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

Nicole Guiso<sup>1</sup>, Guy Berbers<sup>2</sup>, Norman K. Fry<sup>3</sup>, Qiushiu He<sup>4</sup>, Marion Riffelmann<sup>5</sup>, Carl Heinz Wirsing von König<sup>5</sup> for the EU Pertstrain group\*

(\*Members of the EU Pertstrain group are listed in the appendix)

- 1: Institut Pasteur, Paris, France
- 2: National Institute for Public Health and the Environment, Bilthoven, the Netherlands
- 3: Health Protection Agency Centre for Infections, London, UK
- 4: National Institute for Health and Welfare, Turku, Finland
- 5: HELIOS Klinikum Krefeld, Germany

§ Corresponding author:  
Prof. Dr. Carl Heinz Wirsing von König  
Institut für Hygiene und Labormedizin  
HELIOS Klinikum Krefeld  
Lutherplatz 40  
D-47805 Krefeld, Germany  
Phone +49 2 151 32 24 67  
Fax: +49 2 151 32 20 79  
e-mail: [carlheinz.wirsingvonkoenig@helios-kliniken.de](mailto:carlheinz.wirsingvonkoenig@helios-kliniken.de)

## **Abstract**

*Bordetella pertussis* specific antibodies can be detected by ELISA or multiplexed immunoassays. Assays use purified or mixed antigens, and only pertussis toxin (PT) is specific for *Bordetella 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 multiplexed 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 IU/ml and 120 IU/ml should be used, and diagnostic serology cannot validly be 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 and (iv) other methods, such as complement fixation or indirect immunofluorescence, due to their low sensitivity and/or specificity

Word count: 195

## **Indications for pertussis diagnostics**

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, diagnosis of pertussis requires laboratory methods for confirmation.

Direct and indirect tests are available. Direct tests are real-time PCR and culture whereas indirect tests measure specific antibodies in oral fluid or sera. Here, we focus on the detection of antibodies against *B. 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 in the Istituto Superiore di Sanita (ISS) in Rome 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 in the Rijksinstituut voor Volksgezondheid en Milieu (RIVM) 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 EDTA-plasma. Capillary blood samples may be used if a sufficient volume can not be obtained otherwise. Serum or plasma must be separated as soon as possible after blood sampling (24h 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 reanalyzed later together with a possible second sample.

**ELISA in serum samples:** In preparation for the acellular vaccine studies in the 1990s, the ELISA methodology, the type of antigens as well as the reference sera have been standardized, 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 PT, and FHA, and to a lesser extent Pertactin (PRN) and Fimbriae (FIM). Sometimes Adenylate Cyclase-hemolysin 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 (eg. other *Bordetella* species, *Haemophilus* species, *Mycoplasma pneumoniae*, *Escherichia coli*). For this reason, in routine diagnosis, only measurement of anti-PT antibodies is recommended. However, 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 (Riffelmann et al. submitted).

**Reference sera:** WHO references are available from the 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 IU/ml [6].

**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 [7].

**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 [8] or on xMAP technology from Luminex™ [9]. 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 a lot of cross-reactivity with other bacteria (eg. other *Bordetella* species, *Haemophilus species*, *Mycoplasma pneumoniae*). Antigens used in these kits have to be validated concerning their purity, as contaminations with other antigens or antigen aggregates can also produce false positive results. A quantitative interpretation of blot results is not possible; 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 1<sup>st</sup> International Reference preparation (Riffelmann 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 [10]. 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 neutralization:** 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 neutralized by the presence of anti-PT antibodies. The titres of CHO-cell neutralizing antibodies correlate well with the IgG-anti-PT ELISA values, and thus CHO cell assays are rarely used for diagnostic purposes [7,11]. The interpretation of CHO assays cannot be standardized, 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 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 standardized means, e.g.. Oracol swab (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 [12]. 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 [6]. 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 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 [13]. 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  $\geq 100\%$  increase in antibody concentration or on  $\geq 100\%$  decrease in antibody concentration is a sensitive and specific way 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 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 cutoff-values for IgG-anti-PT have been proposed (Table 2): In Massachusetts, 100 EU/ml was used, and later, in order to increase specificity  $> \sim 200$  EU/ml IgG-anti-PT ( $> 20 \mu\text{g/ml}$ ) was employed. In the Netherlands, a cutoff  $> \sim 125$  EU/ml IgG-anti-PT with higher specificity or 62 EU/ml with slightly lower specificity has been used [14,15]. In Germany, a cut-off of  $\sim 50$  EU/ml IgG-anti-PT was suggested, together with  $\sim 50$  EU/ml IgA-anti-FHA. A seroepidemiological 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 [13]. 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 [16]. 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 adapt for diagnosis rather than a higher cut-off with lower sensitivity [17].

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

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

It may be sensible to use dual a cut-off between 62-125IU/ml according to countries as a proof of a recent infection with *B. pertussis* provided that the patient was not vaccinated during the last twelve 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 IU/ml and 20 IU/ml may seem reasonable [18].

### **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 aspirate (NPAs), as soon as possible post-onset of symptoms. Measurement of 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 less than three weeks PCR and measurement of IgG-anti-PT should be performed [1]. If coughing lasted at least 2-3 weeks, measurement of IgG-anti-PT should be sufficient. In outbreak situations, PCR and culture should be done from nasopharyngeal samples and IgG-anti-PT should be measured in serum samples.

Word count: 1998

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**Table 1: Reference preparations from WHO and 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 [##]

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

ND: not declared

**Table 2 Suggested cut-off values for IgG-anti-PT for adolescents and adults**

Adapted from WHO Immunological basis for vaccination series ([http://whqlibdoc.who.int/publications/2010/9789241599337\\_eng.pdf](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%	[19]
MA, USA	Population study	~200 IU/ml	67%	99.9%	[20]
NL	Population study	125 IU/ml	70%	99%	[21]
		62 IU/ml	80%	95%	
D	Population study	50 IU/ml	80%	95%	[22]
EU	Epid. survey	125 IU/ml	n.a.	n.a.	[13]
USA	Epid. survey, model	94 IU/ml	n.a.	n.a.	[16]
		46 IU/ml	n.a.		
AUS	Clinical validation	50 IU/ml	better than 100 IU/ml		[17]

MA, Massachusetts.

n.a., not applicable.

## Appendix

### List of participants of the EUpertstrain network

Members	Email address	Name of organization	Town, Country
Qiushui He and Jussi Mertsola	<a href="mailto:qiushui.he@utu.fi">qiushui.he@utu.fi</a> ; <a href="mailto:Jussi.Mertsola@utu.fi">Jussi.Mertsola@utu.fi</a>	National Institute for Health and Welfare University of Turku	Turku, Finland
Carl Heinz Wirsing von König and Marion Riffelmann	<a href="mailto:carlheinz.wirsingvonkoenig@helios-kliniken.de">carlheinz.wirsingvonkoenig@helios- kliniken.de</a> ; <a href="mailto:marion.riffelmann@helios-&lt;br/&gt;kliniken.de">marion.riffelmann@helios- kliniken.de</a>	HELIOS Klinikum Krefeld	Krefeld, Germany
Nicole Guiso and Sophie Guillot	<a href="mailto:nicole.guiso@pasteur.fr">nicole.guiso@pasteur.fr</a> <a href="mailto:sophie.guillot@pasteur.fr">sophie.guillot@pasteur.fr</a>	Institut Pasteur	Paris, France
Hans Hallander and Abdolreza Advani	<a href="mailto:hans.hallander@smi.se">hans.hallander@smi.se</a> <a href="mailto:reza.advani@smil.se">reza.advani@smil.se</a>	Swedish Institute for Infectious Disease Control	Solna, Sweden
Frits R Mooi and Guy Berbers	<a href="mailto:frits.mooi@rivm.nl">frits.mooi@rivm.nl</a> <a href="mailto:guy.berbers@rivm.nl">guy.berbers@rivm.nl</a>	National Institute of Public Health and the Environment	Bilthoven, the Netherlands
Anna Lutyńska	<a href="mailto:agzyl@pzh.gov.pl">agzyl@pzh.gov.pl</a>	National Institute of Hygiene	Warsaw, Poland
Karen Krogfelt and Tine Dalby	<a href="mailto:kak@ssi.dk">kak@ssi.dk</a> ; <a href="mailto:tid@ssi.dk">tid@ssi.dk</a>	Statens Serum Institut	Copenhagen, Denmark
Norman Fry	<a href="mailto:norman.fry@hpa.org.uk">norman.fry@hpa.org.uk</a>	Health Protection Agency	London, UK
Dorothy Xing	<a href="mailto:dxing@nibsc.ac.uk">dxing@nibsc.ac.uk</a>	National Institute for Biological Standards and Control	Potters Bar, UK
Camille Locht and David Hot	<a href="mailto:camille.locht@pasteur-lille.fr">camille.locht@pasteur-lille.fr</a> <a href="mailto:david.hot@pasteur-lille.fr">david.hot@pasteur-lille.fr</a>	Institut Pasteur de Lille	Lille, France
Clara Ausiello	<a href="mailto:ausiello@iss.it">ausiello@iss.it</a>	Istituto Superiore di Sanita	Rome, Italy
Per Sandven and Jann Storsaeter	<a href="mailto:per.sandven@fhi.no">per.sandven@fhi.no</a> <a href="mailto:jann.stosaeter@fhi.no">jann.stosaeter@fhi.no</a>	Norwegian Institute of Public Health	Oslo, Norway