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REVIEW

What to do and what not to do in serological diagnosis of pertussis: recommendations from EU reference laboratories

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Abstract *Bordetella pertussis*-specific antibodies can be detected by enzyme-linked immunosorbent assays (ELISAs) or multiplex immunoassays. Assays use purified or mixed antigens, and only pertussis toxin (PT) is specific for *B. pertussis*. The interpretation of results can be based on dual-sample or single-sample serology using one or two cut-offs. The EU Pertstrain group recommends that: (i) ELISAs and multiplex immunoassays should use purified non-detoxified PT as an antigen, that they should have a broad linear range and that they should express results quantitatively in International Units per millilitre (IU/ml); (ii) a single or dual diagnostic cut-off for single-serum serology using IgG-anti-PT between 50 and 120 IU/ml should be used, and diagnostic serology cannot be validly interpreted for one year after vaccination with acellular pertussis (aP) vaccines; (iii) IgA-anti-PT should only be used with indeterminate IgG-anti-PT levels or when a second sample cannot be obtained. This group discourages using: (i) other antigens in routine diagnostics, as they are not specific; (ii) micro-agglutination, due to its lack of sensitivity; (iii) immunoblots for pertussis serodiagnosis, as results cannot be quantified; (iv) other methods, such as complement fixation or indirect immunofluorescence, due to their low sensitivity and/or specificity.

Members of the EU Pertstrain group are listed in the appendix.

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Indications for pertussis diagnostics

The diagnosis of pertussis should only be attempted in patients with symptoms compatible with pertussis, such as prolonged coughing with paroxysms and/or whooping or choking. In infants, older vaccinated children, adolescents and adults, the clinical course may not be typical, and prolonged coughing may be the only symptom. In these cases, the diagnosis of pertussis requires laboratory methods for confirmation.

Direct and indirect tests are available. Direct tests are real-time polymerase chain reaction (PCR) and culture, whereas indirect tests measure specific antibodies in oral fluid or sera. Here, we focus on the detection of antibodies against *Bordetella pertussis* antigens.

Development of the recommendations

The EU Pertstrain group consists of representatives of the *Bordetella* reference laboratories in their respective EU countries. In a face-to-face meeting at the Istituto Superiore di Sanità (ISS), Rome, Italy, in 2009, the outline of a manuscript was discussed and agreed upon. After a search of relevant databases, a first draft was written (NG) and intensively reviewed by members of the group. In a second face-to-face meeting, at the Rijksinstituut voor

Volksgezondheid en Milieu (RIVM), Bilthoven, the Netherlands, in June 2010, the draft recommendations were broadly discussed and agreed upon. A second version of the recommendations was drafted (NG, CHWvK). This version was then reviewed again by all authors.

Serological tests

Blood/serum

Most serological assays are validated to test serum; some may also be validated to test heparinised plasma or ethylenediaminetetraacetic acid (EDTA) plasma. Capillary blood samples may be used if a sufficient volume cannot be obtained otherwise. Serum or plasma must be separated as soon as possible after blood sampling (24 h at room temperature). If acute and convalescent serum samples taken at least three weeks apart from one individual are available, they should be tested together in one run. All serum samples may be frozen (at −20°C) after the primary assay and re-analysed later together with a possible second sample.

ELISA in serum samples

In preparation for the acellular vaccine studies in the 1990s, the enzyme-linked immunosorbent assay (ELISA) methodology, the type of antigens, as well as the reference sera have been standardised, and they have been used in all acellular vaccine trials, in sero-epidemiological studies in many countries and for diagnostic purposes in various laboratories [1–5].

Antigens

ELISA is normally done with highly purified antigens. The antigens most frequently used are pertussis toxin (PT) and filamentous haemagglutinin (FHA), and to a lesser extent, pertactin (PRN) and fimbriae (FIM). Sometimes, adenylate cyclase-haemolysin toxin (ACT) is also used. These antigens are used in their active, i.e. non-detoxified, form. The storage conditions of the antigens as well as the duration of storage can vary significantly according to the manufacturers who provide the purified antigens. Anti-PT antibodies are specific for *B. pertussis*, whereas anti-FHA, anti-PRN, anti-FIM and anti-ACT are less specific due to cross-reactivity with other microbial antigens (e.g. other *Bordetella* species, *Haemophilus* species, *Mycoplasma pneumoniae*, *Escherichia coli*). For this reason, in routine diagnosis, only the measurement of anti-PT antibodies is recommended. However, the measurement of other antibodies may be used for transmission, immunogenicity or vaccine non-inferiority studies. Some ELISAs, especially commercial kits, use a mixture of antigens or supernatants or sonicates of whole *B. pertussis* cells. The use of kits with mixed antigens is not recommended [6].

Reference sera

World Health Organization (WHO) references are available from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK) ("WHO International Standard (06/140)" and "WHO Reference Reagent (06/142)") for the measurement of human antibodies to *B. pertussis* antigens, and, thus, quantitative results should be reported in IU/ml [7].

Plates

For in-house ELISAs, differences between plates were observed, and Thermo Scientific Nunc MaxiSorp (Nunc AS, Copenhagen, Denmark) plates and Greiner MICROLON High Binding (Greiner Bio-One, Frickenhausen, Germany) plates seemed to work better than others [8].

Flow cytometry

Bead-based multiplex assays that simultaneously measure antibodies to various antigens have also been applied to *B. pertussis* antigens. The assays use fluorescent microparticles to which purified antigens are covalently coupled and are based on a proprietary method patented in 1983 in Germany [9] or on xMAP Technology from Luminex™ [10]. These assays seem to produce results comparable to conventional ELISA systems.

Immunoblots, line and dot blots

Many commercial kits detect anti-*B. pertussis* and/or anti-PT, anti-FHA, anti-PRN and also ACT antibodies in serums using blotting techniques. Kits using whole-cell suspensions as antigens are not specific for the detection/diagnosis of pertussis infections due to the great cross-reactivity with other bacteria (e.g. other *Bordetella* species, *Haemophilus* species, *Mycoplasma pneumoniae*). Antigens used in these kits have to be validated concerning their purity, as contamination with other antigens or antigen aggregates can also produce false-positive results. A quantitative interpretation of blot results is not possible, but a semi-quantitative reporting may be possible. Although various CE-marked commercial kits are available, a recent comparison showed significant differences between kits and an insufficient correlation to the values of the 1st International Reference Preparation (Kennerkuecht et al. submitted). Consequently, the use of immunoblots for pertussis serology is not recommended by the EU Pertstrain group.

Micro-agglutination

Micro-agglutination has been widely used before ELISA systems became available [11]. As with other bacterial agglutination assays, micro-agglutination with *B. pertussis* cells mainly measures IgM antibodies to outer surface antigens such as FIM, PRN and lipooligosaccharide (LOS). Results are reported in titres starting at 1:20 or 1:40. No agglutinin titre has been attributed to the WHO reference preparation so far. Agglutinating antibodies can be used to measure the exposure of a population to *B. pertussis* antigens; on an individual level, the micro-agglutination method is not useful for confirming the clinical diagnosis of pertussis.

CHO cell neutralisation

This test used the ability of PT to induce characteristic morphological changes in a culture of Chinese hamster ovary (CHO) cells. These changes can be neutralised by the presence of anti-PT antibodies. The titres of CHO cell-neutralising antibodies correlate well with the IgG-anti-PT ELISA values, and, thus, CHO cell assays are rarely used for diagnostic purposes [8, 12]. The interpretation of CHO assays cannot be standardised, and it is difficult to interpret the morphological changes and to score the results objectively.

Complement fixation

Complement fixation using whole cells of *B. pertussis* has been rarely used and suffers from a lack of sensitivity and specificity.

Indirect immunofluorescence

Indirect immunofluorescence using whole cells of *B. pertussis* has the same disadvantages as complement fixation

Oral fluid

Oral fluid can be sampled by standardised means, e.g. Oracol swabs (Malvern Medical Developments) or OraSure oral specimen collection devices (OraSure Technologies Inc.). The diagnostic sensitivity of measuring antibodies in oral fluid samples is lower (~80%) as compared to serum samples, but the samples can easily be obtained and offer an adjunct to diagnostic serology

ELISA in oral fluid

An IgG-capture ELISA capable of detecting anti-PT IgG in oral fluid has been developed. The assay was evaluated by comparison to a serum ELISA. The results showed that the oral fluid assay detected seropositive subjects with a sensitivity of 79.7% [95% confidence interval (CI) 68.3–88.4] and a specificity of 96.6% (95% CI 91.5–99.1) relative to the ELISA [13]. This assay has since been modified to include a monoclonal capture antibody; however, no commercial assays for measuring antibodies in oral fluid are available.

Reporting of serological results using ELISA or flow cytometry

Concentrations of antibodies to *B. pertussis* antigens should be quantitatively expressed in IU/ml as reference preparations are available [7]. The reference preparation defines values for antibodies of isotypes IgG and IgA to PT, FHA, PRN and to FIM. Assigned values of the reference preparation are described in Table 1. The numerical values of IU/ml are equivalent to the previously used ELISA units/ml (EU/ml) derived from the human reference preparations lot 3, lot 4 and lot 5 from the Center for Biologics Evaluation and Research/Food and Drug Administration (CBER/FDA), Bethesda, MD, USA.

Interpretation of serological results

PT and FHA are contained in substantial amounts in all acellular vaccines licensed in Europe (except in Denmark), and PRN and FIM are also components of licensed acellular pertussis vaccines. Thus, the immune response against infection or vaccination cannot be distinguished and pertussis vaccination may interfere with the interpretation of serological results. Due to a continuous circulation of *Bordetellae* in the population, IgG-anti-PT and other antibodies to *Bordetella* antigens are detectable in the majority of all adolescent and adult populations tested so far [14]. Thus, serological diagnosis of pertussis must be performed in immunological non-naïve populations with different kinetics of antibody production.

Additionally, pertussis serology suffers from various other drawbacks:

- Commercial ELISAs are of different antigen composition and quality
- Reference antigens with good purity are not easily available
- Population-based cut-offs for single-sample serology may need verification after changes in the vaccination schedule
- Interpretation of anti-PT concentrations in recently vaccinated persons is difficult

Dual-sample serology based on ≥100% increase in antibody concentration or on ≥50% decrease in antibody concentration is a sensitive and specific method for serological diagnosis [1, 4]. However, even in paired sera, no antibody increase may be seen after infection due to the secondary immune response, and the diagnosis may also be

Table 1 Reference preparations from the World Health Organization (WHO) and the Center for Biologics Evaluation and Research/Food and Drug Administration (CBER/FDA). Values are given in IU/ml for the WHO preparations and in ELISA units (EU)/ml for the CBER/FDA preparations; IU/ml and EU/ml are numerically identical [7]

	IgG-anti-PT	IgA-anti-PT	IgG-anti-FHA	IgA-anti-FHA	IgG-anti-PRN	IgA-anti-PRN
WHO international standard (06/140)	335 IU/ml	65 IU/ml	130 IU/ml	65 IU/ml	65 IU/ml	42 IU/ml
WHO reference reagent (06/142)	106 IU/ml	18 IU/ml	122 IU/ml	86 IU/ml	39 IU/ml	38 IU/ml
CBER/FDA #3	200 EU/ml	15 EU/ml	200 EU/ml	100 EU/ml	ND	ND
CBER/FDA #5	ND	140 EU/ml	ND	280 EU/ml	ND	90 EU/ml
CBER/FDA #4	ND	ND	ND	ND	90 EU/ml	25 EU/ml

ND, not declared

based on a decrease of antibodies, which may be too slow to reach 50% between the acute and convalescent sample.

In clinical practice, diagnosis is mostly based on single-sample serology using a single or a more continuous cut-off. For single-sample serology, various cut-off values for IgG-anti-PT have been proposed (Table 2). In Massachusetts, 100 EU/ml was used, and, later, in order to increase specificity, >~200 EU/ml IgG-anti-PT (>20 μg/ml) was employed. In the Netherlands, a cut-off >~125 EU/ml IgG-anti-PT with higher specificity or 62 EU/ml with slightly lower specificity has been used [15, 16]. In Germany, a cut-off of ~40 EU/ml IgG-anti-PT was suggested, together with ~50 EU/ml IgA-anti-FHA. A sero-epidemiological survey was recently performed in various countries in Western Europe using a cut-off of 125 EU/ml for recent infection, and it can be used as a population-based reference [14]. Another recently completed sero-surveillance in US sera found that three populations could be separated according to their levels of IgG-anti-PT: one cut-off was estimated at 94 EU/ml and a lower cut-off was estimated at 48 EU/ml [17]. Using the lower 50 EU/ml cut-off may be especially useful in outbreak situations, and, overall, this cut-off of 50 EU/ml seems to be more appropriate for diagnosis rather than a higher cut-off with lower sensitivity [18].

Overall, it is astonishing that, irrespective of various vaccination strategies, cut-offs for single-sample serology are comparable throughout the countries in which they were evaluated.

A comparison of receiver operating characteristic (ROC) curve analyses with data from Denmark, the Netherlands and the UK showed that, for all three countries, the single cut-off with optimal sensitivity and specificity may be in the range between 60 and 75 IU/ml.

It may be sensible to use a dual cut-off between 62 and 125 IU/ml according to countries as a proof of a recent infection with *B. pertussis*, provided that the patient was not vaccinated during the last 12 months (Table 2). If diagnosis cannot be established with certainty from a single serum, but is deemed to be necessary according to the clinical symptoms, antibodies should be measured in a second (convalescent) serum sample at two to four weeks interval. In case of non-availability of a second serum sample, measurement of IgA anti-PT antibodies can be an alternative, but no broadly accepted cut-off is available for this antibody subclass. Considering its relatively high specificity and low sensitivity, a cut-off near the minimal level of quantification, which may be between 10 and 20 IU/ml, may seem reasonable [19].

Recommendations for serological testing with suspected pertussis

In neonates and young infants, PCR and/or culture should be performed on nasopharyngeal samples, nasopharyngeal swabs (NPSs) or nasopharyngeal aspirates (NPAs) as soon as possible post-onset of symptoms. The measurement of

Table 2 Suggested cut-off values for IgG-anti-PT for adolescents and adults. Adapted from WHO "The Immunological Basis for Immunization Series" (available online at: http://whqlibdoc.who.int/publications/2010/9789241599337_eng.pdf)

Country	Type of study	Cut-off	Sensitivity	Specificity	Reference
MA, USA	Population study	~100 IU/ml	78%	98%	[20]
MA, USA	Population study	~200 IU/ml	67%	99.9%	[21]
NL	Population study	125 IU/ml 62 IU/ml	70% 80%	99% 95%	[15]
D	Population study	40 IU/ml	80%	95%	[22]
EU	Epidemiological survey	125 IU/ml	n.a.	n.a.	[14]
USA	Epidemiological survey, model	94 IU/ml 46 IU/ml	n.a. n.a.	n.a.	[17]
AUS	Clinical validation	50 IU/ml	Better than 100 IU/ml		[18]

MA, Massachusetts

n.a., not applicable

IgG-anti-PT is only meaningful for older children/adults, including parents and other household members. In vaccinated children, adolescents and adults with less than two weeks of coughing culture and PCR from nasopharyngeal samples should be done. For adolescents and adults with coughing of less than three weeks, PCR and the measurement of IgG-anti-PT should be performed [1]. If coughing lasted at least 2 to 3 weeks, the measurement of IgG-anti-PT should be sufficient. In outbreak situations, PCR and culture should be performed from nasopharyngeal samples and IgG-anti-PT should be measured in serum samples.

Appendix

Table 3 shows the members of the EU Pertstrain group.

Table 3 List of participants of the EU Pertstrain network

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