolate S3 (Fim3) (can be purchased from NIBSC)
Normal sheep sera (NSS)
Phosphate-buffered saline (PBS)
06/128 monoclonal mouse antibodies (anti-Fim3), NIBSC
06/124 monoclonal mouse antibodies (anti-Fim2), NIBSC
Anti-mouse conjugate
Substrate (phosphatase or other, depending on the conjugate of choice)
Optical density (OD) reader
Charcoal agar plates

**Preparation of bacterial suspension**
Bacterial suspension for coating:

- Grow bacteria for 48-72 h on a charcoal agar plate

- Suspend bacterial growth in PBS
- Dilute in PBS to give an OD of 0,1 at 620nm for Fim2 and Fim3 isolates
- Heat inactivate the suspension at 56°C for 1 hour
- Store at +4°C until testing

**ELISA protocol**

Day 1

1. **Coating** (2 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 in the end of the protocol)

Day 2

1. Start with 3x wash 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 1 h + 37 °C**
   - Add 1% NSS-PBS to all wells
   - (0.5 ml Lamb serum + 49.5 ml PBS)

3. After 1h, wash 3 x with NaCl-0,05% Tween 200 µl / well

4. **Monoclonal antibodies 100 µl / well 2 h + 37 °C**
   - Monoclonal antibodies 06/124 (Fim2) and 06/128 (Fim3) diluted in 1% NSS-PBS
   - Dilution 1:1000

5. After 2h, wash 3 x with NaCl-0,05% Tween 200 µl / well

**6. Anti-Mouse Conjugate 100 µl / well 2 h + 37 °C**
   - Anti-mouse conjugate diluted in 1% NSS-PBS buffer
   - Dilution 1:1000

7. After 2h, wash 3 x with NaCl-0,05% Tween 200 µl / well

**8. Substrate 100 µl / well 30min/1h in RT**
   - Phosphatase substrate or similar diluted in Diethanolamine-MgCl2-buffer (or the one recommended for your substrate)
   - 5mg / 5ml buffer = 1 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 405nm.

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

**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 colonization 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* - because of 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 1, select standard purification as supplied my 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 1. 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 | | gtggggaaacaacgacaa | | X |
| | PTS1R2 | | aga gcg tct tgc ggt cga tc | X | |
| | ptxS1-seqR | | gtgtaggggttgggattg | | 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 | | tcatcaccggcaccatca | | X |
| | fim2-R | | ggg ggg ttg gcg att tcc agt ttc tc | X | |
| | fim2-seqR | | gttctttttgacgtaggagg | | X |
| *fim3* | fim3-F | 713 | gac ctg ata ttc tga tgc cg | X | |
| | fim3-seqF | | caccctcaaccatatcaa | | X |
| | fim3-R | | cgc aag gct tgc cgg ttt ttt ttg g | X | |
| | fim3-seqR | | tcttgctgccattggtga | | 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 5 minutes, and centrifuged for 2 minutes at maximum speed in a micro centrifuge.
- 1µl of the lysate supernatant is then used for PCR.
- Lysates of *B. pertussis* can be stored and used for up to 2 years at 4°C.

**Using DNA:**

- 5-10ng 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

**Instruments used:**

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

## Method

General PCR setup

- Use filter tips to minimize PCR product contamination. Further precautions are not necessary because abundant target DNA (cells) are used in the PCR reaction.
- Dilute primer stocks to 10pmol/µl.
- Prepare PCR mix for the amount of samples desired + 2 (See Table 2).
- Add 1µl DNA or lysate dilution to the bottom of the reaction tubes.
- Add 19µl of the corresponding PCR mix (see Table 2).
- Prepare extra sample mix for use as a negative control. Use at least 2 extra reactions without DNA or lysate.

**Table 2. 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 3 and Table 4

**Table 3. PCR program**

| 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 4. *ptx*S1 touch down PCR**

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

**Sequencing**

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

**Table 5. 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 6. PCR program 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 on gel according to Table 1.
- 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. Protocol for Antigen Expression ELISA method and sequencing primers

The ELISA protocol has been described previously by Barkoff et al. (149)

**Materials**

96-well plate (polystyrene, high binding), plate is sealed during the incubation

Plate cover

PRN-negative isolate, OD = 0.1

FHA-negative isolate, OD = 0.1

PT-negative isolate, OD = 0.2

Monoclonal antibodies (see Table 1 below)

Normal sheep sera (NSS)

Phosphate-buffered saline (PBS)

Anti-mouse conjugate

Substrate (phosphatase or other, depending on the conjugate of choice)

Optical density (OD) reader

Charcoal agar plates

**Table 1. Monoclonal mouse antibodies**

| Antigen | mAb | Target | Source |
|---|---|---|---|
| **PT** | 99/512*<br>99/542* | S1 subunit<br>S3 subunit | NIBSC |
| **FHA** | 99/572 | NK** | NIBSC |
| **PRN** | PeM4<br>(97/558)*** | Region I of *prn* gene<br>(Polyclonal) | RIVM<br>(NIBSC) |

** A mixture of the two monoclonal antibodies is used*
*** Not known*
**** Can cause higher background*

**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% CO2)
- Suspend bacterial growth in PBS
- Dilute in PBS to give an OD of 0.1 at 620nm for FHA and PRN isolates
- Dilute in PBS to give an OD of 0.2 at 620nm for PT isolates
- Heat inactivate the suspension at 56 °C for 1 hour
- Store at +4°C until testing

**ELISA PROTOCOL**

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

Day 1 1. **Coating** (2 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 620nm)
- 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 in the end of protocol)

Day 2 1. Wash 3 x with NaCl-0,05% Tween 200 µl/well

- (NaCl-0,05% Tween: 0,5ml Tween 20 + 1000 ml 0,9% NaCl)

**2. Blocking 150µl / well 1h + 37°C**

- Add 1% NSS-PBS to all wells
- (0.5ml Lamb serum + 49.5ml PBS)

3. Wash 3 x with NaCl-0,05 % Tween 200µl / well

4. **Monoclonal antibodies 100µl / well 2h + 37°C**

- Monoclonal antibodies PeM4 (PRN), 99/572 (FHA), 99/512 (anti-PT S1) & 99/542 (anti-PT S3), 06/124 (Fim2) and 06/128 (Fim3) diluted in 1% NSS-PBS
- Dilution 1:1000

5. Wash 3 x with NaCl-0,05 % Tween 200µl / well

**6. Anti-Mouse Conjugate 100µl / well 2h + 37°C**

- Anti-mouse conjugate diluted buffer 1 % NSS-PBS
- Dilution 1:1000

7. Wash 3 x with NaCl-0,05% Tween 200µl / well

**8. Substrate 100µl / well 30min/1h in RT**

- Phosphatase substrate diluted in Diethanolamine-MgCl2-buffer (or according to conjugate recommendations)
- 5mg / 5ml buffer = 1 tablet/ 5ml buffer (sensitive to light, cover the plate with aluminium foil after addition of substrate)

**9. STOP-solution** 100µl / well

- 3M NaOH is added to wells with substrate to stop the reaction (or the one recommended for your substrate)
- After 60min for all antigens (PT, FHA, PRN)
- Measure absorbance at wavelength 405nm.

10. Analysing the results:

- compare sample values to negative control to identify negative isolates (confirm with sequencing).

**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 (2019) [115]. Here we present a table of primers, which can be used for sequence purposes. Note that these are suggestions and other in-house primers can be used as well.

**Table 2. 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 | GCTCAAAGCAGGAAAAAGCA | *prn* Promoter (+seq) (1097635-1097654) |
| PrnProm REV | CGCTTACCTTGATGGTGGTT | *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 | GACAGCGGTTTCTACCTGGA | *prn* gene, sequencing (1100269-1100288) |

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

The MIC protocol has been described previously by Lönnqvist et al. (2018) [127].

**Materials**

Etest® Erythromycin (BioMérieux)

Etest® Azithromycin (BioMérieux)

Densitometer

Petri Dish Rotator

Regan-Lowe Agar

**MIC protocol**

1. Culture *B. pertussis* on Regan-Lowe agar (charcoal blood agar) 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 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 sterilized 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 down the Etest on the plate and remove any air bubbles by pressing the Etest gently with the tweezers. Let it rest 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 1).
7. Read the plate after 48 hours and 72 hours. For pertussis, the growth is slow, so usually incubation times of 72 hours is needed.
8. Results from sensitive and resistant isolates are presented in Figures 1 and 2, respectively. NB! There is currently no approved MIC from the EUCAST.

*Figure 1. Erythromycin Etest on macrolide-sensitive control isolate ATCC 9797. The arrow shows the edge of the inhibition zone that determines the MIC.*

*Figure 2. Erythromycin Etest on macrolide-resistant control isolate NAP-12-30 (China). No inhibition zone can be detected.*

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