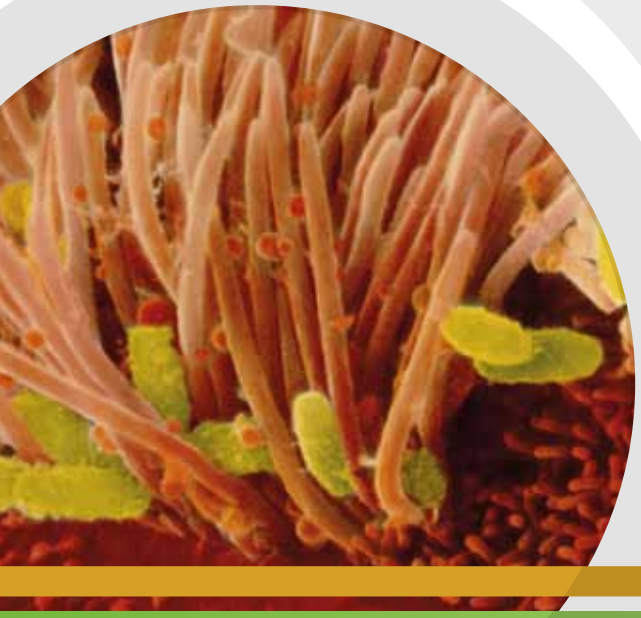

TECHNICAL REPORT

External quality assurance scheme for *Bordetella pertussis* serology

as part of the EUpert-Labnet surveillance network

2013

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ECDC TECHNICAL REPORT

External quality assurance scheme for *Bordetella pertussis* serology 2013

As part of the EUpert-Labnet surveillance network

This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Dr Assimoula Economopoulou and produced by Dr Norman Fry, Health Protection Agency (HPA) (London, UK), Dr Kevin Markey, Dr Dorothy Xing, National Institute for Biological Standards and Control (NIBSC) (Potters Bar, UK) and Dr Qiushui He, National Institute for Health and Welfare (THL) (Turku, Finland), on behalf of the EUpert-Labnet consortium as part of the coordination of activities for laboratory surveillance of whooping cough in Member States/EEA countries (referring to Specific Contract ECDC/2011/013).

From 1 April 2013, the HPA's functions were transferred to Public Health England. The National Institute for Biological Standards and Control (NIBSC), previously part of the HPA, is now a new centre of the Medicines and Healthcare Products Regulatory Agency (MRHA).

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Abbreviations

DSN	Dedicated surveillance network
ECDC	European Centre for Disease Prevention and Control
EDQM	European Directorate for the Quality of Medicines and Healthcare
EEA	European Economic Area
ELISA	Enzyme-linked immunosorbent assay
EQA	External quality assurance
EUpert-Labnet	Consortium of European pertussis experts funded by ECDC for this programme
EUVAC.NET	A former European surveillance network for selected vaccine-preventable diseases hosted at the Statens Serum Institut, Denmark
FHA	Filamentous hemagglutinin
GCV	Geometric coefficient of variation
GM	Geometric mean
HPA	Health Protection Agency (UK)
IgG	Immunoglobulin G
IHR	In-house reference serum
IS	International Standard
IU	International Unit
LPS	Lipopolysaccharide
MIA	Multiplex immunoassay
NIBSC	National Institute for Biological Standards and Control (UK)
RSIL	Respiratory and Systemic Infection Laboratory (of the HPA, London)
PT	Pertussis toxin
THL	Terveyden ja Hyvinvoinnin Laitos/National Institute for Health and Welfare (Finland)

Executive summary

As part of the contract with ECDC (Coordination of activities for laboratory surveillance of whooping cough in Member States and EEA countries), an EQA collaborative study was organised during the period July–October 2012. The main aim of the study is to assess the ability of national reference laboratories from EU/EEA Member States to correctly perform laboratory serodiagnostic tests for pertussis. This will make it possible to identify any laboratories producing results which differed significantly from the values obtained by the majority of participants. Furthermore, as a result of the scheme it will be possible to make recommendations for improvements to methodologies and identify areas for training to enable particular laboratories to improve their methods, procedures and global performance. Human plasma containing different concentrations of anti-pertussis toxin (PT) antibodies (IgG) were kindly supplied to NIBSC by Professor Carl Heinz Wirsing von König and Dr Marion Riffelmann of HELIOS Klinikum, Krefeld, Germany. From these a panel of five freeze-dried sera containing different concentrations of anti-PT IgG was prepared, which also included the Pertussis Antiserum (Human) 1st WHO Reference Reagent which has a defined anti-PT IgG concentration of 106 IU/ml. This was also blinded and labelled as Sample D in the present study. The concentrations ranged from undetectable levels of anti-PT IgG to concentrations clinically associated with infection. The panel was blinded and sent to participants who used their own routine diagnostic ELISA assays to determine the anti-PT IgG concentrations. Raw data were then returned to NIBSC for analysis. Twenty-one laboratories from twenty countries agreed to take part in the study. A total of 76 data sets were collected. The majority of the 21 participating laboratories used only one method (either an in-house ELISA or commercial kit) to determine the anti-PT IgG concentration of the sera panel. One laboratory used a multiplex immunoassay. Another laboratory used two different in-house ELISA methods. One laboratory also submitted results obtained from an in-house ELISA and two different commercial kits. In total, nine participants determined the anti-PT IgG concentrations using in-house ELISA methods. Thirteen laboratories provided results using six different commercial kits. Sixteen data sets had a sufficient range of dilutions to enable statistical analysis using a four-parameter logistic model and fifteen of these arranged the samples in the same rank of increasing anti–PT IgG concentration (the data for one laboratory was non-linear for all assays). The geometric mean of all data sets for Sample D was 103.4 IU/ml and close to the expected value of 106 IU/ml, indicating the accuracy of the assays used by most of the participants. However, one laboratory produced results that were significantly higher than the geometric means. This participant and the laboratory that produced non-linear results may require training or to use alternative kits. Participants also used their own in-house ELISAs to calculate the anti-PT IgG concentrations of the samples. Of the 15 assays that returned such calculations 13 ranked the samples in the same order as those using the four-parameter logistic model. One laboratory reversed the two highest samples while the remaining laboratory could not distinguish between two samples and also had a different ranking. This may also indicate that training is required.

This report presents the results of the external quality assurance (EQA) scheme for *Bordetella pertussis* serology funded by the European Centre for Disease Prevention and Control (ECDC).

1. Background

The European Centre for Disease Prevention and Control (ECDC) is a European Union (EU) agency with a mandate to operate the dedicated surveillance networks (DSNs) and to identify, assess and communicate current and emerging threats to human health from communicable diseases. As part of its mission, ECDC shall 'foster the development of sufficient capacity within the Community for the diagnosis, detection, identification and characterisation of infectious agents which may threaten public health. The Centre shall maintain and extend such cooperation and support the implementation of quality assurance schemes.'

In September 2011, the responsibilities of the EUVAC.NET were transferred to ECDC and subsequently a consortium of pertussis experts from nine countries, the EUpert-Labnet network, was established and awarded the Framework Contract 'Coordination of activities for laboratory surveillance of whooping cough in Member States/EEA countries' (ECDC/2011/013). The EUpert-Labnet network is led and coordinated by Dr Qiushui He and Professor Jussi Mertsola (Turku, Finland).

The UK Health Protection Agency's Respiratory and Systemic Infection Laboratory (RSIL) Colindale, UK, and the National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK, were contracted to deliver Work package 2: EQAs for laboratory diagnostics and molecular typing of pertussis. Work package 2 (coordinated by Dr Norman Fry, HPA – Colindale, Dr Dorothy Xing, HPA - NIBSC and Dr Kevin Markey, HPA –NIBSC) comprises three EQAs for PCR, serology, and strain typing. This report details the second of these EQAs.

External quality assurance (EQA) is an element of quality management systems involving the evaluation by an outside agency of laboratory performance on material that is supplied specially for the purpose. ECDC organises a series of EQAs for EU/EEA countries. Non-EU/EEA countries are also involved in the EQA activities organised by ECDC. The aim of the EQA is to identify areas for improvement in laboratory diagnostic capacities relevant to the surveillance of the diseases listed in Decision No. 2119/98/EC 2, and to ensure comparability of results between laboratories from all EU/EEA countries. The main purposes of EQA schemes include:

- assessment of the general standard of performance; and to ensure comparability of results between laboratories from all EU/EEA countries;
- assessment of the effects of analytical procedures (method principle, instruments, reagents, calibration);
- evaluation of individual laboratory performance;
- identification of problem areas;
- provision of continuing education;
- identification of training needs.

Whooping cough (pertussis) is an acute respiratory infection most commonly caused by the bacterium *Bordetella pertussis* and, to a lesser extent, by *B. parapertussis*. Pertussis remains endemic worldwide and is an important public health issue. Infants are most vulnerable to pertussis infection but older children and adults can display milder symptoms and be responsible for transmission. In recent years there has been a marked increase in the number of cases in areas such as the EU and North America where vaccination coverage is high. Laboratory diagnosis of pertussis infection is important for surveillance, treatment and prevention. It is noted that there have been wide variations in the reporting of laboratory-confirmed pertussis cases globally and one of the reasons could be the differences in the methods used for diagnosis which have highlighted the importance of harmonisation and standardisation of diagnostic methods in a successful laboratory-based surveillance system.

Serological analysis by Enzyme-linked Immunosorbent Assays (ELISAs) has been widely used to evaluate antibody responses to pertussis vaccination and infection. As part of the EUVAC.NET contract with ECDC (Pertussis Work Area 4), a collaborative study was organised in July–October 2010 to assess current laboratory performance of serological assays for pertussis, to compare in-house references that were being used and to identify any needs for standardisation of the serological assays (Xing et al 2011). In that study participants were asked to use their regular ELISA methods to differentiate between two well-defined reference preparations with high (335 IU/ml) and low (106 IU/ml) IgG antibodies to pertussis toxin (PT). Seventeen reference laboratories in Europe took part in the study, nine of them using in-house ELISA methods and 10 using commercial kits. It was found that all ELISA methods which used only purified PT as a coating antigen could distinguish between the two preparations, yielding the expected results. Six commercial kits were used in the study and it was found that those applying a mixture of *B. pertussis* antigens to coat the ELISA plates did not produce the expected results. This indicates that antigen coating could be an important factor affecting laboratory performance. It was therefore recommended that only purified PT should be used to coat ELISA plates (Xing et al 2011). Recently the members of the EU Pertstrain group published recommendations for serological diagnosis of pertussis and they also recommended only using non-detoxified purified PT as coating antigen. The assays should have a broad linear range and should express results quantitatively in International Units per millilitre (IU/ml) (Guiso et al 2011). Unfortunately, a subsequent EQA assessment of pertussis serology in Germany that collected data from 183 German laboratories found that only one third (69 laboratories) were following the EU Pertstrain recommendations, while the remaining two thirds still used mixed coating antigens, making it difficult to interpret the data from these laboratories (Riffelmann et al 2012). More recently, there have been reports that manufacturers are starting to adapt their products according to the recommendations (Guiso et al 2011, Riffelmann et al 2012).

2. Introduction

Pertussis remains endemic worldwide and is an important public health issue. In the past few years, there has been a marked increase in reported pertussis cases in countries with high vaccination coverage (Gabutti and Rota, 2012). Laboratory diagnosis of pertussis is important for treatment, prevention and surveillance. It is noted that there have been wide variations in the reporting of laboratory-confirmed pertussis cases globally and one of the reasons could be the differences in the methods used for diagnosis.

External quality assurance is important to ensure accurate diagnosis and good laboratory performance. As part of the new contract with ECDC (Coordination of activities for laboratory surveillance of whooping cough in Member States and EEA countries, Work Package 2), this EQA programme builds upon the previous collaborative studies on serological assay organised by the EUVAC.NET and EU Pertstrain consortia.

The current programme is designed to develop and standardise diagnostic techniques for the identification of pertussis outbreaks, and studying impact of the changes on pertussis vaccines and vaccination schedules on incidence of disease, immunity of the human population and on the bacterial population. Through EQA schemes, the national reference laboratories from each EU/EEA Member States will be assessed on their ability to correctly perform laboratory diagnostic tests and molecular typing for pertussis. Furthermore, as a result of the scheme it will be possible to make recommendations for improvements to methodologies and identify areas for training to enable particular laboratories to improve their methods, procedures and global performance.

The specific aims of this *Bordetella pertussis* serology EQA were:

- to assess the current performance of laboratories participating in the EQA;
- to assess the ability of assays to distinguish a panel of serum samples with different concentrations of anti-PT IgG;
- to assess the possibility of setting up assay validity criteria to standardise serological tests using the International Standard Serum.

3. Materials and methods

3.1. Organisation

The EUpert-Labnet *B. pertussis* serology EQA was organised by the National Institute for Biological Standards and Control (UK) and was intended for national reference laboratories in EU Member States, Iceland, Liechtenstein and Norway. In June 2012, national reference laboratories in Europe currently performing serological assays for the diagnosis of pertussis infection by measuring sera antibody to PT were invited to participate in the study (Annex 1). A total of 21 laboratories from 20 countries participated, 19 from EU countries and one from Norway (Annex 2).

Throughout this report, where assay results are attributed to the various laboratories, each laboratory is identified only by a randomly assigned code number ranking from 1 to 21, so that only they can identify which set of assay results they contributed. Separate experiments have been numbered sequentially within laboratories.

3.2 Selection and preparation of sera panel

All human plasma samples were collected in Germany and kindly donated by Professor Carl-Heinz Wirsing von König and Dr Marion Riffelmann of HELIOS Klinikum, Krefeld, Germany. All procedures for sample collection and preparation were carried out according to the current guidelines of the German Medical Association (Bundesärztekammer) and the standard operating procedures of INSTAND for proficiency testing in infection serology (Müller et al 2012, Müller et al 2006). No clinical information was provided about the 'patients'.

Primary screening was done with IgG-anti-PT in a single dilution protocol. Plasma donations were frozen within a maximum of two hours following plasmapheresis. Whole blood was processed within 16 hours of collection (intermediate storage at +4°C), and the separated plasma was frozen within 4 hours. Samples were stored at -30°C. The frozen plasma samples were transported to NIBSC in April 2012. Before re-calcification the plasma samples were pooled according to IgG-anti-PT antibody content as described below. Each sample contained approximately 250 ml plasma.

Four different groups were defined according to their antibody content: <2 EU/ml, ~22 EU/ml, ~47 EU/ml and ~137 EU/ml IgG-anti-PT respectively.

Serum was prepared following re-calcification. In brief, the plasma was thawed at 4ºC. Samples were pooled in sterile glass beakers, 1 M $CaCl_2$ solution (Sigma-Aldrich, Poole, UK) was added at a concentration of 20 µl $CaCl_2$ per 1 ml of plasma. Samples were then incubated at 37ºC in a water bath for 30 minutes and left overnight at 4ºC. Sera were extracted from the clot by squeezing the clot with a flat surface and decanting the sera which was then centrifuged 3,000 x g for 30 minutes. An Activated Plasma Clotting Time (APCT) test was performed to confirm the absence of any remaining clotting agents.

Each sera sample with no dilution or additions was filled in 0.5 ml aliquots into 3ml glass ampoules and then freeze-dried on a five-day cycle (starting shelf temperature of -50°C), after which the ampoules were back-filled with high-purity nitrogen before sealing. The detailed information on plasma samples and resulting sera panel appear in Table 1. Previously freeze-dried ampoules of Pertussis Antiserum (Human) 1st WHO Reference Reagent (NIBSC 06/142) were blinded and relabelled as Sample D.

The set of materials included in the study contains a panel of five sera preparations with blinded concentrations of anti-pertussis toxin IgG (coded by letter) plus the Pertussis Antiserum (Human) 1st WHO International Standard (06/140) for use as a reference when calculating the anti-PT IgG concentrations in the sample panel.

Table 1. Preparation of the sera panel

Sera panel code	Plasma sample number	Estimation of IgG-PT in plasma sample	Proposed mean IgG-PT in pooled sample	Filling number after freeze-drying	Number of ampoules produced
Sample A	556066	<2	<2	SS-369	900
	556055	<2			
Sample B	556035	22	~22	SS-373	800
	556083	22			
Sample C	556050	43	~47	SS-366	900
	556079	46			
	556065	48			
	556058	50			
Sample D	Relabelled- Pertussis Antiserum (Human) 1st WHO Reference Reagent (106 IU/ml (94-118) of anti-PT IgG)			06/142	n/a
Sample E	556049	128	~137	SS-372	900
	556061	130			
	556041	152			
Pertussis antiserum (human) 1st WHO international standard (335 IU/ml of anti-PT IgG)				06/140	n/a

3.3. Sample shipment

Each participating laboratory received two sets of ampoules consisting of the panel of sera samples of human serum coded by letter together with one ampoule of 06/140. The details of these samples are set out in Table 1.

The panels were prepared, packed according to local regulations, collected by courier on 24 July 2012 from NIBSC South Mimms, Hertfordshire, UK and dispatched to the 21 laboratories (Annex 2). All packages were received in a timely manner, allowing laboratories sufficient time to meet the deadline of Friday 26 October 2012.

3.4. Assay methods

The primary goal of the study was to evaluate the panel of sera preparations provided in terms of IgG-anti-PT concentrations using ELISA.

Serological assays by in-house enzyme-linked immunosorbent assays (ELISAs) were used by nine laboratories in the study and 12 laboratories used commercial ELISA kits. One laboratory (Lab 16) performed the assay using two commercial ELISA kits and an in-house ELISA method. Another (Lab 12) performed two different in-house ELISA methods. Laboratories who performed in-house ELISA used their own methodology, reagents and calculation methods, including their own in-house references and controls. For laboratories that used commercial ELISA kits, all reagents were supplied with the kits. A summary of assay information is given in Annexes 5a and 5b. One participant (Lab 14) performed a multiplex immunoassay (MIA) instead of an ELISA.

3.4.1. Antiserum preparations

It was recommended that ampoules of lyophilised sera be stored at -20°C. A recommendation on volumes for reconstituting each sera sample was also included in the shipment (Annex 4). Samples should be divided into aliquots and stored at -20°C. The aliquots should be used only once as freeze-thawing was not advised. An initial dilution of approximately 1/100 of the reconstituted Pertussis Antiserum (Human) 1st WHO International Standard (06/140) was recommended, with Sample D in the first row for IgG ELISA assays followed by ½ dilutions. For Samples A, B, C and E 1/10 dilutions were suggested in the first row for IgG ELISA assays, followed by ½ dilutions. However, individual laboratories were encouraged to perform a pilot study to choose the suitable dilution for the samples under their own experimental conditions.

3.5. Study design

Participants were asked to complete and return to NIBSC assay sheets detailing their assay procedure/conditions together with the raw data for each assay.

3.5.1. For laboratories using their own in-house ELISA

The participating laboratories were asked to coat ELISA plates using their own in-house sourced PT antigen and perform a minimum of three independent assays on the six serum preparations. Participants were also asked to include their in-house reference serum (IHR) in the assays. The three independent assays were to be run on three different days. On each assay, dilution curves for each preparation should have at least two or three replicates per assay and preferably each dilution curve should include at least four doses in the linear region. All preparations should be included in each assay. As far as possible, each plate should also include the appropriate IHR where applicable.

Laboratories should use their own methodology, reagents and calculation methods, include their in-house references and controls and use assay runs that meet their internal validity criteria.

3.5.2. For laboratories using commercial ELISA kits

The participating laboratories were asked to perform a minimum of three independent assays on the panel of six sera preparations provided in this study using the commercial ELISA kit that is routinely used in their laboratory. They were also asked to include the reference serum provided in the kit in the assay. The three independent assays were to be run on three different days. On each assay, dilution curves for each preparation were to have at least two replicates per assay and preferably include at least four doses in linear region for each dilution curve. Laboratories were to use the methodology and validity criteria recommended by the kit manufacturer.

3.6. Statistical methods

Raw data were returned to NIBSC for analysis, to ensure as far as possible consistent calculation of results. All analysis was performed using EDQM's CombiStats software (version 5.0). For all assays the data for each test preparation were analysed separately against the reference preparation.

Where possible a four-parameter logistic model was used, including the full range of assay responses. If this was not possible, parallel line analysis of assay response against log dose was performed, selecting a linear section of the dose-response curve.

Deviations from the fitted model (non-linearity and non-parallelism) were considered significant at the 1% level and these assays were considered invalid.

All laboratory mean potency estimates shown are weighted or semi-weighted geometric mean estimates calculated according to the methods described in Ph. Eur. General Chapter 5.3. Semi-weighted means have been used where significant heterogeneity of assay estimates was detected ($p<0.1$). Overall mean potencies and confidence intervals are calculated as unweighted geometric mean values. Variability is expressed as a percentage using a geometric coefficient of variation (GCV = $\{10^{s}-1\}\times100\%$ where s is the standard deviation of the log10 transformed potency estimates).

4. Results

4.1. Characterisation of freeze-dried ampoules

The freeze-drying of the samples was successful and each sample generated a homogenous, pale yellow, robust, loose cake, as expected. The mean fill weights for the sera filled for this study ranged from 0.5096 to 0.5150g (CV% 0.45 to1.00), the mean dry weights ranged from 0.0395 to 0.0411g (CV% 1.4 to 2.16), the residual moisture range from 0.26 to 0.89%w/w (CV% 25.99 to 39.60) and the mean oxygen content was from 0.40 to 0.89%w/w (CV% 24.14 to 0.89).

However, following the EQA the anti-PT titers were found to be much lower than proposed in the original plasma samples (Table 1). The levels were in the region of 30% of the expected values for samples B, C and E. Investigations were carried out on the protease levels in the freeze-dried samples using two chromogenic substrates, S2288 a wide-specificity substrate; and S2251, a substrate with specificity for plasmin (and kallikrein), known to cleave antibodies. In addition, Thioflavin T (ThT) was used to detect cross-beta structures associated with protein polymers and aggregates. The results obtained from the sera samples were found to be similar to those in 06/142 for all three tests, indicating that enhanced levels of proteases were not responsible for the lower-than-expected anti-PT titers.

4.2 Summary of data returned

All 21 laboratories completed questionnaires and submitted results by the relevant deadlines. All participants performed either an in-house ELISA, MIA or ELISA with commercial kit with the exception of two laboratories (Laboratories 12 and 16). Laboratory 12 used two different ELISA methods and Laboratory 16 performed an in-house ELISA and also used two different commercial kits to return three datasets. Thus, the total number of datasets presented was 24. Nine laboratories used their own in-house ELISA methods; twelve laboratories used a total of six different commercial kits (Savyon, Virotech, EuroImmun, Novatech, Euro Diagnostica and Virion) and one laboratory used a multiplex immunoassay. Detailed assay information is presented in Annexes5A and 5B. The majority of laboratories that performed in-house ELISA returned assay raw data which enabled recalculation at NIBSC using a common statistical analysis. The exception was Laboratory 21 which did not submit raw data for the reference (06/140). For laboratories that performed assays using commercial kits, six used single point assays and therefore NIBSC could not perform common analysis on these data. Two laboratories (16b and 19) reported results as positive/negative. Laboratory 19 also sent raw data to NIBSC which could be analysed using the common statistical analysis. One participant (Laboratory 17) submitted results that could be analysed using the four-parameter logistic model but all assays were found to be non-linear and therefore the results cannot be calculated and presented here.

4.3. Estimates of sera sample panel

Table 2 presents the statistical analysis of data supplied by participants who used in-house ELISA methods that could be analysed using the four parameter logistic model in NIBSC, with the exception of Laboratory 21 for which in-house calculated results are presented. Sample A was found to be outside the response range of the reference or under detection limits in all assays analysed by NIBSC, with the exception of those presented by Laboratory 21 which used an in-house calculation where the mean estimate was shown to be less than 4 IU/ml.

Table 3 shows results from commercial kits calculated by NIBSC using the four parameter logistic model (five laboratories) and participants' own results as calculated by the remaining seven laboratories that performed assays unsuited to the four parameter logistic model. Overall, all assays correctly identified Sample A as negative – indicated by the fact that this sample gave a non-parallel response/or less than 5 IU/ml. The geometric mean for Sample B was found to be 6.00 IU/ml, Sample C 13.85 IU/ml, Sample D 101.66 IU/ml and Sample E 31.18 IU/ml for the in-house ELISA assays. Slightly higher geometric mean estimates were found for commercial kit assays with Sample B: 7.98 IU/ml, Sample C: 19.76 IU/ml, Sample D 101.67 IU/ml and Sample E 47.85 IU/ml. The geometric mean of Sample D, which is the WHO International Reference Reagent, was found to be close to the expected value of 106 IU/ml, as determined in the collaborative study to establish the reference material (Xing et al 2008). Laboratory 1 produced values that were significantly higher than the geometric means for Samples B, C, D and E.

In general, there was agreement among laboratories using in-house ELISA assays and the between-laboratory GCVs were 20–64% for different samples (Table 2). However, assays using commercial kits showed larger between-laboratory GCVs: 46–77% (Table 3). NIBSC calculated a histogram of geometric means for sera samples B to E from each replicate using the four-parameter logistic model against the IS (06/140) (Figure 1). Variability of assay performance for individual laboratories is presented in Figure 2a for in-house ELISA methods and Figure 2b for commercial kits. In general, variation among assays was found to be participant-dependent. For a few laboratories, a two-to-three fold variation was observed.

Table 2. Results of in-house ELISA calculated by NIBSC against IS where possible[a] or reported by participants

Lab.	Source of Ag	Sample A	Sample B	Sample C	Sample D	Sample E	Comments
4	In-house		5.59	13.89	131.72	39.72	Using IHR curve
			4.36	6.52	77.73	82.37	
			4.78	11.99	97.30	30.38	
		GM	***5.16***	***12.87***	***106.99***	***46.18***	
6	GSK		4.07	8.41	85.45	18.58	
			3.62	7.40	67.75	16.12	
			4.42	11.83	94.92	15.78	
			3.39	7.83	79.42	14.57	
		GM	***3.88***	***8.37***	***79.32***	***16.23***	
7	Sanofi		10.75	18.71	96.85	34.86	
			5.33	13.53	NP	21.46	
			2.38	NP	89.74	21.46	
		GM	***5.05***	***15.61***	***94.39***	***29.98***	
8	GSK		13.23	18.61	139.99	36.83	
			9.77	15.99	103.99	26.58	
			8.00	20.81	79.50	33.81	
		GM	***10.38***	***18.11***	***104.20***	***32.05***	
12a	g-PT		9.63	15.65	95.36	37.37	
			16.86	19.16	NP	30.72	
			6.98	11.22	68.90	26.19	
			5.13	10.69	72.82	28.91	
			6.10	11.41	79.24	24.72	
		GM	***8.52***	***13.92***	***77.70***	***28.90***	
12b	GSK		5.87	14.73	108.79	33.08	
			NP	15.17	114.24	31.17	
			6.81	14.78	126.73	31.16	
			8.21	11.02	108.74	25.62	
		GM	***6.80***	***14.02***	***113.33***	***31.18***	
14	In-house or		3.41	7.78	77.18	24.80	
	Kaketsuken		NL1%	8.69	115.27	26.12	
			NL0.1%	9.15	97.35	24.84	
			NL0.1%	9.24	112.37	25.86	
			4.18	8.80	81.79	21.77	
			4.05	9.32	84.67	23.16	
		GM	***4.00***	***8.61***	***94.46***	***24.55***	
15	1049 PT		2.29	9.27	101.49	20.84	
			3.31	11.08	95.27	20.57	
			2.85	8.67	76.77	17.39	
		GM	***2.89***	***9.55***	***86.81***	***19.90***	
16a	List Biologicals		5.39	13.06	121.79	33.98	
			4.50	12.00	107.82	32.31	
			4.37	12.58	101.87	34.76	
		GM	***4.78***	***12.73***	***109.31***	***33.71***	
20	GSK		**6.60**	**20.88**	129.03	27.12	
			6.42	20.18	136.66	31.55	
			6.81	18.84	130.41	35.00	
		GM	***6.63***	***20.27***	***130.63***	***31.03***	
21	Kaketsuken	4	19.00	34.00	151.00	81.00	Participant reported results
		4	15.00	27.00	127.00	78.00	
		3	15.00	25.00	141.00	86.00	
		GM	***16.23***	***28.42***	***139.32***	***81.60***	
GM of laboratory GMs			***6.00***	***13.85***	***101.66***	***31.18***	
95% limits			***(4.31–8.35)***	***(10.79-17.77)***	***(89.62-115.32)***	***(23.48-41.39)***	
GCV			***63.7%***	***44.9%***	***20.6%***	***52.5%***	

[a] See Section 3.6 Statistical methods
NL: non-linear at 1% level
P: non-parallel at 1% level

Table 3. Results from commercial kits calculated by NIBSC where possible[a] or reported by participants

Lab	Type of kit	Sample A	Sample B	Sample C	Sample D	Sample E	Comments
1	Savyon		19.14	58.19	248.07	172.64	1:100 down four wells
			17.78	59.78	224.57	168.70	
			16.83	66.1	294.19	161.88	
		GM	***18.16***	***59.96***	***248.90***	***166.33***	
2	Virotech		3.93	11.64	100.28	25.73	Diluted down duplicates
			3.85	12.32	108.27	22.50	
			3.82	13.05	103.55	27.06	
		GM	***3.84***	***12.56***	***104.09***	***25.13***	
3	EUROIMMUN	<5	7.25	13.75	86.25	46.75	Single quadruple points. Participant reported results as IU/ml
		<5	8.00	16.5	88	50.75	
		<5	8.00	18	81.75	49.75	
		GM	***7.74***	***15.98***	***85.29***	***49.05***	
5	NovaTec	2.47	13.21	22.75	39.58	33.52	Single point duplicates. Participant reported results as NU/ml.
		2.44	11.85	21.5	35.19	32.44	
		2.42	10.36	17.23	32.82	29.42	
	GM	***2.44***	***11.75***	***20.35***	***35.76***	***31.75***	
9	NovaTec	3.5	6.5	13.5	11	13.5	Single point with duplicates. Participant reported results as Units/ml
		3	4	9.5	11	14.5	
		3	4.5	8	9	11.5	
	GM	***3.16***	***4.89***	***10.09***	***10.29***	***13.11***	
10	Virotech		NL	NL	NL	30.96	Two to three dilutions down with duplicates
			NP	13.28	105.2	32.80	
			NL	12.57	126.99	29.28	
		GM		***12.98***	***118.36***	***30.73***	
11	EUROIMMUN		6.47	13.30	66.25	34.83	Participant reported results as IU/ml
			6.35	12.88	81.77	35.78	
			6.59	13.09	72.04	35.38	Dilution down 4 wells,
		GM	***6.47***	***13.09***	***73.08***	***35.33***	duplicates
13	EUROIMMUN		4.68	8.06	106.71	24.62	Dilution down 4 wells, duplicates
			9.41	19.75	NL	52.40	
			5.35	10.98	96.71	26.79	
		GM	***6.19***	***12.09***	***99.92***	***32.91***	
16b[#]	Euro Diagnostica*	Neg	Neg	Neg	positive	Neg	Single point with duplicates Participant reported results
		Neg	Neg	Neg	positive	Neg	
		Neg	Neg	Neg	positive	Neg	
16c	Serion	2	16	32	104	61	Single point with duplicates. Participant reported results as IU/ml.
		2	13	30	98	55	
		2	14	34	111	64	
		GM	***14.28***	***31.96***	***104.20***	***59.88***	
17	NovaTec		NL	NL	NL	NL	Dilution down four wells, duplicates
			NL	NL	NL	NL	
			NL	NL	NL	31.61	
		GM				***31.61***	
18	EUROIMMUN	<5	7	17	75	42	Single point with duplicates. Participant reported results as IU/ml.
		<5	7	19	85	55	
		<5	7	15	85	54	
		GM	**7.00**	**16.92**	**81.53**	**49.97**	
19	NovaTec		7.58	22.59	120.94	38.56	Results expressed as positive/negative. Raw data returned to NIBSC. Dilution down four wells, duplicates.
			NL	NL	120.84	NL	
			NL	NL	125.95	NL	
		GM	***7.58***	***22.59***	***122.30***	***38.56***	
GM of laboratory GMs			***7.98***	***19.76***	***106.67***	***47.85***	
95% limits			***(5.3-12.01)***	***(12.41-31.47)***	***(77.68-146.48)***	***(29.64-77.26)***	
GCV			***63.2%***	***74.4%***	***46.1%***	***77.4%***	

[a] See Section 3.6 Statistical methods

NL: non-linear at 1% level

NP: non-parallel at 1% level.

RR: outside the response range for the reference

* Negative at OD <0.3; positive OD at 0.8-1.4 reported by the participant.

Figure 1. Histogram of individual assay estimates (IU/ml) for sera samples B to E, calculated relative to the International Standard at NIBSC (laboratory numbers in cells)

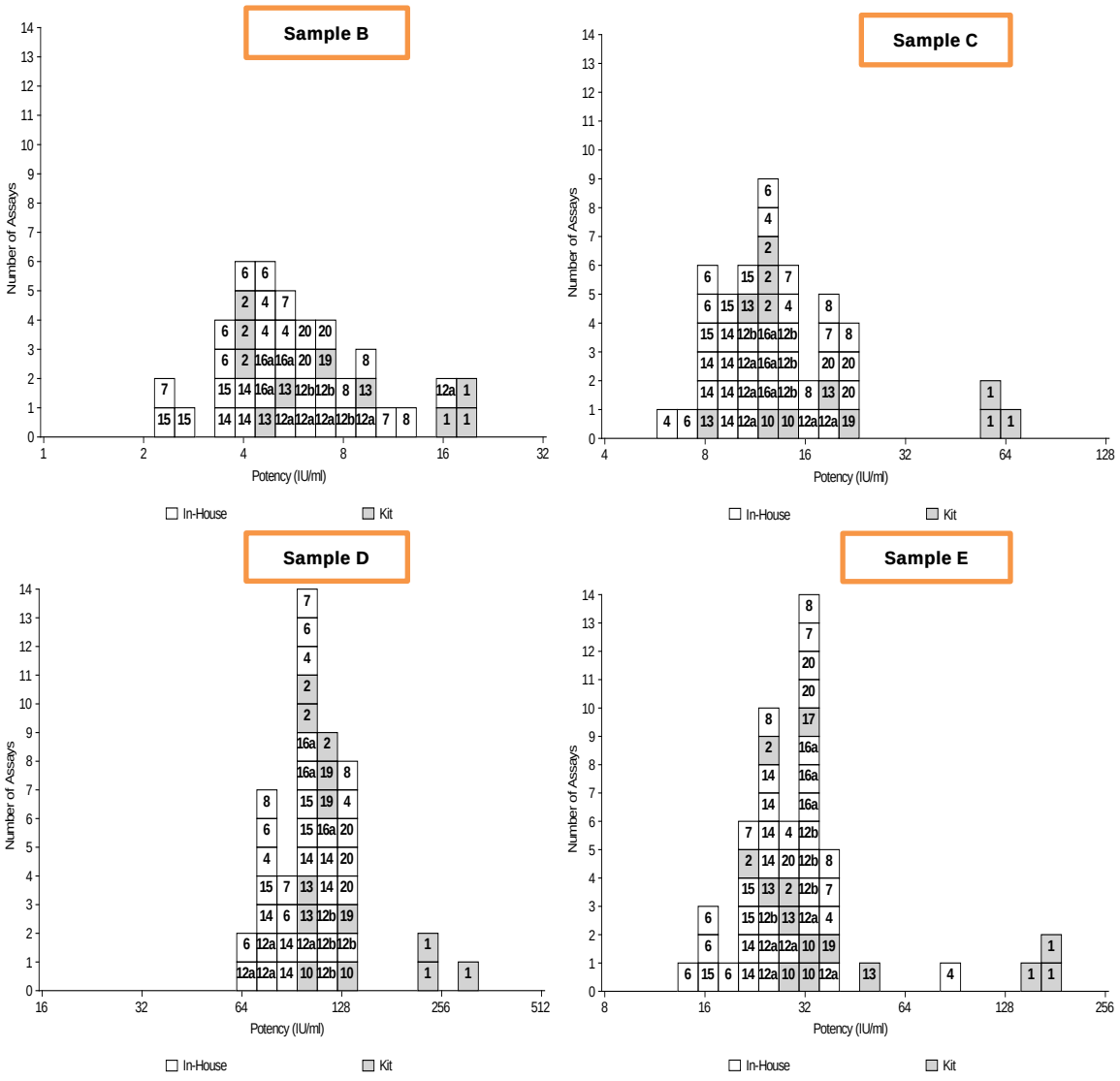
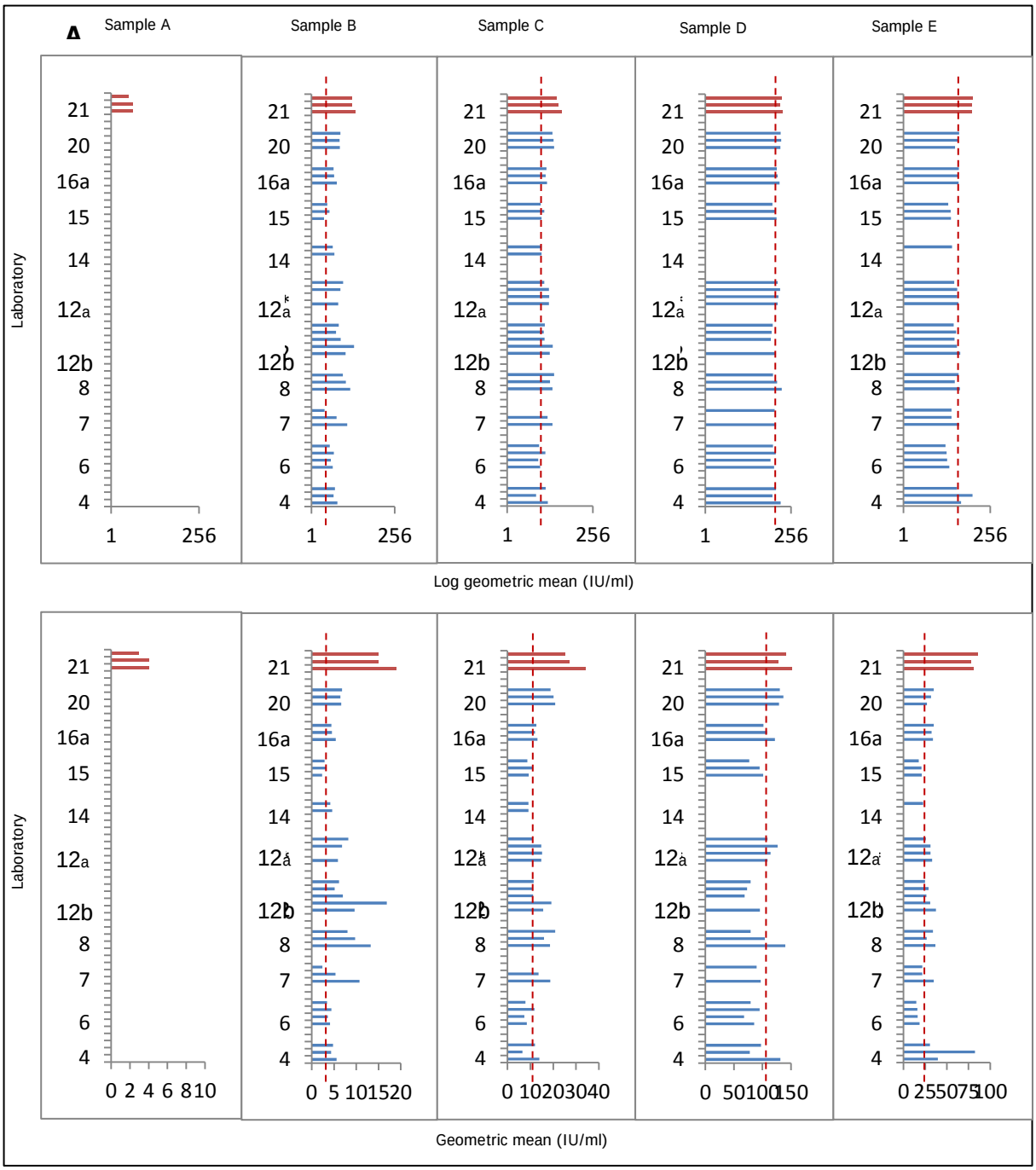


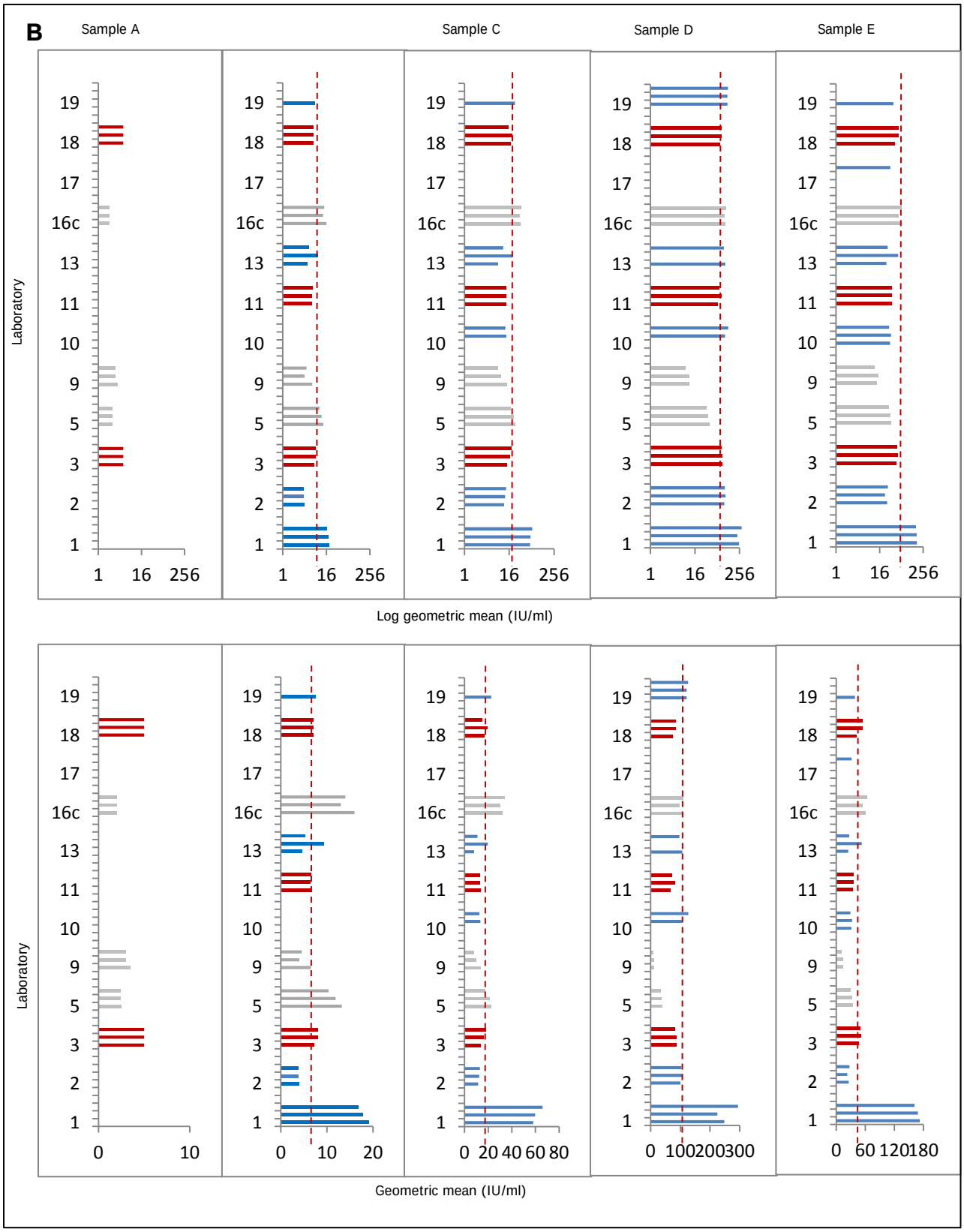
Figure 2a. Assay performance of individual laboratories (in-house ELISA)
In each case the top figure presents the log of the geometric mean for each assay, while the bottom figure illustrates the absolute geometric mean values for each assay.



□= NIBSC calculation expressed in 'IU'
□= in-house calculation expressed in 'IU'
□ = in-house calculation, expressed as units
* Different protocols were used. Dotted lines show GM or expected value for Sample D.

Figure 2b. Assay performance of individual laboratories (commercial kits)

In each case the top figure presents the log of the geometric mean for each assay, while the bottom figure illustrates the absolute geometric mean values for each assay.



□= NIBSC calculation expressed in 'IU'

□= in-house calculation expressed in 'IU'

□ = in-house calculation, expressed as units

* Different protocols were used. Dotted lines show GM or expected value for Sample D.

4.4 Ranking of sera samples in increasing IgG concentrations

Another aim of the present study was to assess that ability of the participants to differentiate the sera samples and rank them in terms of increasing anti-PT IgG concentration. Table 4 shows the order in which the participants ranked the panel in terms of increasing anti-PT IgG concentrations, from data that could be analysed using the four-parameter logistic model by NIBSC and data submitted by the participants from their own calculations. All laboratories were able to distinguish between the samples in the panel and provided suitable data to be able to rank them in the same order. The exceptions were Laboratory 1, which reversed Samples D and E in their own calculations, and Laboratory 9, which failed to distinguish between Samples C and D and had Sample E as the highest concentration. The data can be used to ascertain how well the assays could distinguish between samples of different concentrations.

Table 4. Sera samples ranked in order of increasing anti-PT IgG concentrations, based on results calculated at NIBSC using raw data fitting the four-parameter logistic model (where applicable)[1] and final concentrations determined by the participants using their routine in-house methods[2]

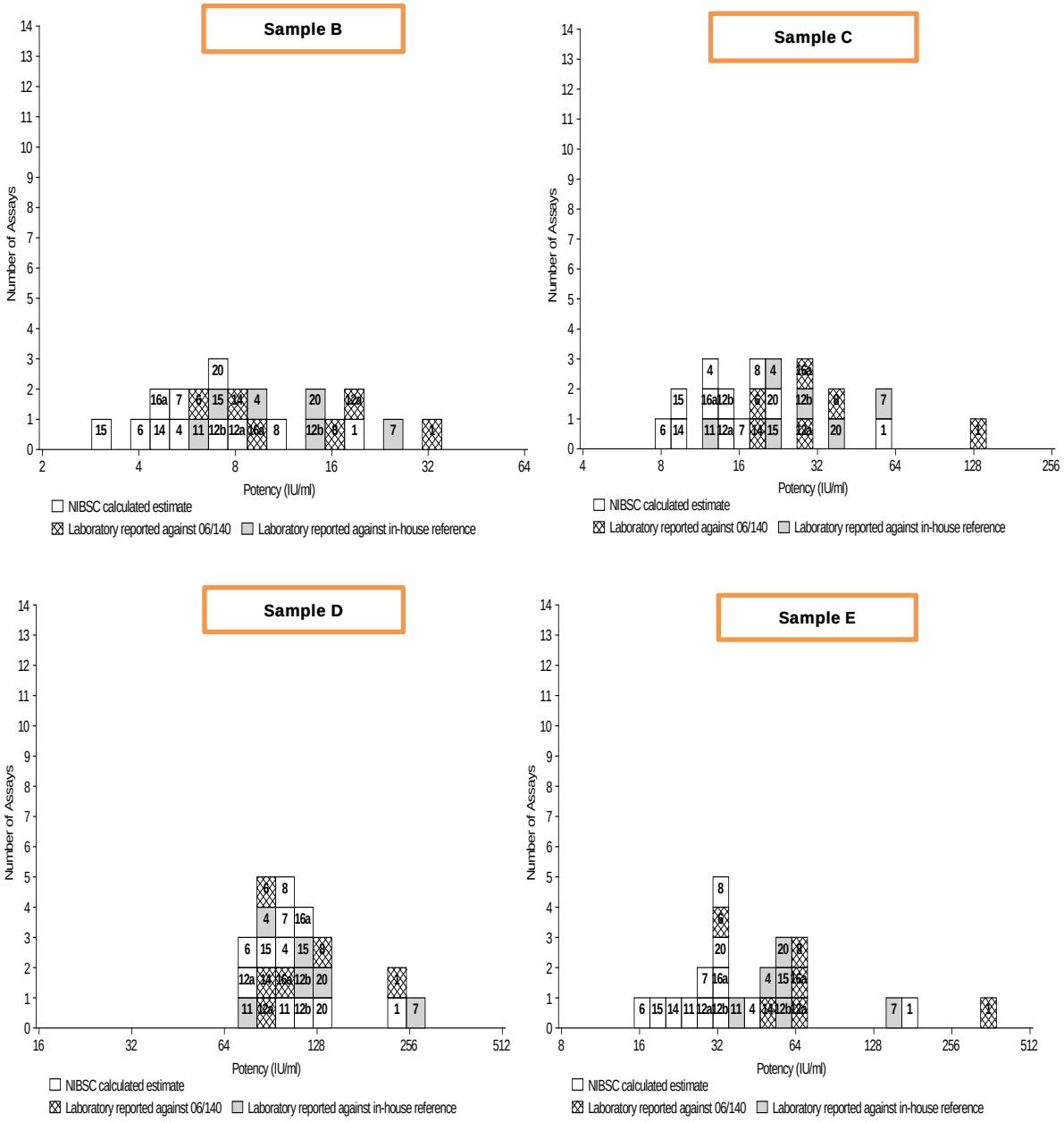
		Increasing anti-PT IgG concentration				
Lab	Method of calculation	1	2	3	4	5
1	NIBSC- four parameter model	A	B	C	E	D
	Participant's in-house results	A	B	C	D	E
2	NIBSC four-parameter model[1]	A	B	C	E	D
3	Participant's in-house results[2]	A	B	C	E	D
4	Both NIBSC and in-house	A	B	C	E	D
5	Participant's in-house results[2]	A	B	C	E	D
6	Both NIBSC and in-house	A	B	C	E	D
7	Both NIBSC and in-house	A	B	C	E	D
8	Both NIBSC and in-house	A	B	C	E	D
9	Participant's in-house results[2]	A	B	C/D	C/D	E
10	NIBSC four-parameter model [1]	A	B	C	E	D
11	Participant's in-house results[2]	A	B	C	E	D
12a	Both NIBSC and in-house	A	B	C	E	D
12b	Both NIBSC and in-house	A	B	C	E	D
13	NIBSC- four parameter model[1]	A	B	C	E	D
14	Both NIBSC and in-house	A	B	C	E	D
15	Both NIBSC and in-house	A	B	C	E	D
16a	Both NIBSC and in-house	A	B	C	E	D
16b	Results reported as positive/negative, could not be ranked					
16c	Participant's in-house results[2]	A	B	C	E	D
17	NIBSC- four parameter model[1]	A	B	C	E	D
18	Participant's in-house results[2]	A	B	C	E	D
19	NIBSC four-parameter model[1]	A	B	C	E	D
20	Both NIBSC and in-house	A	B	C	E	D
21	Participant's in-house results[2]	A	B	C	E	D

[1] Participant did not return calculated values for each sera sample.
[2] Raw data did not fit the four-parameter logistic model.

4.5 Comparison of results calculated by NIBSC using IS and by participants using IS/or in-house references

A number of participants also calculated the anti-PT IgG concentrations in the panel from their own data using their routine methods. Comparisons of results calculated by NIBSC using IS as reference and by participants using IS/or in-house references are presented in Figure 3. Laboratories using in-house references showed similar or up to five-fold higher values than using IS. Annex 6 shows the geometric mean of multiple assays calculated by NIBSC and participants using raw data.

Figure 3. Comparison of results calculated by NIBSC using IS and by participants using IS or in-house references

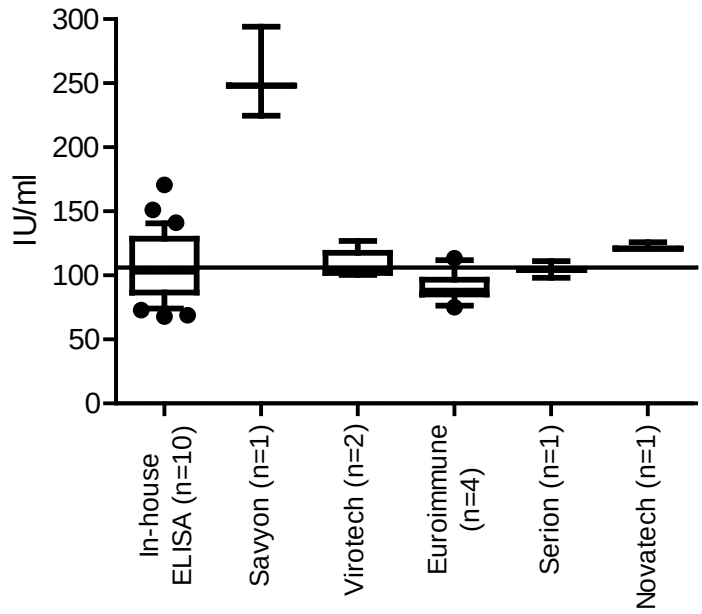


4.6 Comparison of results by in-house ELISA and commercial kits

Figure 4 presents box plots of the quantitative results of anti PT-IgG ELISAs reported for Sample D in IU/ml. Sample D is the 1st WHO Reference Reagent Pertussis Antiserum (Human) (NIBSC code: 06/142) which was blinded in the present study. The straight line at 106 IU/ml represents the expected value; the boxes show the 25–75% interval (where applicable); the whiskers show the 10–90% interval; the dots are outliers and the solid line represents the mean.

A previous collaborative study calibrated the anti-PT IgG for this standard at 106 IU/ml when it was being established. Figure 4 compares the different ELISA methods returning data that could be analysed by the four-parameter logistic model based on the type of method used. The means for the in-house methods and Virotech, EuroImmun and Serion kits were all close to the expected value of 106 IU/ml. However, the Savyon kit used by Laboratory 1 returned a result twice as high as the expected value. Figure 4 presents the data for each of the participants using in-house ELISA that could be analysed by the four-parameter logistic model. Values ranged from 170 IU/ml to 73 IU/ml.

Figure 4. Box plots of the quantitative results for anti PT-IgG ELISAs (Sample D) calculated against the IS or expressed as international units (IU) by in-house calculation



The straight line at 106 IU/ml represents the expected value, the box shows the 25–75% interval (where applicable), the whiskers show the 10–90% interval, the dots are outliers and the solid line represents the mean.

n= number of laboratories.

5. Discussion

ELISAs for measuring antibodies to pertussis antigens have been widely employed in acellular vaccine clinical trials and seroepidemiological studies. They have also become more popular for diagnostic purposes (Xing et al 2011). While in-house ELISA methods have been used for years, there has recently been a dramatic increase in the number of diagnostic laboratories using various commercial kits. The main focus of this EQA is to assess the ability of national reference laboratories from EU/EEA Member States to correctly perform laboratory serodiagnostic tests for pertussis. Furthermore, the scheme will identify areas requiring training and enable recommendations to be made for improvements to methodologies and procedures in order to enhance global performance.

The primary aim of the present study was to evaluate five sera samples with respect to concentrations of anti-PT IgG against a reference sera preparation using immunoassays. In this way it was possible to ascertain how well the assays currently being used by European diagnostic laboratories could differentiate between the sera samples. Although the losses in activity of the sera panel after re-calcification and freeze-drying were disappointing, all laboratories were able to distinguish Sample A (estimated concentration of IgG-PT < 2 IU/ml) from the other samples with higher anti-PT IgG concentrations. The majority of laboratories showed comparable results for each sample and ranked them in the same order (Table 4), indicating the satisfactory performance of these assays.

Previous studies have indicated that the use of commercial kits, which are coated with a mixture of antigens and not just purified PTx, are not sufficiently strong enough to distinguish preparations with high (335 IU/ml) and low (106 IU/ml) anti-PT IgG preparations (Xing et al 2011). It is therefore recommended that only kits with purified PT as coating antigen should be used (Guiso et al 2011, ECDC 2012, Xing et al 2011). In this study the majority of participants used purified PT as the coating antigen. However two kits, Savyon and NovaTech, used both mixed PT and FHA. The only laboratory to use the Savyon kit (Laboratory 1) produced results that were considerably higher than the geometric mean for each sera sample in the panel. Of the four laboratories that used NovaTech, one (Laboratory 17) produced results that were non-linear at either the 0.1 or 1.0% level. Laboratory 9, which used the same kit in single point dilutions, could not differentiate between Samples C and D and found Sample E to be higher than both, which does not correspond to the results obtained by the other participants. Laboratory 19 used a range of dilutions in this kit and had the same ranking order as the other participants but two out of three replicates produced non-linear results at the 0.1 or 1.0% level. Laboratory 5 used single point assays with this kit and the results obtained ranked the samples in the order expected, but the differences between Samples C, D and E were not as pronounced as the geometric means. This once again indicates the need to use only purified PT as coating antigen.

Two laboratories used ViroTech kits, which use purified PTx as coating antigen, with a range of dilutions (Laboratories 2 and 10) that could be analysed by the four-parameter logistic model. The results obtained were consistent with the geometric means and for Sample D they obtained 104 and 105 IU/ml respectively, indicating the accuracy of this kit. While four laboratories used EuroImmun kits (Laboratories 3, 11, 13 and 18) only one performed a range of dilutions that was applicable to four-parameter logistic analysis (Laboratory 13). They obtained a GM of 97.3 IU/ml for sera Sample D.

In the present study a total of 19 (79%) assay methods used purified PT as coating antigen while only five assays were performed with PT and FHA as coating antigens. In contrast, a previous EQA found that 13 out of 20 (65%) participants used purified PT while the remaining laboratories used PT plus FHA (n=5), or PT plus FHA with LPS (n=1) or whole cell lysate (n=1) (Xing et al 2011). This may indicate that more laboratories are beginning to follow the ECDC recommendations (ECDC 2012) if they had not already done so.

There were eight different sources of purified PT used by the participants in the present study for in-house ELISA and MIA (Table 2) and there was no particular trend in results obtained by using the various sources of PT coating antigens. Most laboratories used native PTx as coating antigen, however one laboratory used a genetically modified PTx which showed comparable results with other participants. This may suggest that the genetically modified PTx could be used for coating purposes after evaluation. In general, in-house ELISA methods showed less variability than the commercial kits, as evidenced by the GCV percentage: 20–64% for the in-house ELISA compared to 46–77% for commercial kits. This may suggest that standardisation of commercial kits would help improve comparability and harmonisation.

Pertussis Antiserum (Human) 1st WHO International Standard (06/140) has an assigned unitage of 335 IU/ampoule anti-PT IgG in an international collaborative study (Xing et al 2008). Fourteen participants in this EQA also reported calculated values for the 1st IS using their in-house references and six of these laboratories used in-house ELISA. The overall mean of the laboratory means was found to be 325.6 IU/ml, with a variation of ~70–112% corresponding to the assigned value. This may suggest that in-house references used by these laboratories are reasonably well calibrated in terms of the 1st IS, with the exception of Laboratory 4 whose results were ~70% of the assigned value. Of the eight laboratories that used commercial kits, one (Laboratory 18) expressed results as >200 IU/ml and another three laboratories (Laboratories 1, 5 and 9) reported results expressed as arbitrary binding units (BU) or units against kit references with laboratory means of 67.09, 38.38 and 24.27 units/ml respectively. The GM of multiple assay results from the remaining three laboratories (Laboratories 3, 11 and 16c) which used in-house references calibrated in terms of IU/ml were found to be 309.16, 271.79 and 193.30 IU/ml,

which were 92%, 81% and 58% of the assigned value of 335 IU/ml respectively. The results indicate that although all these commercial kits are CE marked in terms of quality, the information on reference sera (e.g. their concentration in relation to the international units/or the US reference lot) were not clearly stated. Commercial ELISA kits for pertussis diagnosis urgently need to be standardised. Since many routine laboratories perform serological diagnosis using commercial kits, guidelines on how to evaluate commercial kits may provide useful information for diagnostic laboratories.

This study also suggested that although values of anti-PT IgG antibodies of the individual sera were known, the pooled sera should be randomly selected and tested to ensure the proposed values apply. This is important for future EQA studies using pooled sera, not only for pertussis but also for serological diagnosis of other infectious diseases. Unfortunately, the test panel was not tested after the sera were pooled in this study.

Other relevant factors that may have an effect on the results of ELISA include blocking buffer, conjugate used and software used for calculations (Guiso et al 2011, Xing et al 2011, ECDC 2012). These factors should be also taken into account when planning further studies.

6. Recommendations

The present study found that using a range of dilutions and comparing sera samples to the Pertussis Antiserum (human) 1st WHO International Standard on plates coated with purified pertussis toxin facilitated accurate determination of anti-PT IgG concentrations in IU/ml. This corresponds to observations made previously. It is therefore recommended that only purified PT is used in immunoassays, whether for in-house ELISA, commercial kits or multiplex immunoassays. Regular EQA studies with a serum panel of different anti-PT IgG concentrations are needed to validate and standardise serological diagnosis of pertussis in Europe. Since commercial kits are increasingly being used, guidelines for the serological diagnosis of pertussis using commercial kits should be included in future standardisation activities in Europe.

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Annex 1. EUpert-Labnet *Bordetella pertussis* serology EQA: study protocol and results submission form

EQA scheme for the laboratory diagnostics of pertussis

Study protocol for laboratories

Participants

Laboratories currently performing serological assays for diagnosis of pertussis by measuring antibodies to pertussis toxin

The aims of the study are:

- to assess current laboratory performance of serological assays for pertussis
- to compare in-house reference preparations currently used by participants for the serological assay/ or reference preparations provided in commercial kits
- to identify needs for standardisation and training in serological assays.

Materials

The set of materials received by participants contains a panel of six sera preparations containing five defined but blinded concentrations of anti-pertussis toxin IgG (coded by letter). Also included is the Pertussis Antiserum (Human) 1st WHO International Standard Reference Reagent (06/140) for use as a reference to determine the anti-PT IgG concentrations.

Antiserum preparations

Vials of freeze-dried sera should be stored at -20°C.
The panel should be reconstituted with the following volumes of sterile distilled water.

Sample	Volume dH_2O (mL)
Pertussis Antiserum (Human) 1st WHO IS Reference Reagent (06/140)	1.0
Sample A	0.5
Sample B	0.5
Sample C	0.5
Sample D	1.0
Sample E	0.5

Samples should be divided into aliquots and stored at -20°C. The aliquots should be used only once as freeze-thawing is not recommended.

In our study, we found an initial dilution at approximately 1/100 of the reconstituted Pertussis Antiserum (Human) 1st WHO Reference Reagent (06/140) and Sample D in the first row of IgG ELISA assays, followed by ½ dilutions to be satisfactory.

For Samples A, B, C and E in the first row of IgG ELISA assays 1/10 dilutions followed by ½ dilutions were found to be satisfactory.

However, individual laboratories may perform a pilot study to choose the suitable dilution for these samples under their own experimental conditions.

Others

Participants are encouraged to include their in-house reference serum and antigen /or reference serum/antigen provided by commercial kits in the assays. (See details in the Methods section).

Methods

The primary goal of the study is to determine the anti-PT IgG concentrations of the sera preparations provided by ELISA.

For laboratories using their own in-house ELISA:

The participating laboratories are asked to coat ELISA plates using their own in-house sourced pertussis toxin antigen and perform a minimum of three independent assays on the six serum preparations. Participants are also asked to include their in-house reference serum (IHR) in the assay. The three independent assays should be run on three different days. For each assay, dilution curves for each preparation should have at least two or three replicates per assay and preferably each dilution curve should include at least four doses in the linear region. All

preparations should be included in each assay. As far as possible, each plate should also include the appropriate IHR, if applicable.

Laboratories should use their own methodology, reagents and calculation methods, include their in-house references and controls and use assay runs that meet their internal validity criteria.

For laboratories using commercial ELISA kits:

The participating laboratories are asked to perform a minimum of three independent assays on the panel of six sera preparations provided in this study using the commercial ELISA kit that is routinely used in their laboratory. Participants should include the reference serum provided in the kit in the assay. The three independent assays should be run on three different days. On each assay, dilution curves for each preparation should have at least two replicates per assay and preferably each dilution curve should include at least four doses in linear region.
For the assay using commercial kits, laboratories should use the methodology and validity criteria recommended by the kit manufacturer.

Collection and analysis of data

NIBSC will be responsible for the collection and analysis of data submitted by the participating laboratories. Samples are available for shipment on 23 July 2012. Laboratories are requested to schedule sample analysis so that all results are ready for submission by 26 October 2012. Participants are required to supply all raw data to NIBSC for analysis using common methods. Participants are also encouraged to submit their calculated results using their own methods. At the NIBSC, data will be collected, coded and entered into a database for analyses. Laboratories will be identified by a code in all analyses to maintain confidentiality.

In order that data can be efficiently analysed and transcription errors minimised, it is requested that data are supplied in electronic format. We recognise that participants will have different plate layouts and plate readers which they use routinely, and that these may not be readily reconfigured. To accommodate this, the data can be accepted in a variety of formats.

It is very important that the data submitted are clearly annotated and that the plate layout, initial dilutions and dilution factors are clearly indicated.

Data as text files, in Excel spread sheets, or incorporated into Word documents can be readily used. Laboratories are also asked to fill in Tables 1 and 2 describing their routine set-up for ELISAs.

Please also use Table 3 to show your plate layouts in addition to annotating your raw data. Table 4 is for use only by participants who score their results as positive and negative.

Legal/ethical factors

Participants are responsible for their own legal/ethical issues according to the regulations in their country for the handling of human samples.

All laboratories participating in the study will be fully acknowledged. In the report, where assay results are attributed to the various laboratories, each laboratory will be identified only by a code number. Each participant will be told the identity of their own code number, so that they alone can identify which set of assay results they contributed.

To be completed for each assay

EQA scheme for the laboratory diagnostics of pertussis

Table 1a. Information form

For laboratories using own in-house ELISA

Name of laboratory:

Antigen information	
Pertussis toxin antigen (e.g. mixture with other antigen(s)/or purified PT) If mixture, please specify Sources (e.g. in-house or/commercial) If commercial source, please specify	
Is any stabiliser used in the antigen solution (if yes, please specify)	
Diluent and pH used for coating antigen	
Conjugate information	
Type of conjugates for IgG assay, please specify the source of the conjugates)	
In-house reference antiserum	
Source	
Calibration against	
Diluent used for anti-sera and pH of the diluent	
Validity criteria used	

To be completed for each assay

EQA scheme for the laboratory diagnostics of pertussis

Table 1b. Information form

For laboratories using commercial ELISA kits

Name of laboratory:

Commercial kit information	
Manufacturer name	
Product code number	
Antigen information	
Coating antigen information (e.g. whole cell suspension, purified PT, PT+FHA, other mixture)	
Reference antiserum provided by the kit	
Is there any unitage for the reference anti-serum? If yes, please specify	
Calibration against which standard?	
Is positive control and negative control included in the kit?	
Validity criteria stated in the kit? If yes, please specify	
Does the kit provide quantitative results?	

To be completed for each assay

EQA scheme for the laboratory diagnostics of pertussis

Table 2. Assay sheet for laboratories

Name of laboratory:

Antigen: own sourced/commercial kit PT*

Sample code	**Assay number (or assay date)**								
	1			**2**			**3**		
	Reconstitution volume per ampoule, if applicable	Initial dilution in 1st row	Dilution factor through the plate	Reconstitution volume per ampoule, if applicable	Initial dilution in 1st row	Dilution factor through the plate	Reconstitution volume per ampoule, if applicable	Initial dilution in 1st row	Dilution factor through the plate
06/142									
A									
B									
C									
D									
E									
IHR/kit ref									

*Please delete as appropriate

IHR= In-house reference

If more than three assays performed, please photocopy this sheet and change the assay number accordingly.

To be completed for each assay

EQA scheme for the laboratory diagnostics of pertussis

Table 3. Plate layout for laboratories

(Please use this sheet to show sample layout and dilutions made down the plate.)

Name of laboratory:

Antigen: own sourced/commercial kit PT*

Please input sample name and dilution into each row and column, if applicable.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Please make copies of this form and use to show the layout for all plates used in each independent assay.

To be completed for each assay

EQA scheme for the laboratory diagnostics of pertussis

Table 4. Assay sheet for positive and negative results

Name of laboratory:

Antigen: Own sourced/commercial kit PT*

Please input sample name and dilution into each row and column if applicable.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Please copy this sheet and fill in for each assay.

Annex 2a. EUpert-Labnet *Bordetella pertussis* serology EQA. Information from participants using in-house ELISA and MIA methods

Lab code	Number of assays	Information (in-house antigen)	Antigen stabiliser	Coating buffer	Conjugate	In-house reference serum	Diluent used	In-house ref serum calibrated against	In-house reference unitage
4	3	In-house production of PT	Ammonium sulfate	Carbonate buffer pH 9.60	IgG #PO214	Human, from pertussis-vaccinated person	PBS pH 7.40 + 0.1% Tween 20 and 1% powdered skimmed milk	WHO IS Pertussis Antiserum (Human) 1st IS NIBSC 06/140	440 IU/ml
6	4	Purified PT 1ug/ml GlaxoSmithKline Rixensart Belgium	Glycerol	Phosphate-buffered saline pH 7.2-7.4	Goat anti-human IgG Art 075-1002 Kirkegaard & Perry Laboratories, USA	In-house positive pooled sera (IH+), made from highly positive serum samples on 17 October 2000	BSA-PBS pH 7.2-7.4	NIBSC 06/142	~500 IU/ml
7	3	Purified PT Sanofi Pasteur	Glycerol 50%	Sodium carbonate 0.05M pH 9.6 +/-0.1	Anti-human IgG antibody phosphatise labelled Kirkegaard & Perry, ref: 075-1002	PTMMH Unit	PBS 1x +0 0.5% Tween + 0.005% PPG	Lot 3	226 EU/ml
8	3	Purified PT GSK Biologicals SA, Rixensart Belgium	Glycerol	Carbonate buffer 50mM pH 9.6	Anti-human IgG antibody phosphatise labelled Kirkegaard and Perry, ref: 075-1002	WHO reference serum high titre	PBS + 0.2% BSA (w/v) + 0.05% Tween 20	n/a	335 EU/ml
12a	5	Genetically modified PT (PT-9k/129G) in-house purified from Novartis: starting material	50% v/v glycerol	Carbonate buffer pH 9.6	HRP labelled rabbit anti-human IgG (DAKO P214), 1:2500 dilution in PBS-T (PBS 1x + 0.1% Tween-20 pH 7.4 + 0.02	Pertussis Antiserum (Human) Internal Reference reagent (06/142 – WHO)	PBS 1x + 0.1% Tween-20 pH 7.4± 0.02 + 0.1% powder milk	Pertussis Antiserum (Human) 1st WHO Reference Reagent (06/140)	Anti-PT IgG 106 IU / ampoule

Lab code	Number of assays	Information (in-house antigen)	Antigen stabiliser	Coating buffer	Conjugate	In-house reference serum	Diluent used	In-house ref serum calibrated against	In-house reference unitage
12b	4	Purified PT TOH 15 from Glaxo Smith Kline Beecham, Belgium	50% v/v glycerol	PBS pH 7.4	Lyophilised conjugate: Goat anti human IgG Reserve AP. #KIRK0751-1002 from Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD, USA	Pertussis Antiserum (Human) Internal Reference reagent (06/142 – WHO)	PBS + 0.1% BSA + 0.05% Tween20	Pertussis Antiserum (Human) 1st WHO Reference Reagent (06/140)	Anti-PT IgG 106 IU / ampoule
14	3	Purified PT Netherlands Vaccine Institute OR Kaketsuken (Kumamoto, Japan)	50 % glycerol	N/A PT is coupled to activated carboxylated microspheres	Goat anti-human IgG (γ chain specific) R-phycoerythrin conjugated, Jackson Immunoresearch # 109-116-098	Pool of diagnostic sera submitted to the RIVM for suspected whooping cough for which the diagnosis was confirmed with high IgG PT concentrations	PBS pH 7.2 containing 0.1% (v/v) Tween-20 and 3% (w/v) BSA	Lot 3	
15	3	Purified PT (code 1049)	4M $MgCl_2$	Pre-coated with 100ul fetuine-solution (50mg/l) in 0.01M PBS pH 7.2, plates are then coated with 100ul PT (0.708ug/ml) in 0.01M PBS, 0.5% BSA, 0.01% Tween 20 pH 7.2	Rabbit-anti human IgG peroxidase labelled (DAKO nr P0216)	REF-991 Pooled sera from patients with B. Pertussis with high IgG-PT titers.	0.01M PBS, 0.5% BSA, 0.01% Tween-20 pH 7.2	Lot 3	1300 U/ml

Lab code	Number of assays	Information (in-house antigen)	Antigen stabiliser	Coating buffer	Conjugate	In-house reference serum	Diluent used	In-house ref serum calibrated against	In-house reference unitage
16	3	Pertussis toxin in glycerol from List Biologicals Laboratories #179B 0.2 mg/ml Lot 179216A1B Coated at 1ug/ml	50% glycerol	0.05M carbonate / bi-carbonate buffer pH 9.6	A3187 anti-human IgG (gamma chain spec) ALP (Lot 066K6001) Sigma/Aldrich	06/140 NIBSC 1st IS	PBS with 0.02% NaN3 / 0.1% skimmed milk / 0.05% Tween pH 7.4	N/A	Anti-PT IgG 335 IU / ampoule
20	3	Purified PT TOH 15 from Glaxo SmithKline Beecham, Belgium	u	PBS pH 7.4	Lyophilised conjugate: Goat anti human IgG Reserve AP. #KIRK0751-1002 from Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD, USA	IgG 42, an immunoglobulin from Statens Bakteriologiska Laboratorium, Sweden	0.1% BSA in PBS and 0.05% Tween20	NIBSC WHO IS Pertussis Antiserum 06/142	930 IU / ml.
21	3	Pertussis toxin from *Bordetella pertussis* Tohama strain phase I (code 670536) Kaketsuken, Japan	200µg/mL in 0.025M Tris buffer (pH8) containing 50% glycerol and 0.5M NaCl	0.05M carbonate coating buffer, pH 9-9.5, trace sodium azide preservative	Anti-Human IgG (whole molecule) – peroxidase Sigma-Aldrich A8792	NIBSC 06/140	Serum diluent, Microimmune Ltd PN 2040 pH 7.2	N/A	Anti-PT IgG 335 IU/ampoule

Annex 2b. EUpert-Labnet *Bordetella pertussis* serology EQA. Information from participants using commercial kit ELISA methods

Lab code	Number of assays	Kit sources	Coating antigen	Reference serum unitage	Ref serum calibrated against	Positive and negative controls	Validity criteria	Quantitative results
1	3	Savyon-Diagnostics Ltd. SeroPertussis IgG Savyon-Diagnostics Ltd., Israel	PT and FHA	NK	NK	NK	0-10 BU/ml below assay limit 10-30 BU/ml low titer 30-50 BU/ml medium titer > 50 BU/ml high titer	NK
2	3	Virotech Pertussis Toxin ELISA IgG Test kit Code: EC215G00	Purified PT	NK	NK	Yes	NC <0.15 Cut-Off > 0.26 PC > 0.49 Calculation of IU automated in Excel file.	Yes, IU/ml
3	3	EuroImmun AG Anti-*Bordetella pertussis* toxin ELISA (IgG) No. EI 2050-9601G	Purified PT from Tohama strain	NK	NK	Yes	Calibrator 1 (200 IU/ml) Calibrator 2 (100 IU/ml) Calibrator 3 (25 IU/ml) Calibrator 4 (5 IU/ml) Cal 1 > 1,000 OD Cal 3 > 0,140 OD OD Cal 1 > OD Cal 2 > OD Cal 3> OD Cal 4	Yes in IU/ml
5	3	NovaTec Immundiagnostica GmbH BOPG0030DB	PT + FHA	No unitage	N/A	Yes	k- < 0,200 and < cut-off; 0,150 < cut-off < 1,300; K+ > cut-off **Results in Novagnost Units** Cut-off: 10 U Grey zone: 8,5 – 11,5 U Negative: < 8,5 U Positive: > 11,5 U	Semi -quantitative
9	5	*Bordetella pertussis* IgG, NovaTec, Germany	NK	NK	NK	NK	Grey zone: 9-11 Units/ml	NK

Lab code	Number of assays	Kit sources	Coating antigen	Reference serum unitage	Ref serum calibrated against	Positive and negative controls	Validity criteria	Quantitative results
10	3	Virotech, Pertussis Toxin IgG ELISA EC215G00, EN218Q60	Purified PT	NK	WHO	Yes	VE(pos.control)=(OD(pos.cont.)/OD(cut-off))*10 VE(patient sera)=(OD(patient sera)/OD(cut-off))*10 OD of blank <0.15 Red: positive >112IU/ml Green: borderline (75-112) IU/ml Black: negative (<75IU/ml)	Semi - quantitative
11	3	Euroimmun AG Anti-*Bordetella pertussis* toxin ELISA (IgG) No. EI 2050-9601 G	Purified PT	NK	In-house ref 06/140 and 06/142 + calibrators Calibration against 06/140	Yes	Calibrator 1 (200 IU/ml) Calibrator 2 (100 IU/ml) Calibrator 3 (25 IU/ml) Calibrator 4 (5 IU/ml) Positive control range 51-95 IU/ml, and negative control range 0-18 IU/ml. Also OD of 200IU/ml (calibrator 1) >1.0, and OD of 25 IU/ml calibrator 3 > 0.14. Also OD values of calibrator 1> OD calibrator 2> OD calibrator 3 > OD calibrator 4.	Yes
13	3	EuroImmun AG Anti-*Bordetella pertussis* toxin ELISA (IgG) No: EI 2050-9601 G	Purified PT	Calibrator 1, 2, 3 and 4. Calibrator 1 (200 IU/ml) Calibrator 2 (100 IU/ml) Calibrator 3 (25 IU/ml) Calibrator 4 (5 IU/ml)	WHO IS Pertussis Antiserum, human, 1st IS NIBSC code 06/140	Positive control 73IU/ml Negative control 1IU/ml	Cal 1 > 1,000 OD Cal 3 > 0,140 OD OD Cal 1.> OD Cal 2 > OD Cal 3> OD Cal 4	Yes Anti-PT-IgG ≥ 100 IU/ml - positive Anti-PT-IgG < 40 IU/ml –negative Anti-PT-IgG ≥40-< 100 IU/ml - borderline

NK = not known

Annex 3. EUpert-Labnet *Bordetella pertussis* serology EQA. Calculation of results performed by NIBSC and individual participants

	Methods	Results calculated by Combistats v 06/140 using raw data by NIBSC					Geometric mean of multiple assays results calculated by participants						
Code		**Sample A**	**Sample B**	**Sample C**	**Sample D**	**Sample E**	**Sample A**	**Sample B**	**Sample C**	**Sample D**	**Sample E**	**06/140**	**Units**
1	Savyon		18.16	59.96	248.90	166.33	2.36	10.31	38.34	57.38	68.70	67.09	BU/ml via Kit ref.
							11.73	34.76	125.13	241.98	339.72		IU/ml via IS
2	Virotech	-	3.84	12.56	104.09	25.13	N/A						
3	EUROIMMUN	-					< 5	7.73	15.97	85.19	48.91	309.16	IU/ml
4	In-house	-	5.16	12.87	106.99	46.18	1.01	9.70	21.27	88.14	51.48	233.12	IU/ml
5	NovaTec	No raw data returned					2.43	11.72	20.25	35.72	31.73	38.38	Units/ml via Kit ref.
6	In-house	-	3.88	8.37	79.32	16.23	0.50	6.48	18.15	89.19	31.85	335.00	IU/ml
7	In House	-	5.05	15.61	94.39	29.98		24.73	53.93	260.06	156.50	377.57	EU/ml
8	In house	-	10.38	18.11	104.20	32.05	<2	17.28	37.39	132.69	68.30	314.93	EU/ml
9	NovaTec	-					2.59	4.34	9.86	10.10	13.01	24.27	Units/ml via Kit ref.
10	Virotech	-		12.98	118.36	30.73	N/A						
11	EUROIMMUN	-					0.22	6.47	13.09	73.08	35.33	271.79	IU/ml
12a	In-house	-	8.52	13.92	77.70	28.90	5.55	18.48	28.99	91.56	62.09		IU/ml
12b	In-house	-	6.80	14.02	113.33	31.18	5.66	14.91	28.41	110.04	58.95		IU/ml
13	EUROIMMUN	-	6.19	12.09	99.92	32.91	N/A						
14	MIA	-	4.38	9.18		22.45	0.27	8.52	18.17	91.58	46.93		IU/ml
15	In-house	-	2.89	9.55	86.81	19.90	<1	7.32	22.25	116.00	54.39	374.17	U/ml via in-house Ref.
16a	In-house	-	4.78	12.73	109.31	33.71	1.00	9.32	27.54	99.58	67.94	318.82	IU/ml
16b	Euro Diagnostica	-					Expressed as positive/negative results						
16c	Virion	-					2.00	14.28	31.96	104.20	59.88	193.30	IU/ml
17	NovaTec	-	All samples were NP or NL		31.61		N/A						
18	Euroimmun	-					< 5	7.00	16.92	81.53	49.97	>200	IU/ml
19	NOVATEC	-	7.58	22.59	122.30	38.56	Expressed as positive/negative results						
20	In-house	-	6.63	20.27	130.63	31.03	_	14.1	40.0	136.0	59.5	343.4	IU/ml
21	In-house	No raw data returned for IS					3.63	16.23	28.42	139.32	81.60		IU/ml

N/A not provided

'-' unable to calculate.