TECHNICAL REPORT

External quality assessment scheme for *Bordetella pertussis* serology 2020 data

On behalf of EUPert-LabNet network

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ECDC TECHNICAL REPORT

External quality assessment scheme for *Bordetella pertussis* serology – 2020 data

As part of the ERLNPert-Net network

This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), as part of the coordination of activities for laboratory surveillance of pertussis (whooping cough) in EU/EEA Member States (ECDC/2019/023), coordinated by Dr Sabrina Bacci and Christiana Carstairs and produced by Dr Kevin Markey and Alex Douglas-Bardsley, National Institute for Biological Standards and Control (NIBSC) (Potters Bar, UK) and Alex-Mikael Barkoff and Prof Qiushui He, University of Turku (Turku, Finland), on behalf of the ECDC European Reference Laboratory Network for Pertussis (ERLNPert-Net) consortium.

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Abbreviations

ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-linked immunosorbent assay
EQA	External quality assessment
ERLNPert-Net	ECDC European Reference Laboratory Network for Pertussis
EU/EEA	European Union/European Economic Area
EUVAC.NET	European Community Network of Vaccine-preventable Diseases
FFHA	Filamentous haemagglutinin
GCV	Geometric coefficient of variation
GM	Geometric mean
IgG	Immunoglobulin G
IHR	In-house reference serum
IU	International Units
MIA	Multiplex immunoassay
NIBSC	National Institute for Biological Standards and Control (United Kingdom)
PT	Pertussis toxin
RR	Outside the response range for the reference
THL	National Institute for Health and Welfare (Turku, Finland)
UK	United Kingdom
WHO	World Health Organization

Executive summary

Laboratory confirmation of pertussis (whooping cough) is integral to surveillance, especially to monitor the effectiveness of vaccination strategies and to inform any changes to national policies. The European Centre for Disease Prevention and Control (ECDC) has organised previous and current studies in order to ensure maximum participation of microbiology reference laboratories across EU/EEA countries, as well as to highlight any issues of concern and make recommendations.

This report presents the results of the ECDC European Reference Laboratory Network for Pertussis (ERLNPert-Net) external quality assessment (EQA) scheme for *Bordetella pertussis* PCR, funded by ECDC on behalf of ERLNPert-Net and conducted from April to September 2020. The EQA was organised by the National Institute for Biological Standards and Control (NIBSC), based in Potter's Bar, United Kingdom (UK).

The primary aim of this EQA scheme was to assess the ability of national reference laboratories in EU/EEA Member States to correctly perform laboratory serodiagnostic tests for pertussis. This was achieved by assessing each participating laboratory's ability to correctly measure the anti-pertussis toxin (anti-PT) immunoglobulin G (IgG) in sera samples and identifying any laboratories that are producing results significantly different from the values obtained from the majority of participants. Furthermore, the scheme helped to identify methodologies in need of further improvement and areas for training (e.g. where particular laboratories may improve their methods, procedures and performance).

Of the 31 laboratories that were invited to participate in the study, 17 agreed to take part. NIBSC sent blinded panels of eight freeze-dried sera samples containing different concentrations of anti-PT IgG (concentrations ranging from no anti-PT IgG to concentrations clinically associated with pertussis infection) to each of the 17 laboratories in 17 EU/EEA Member States. The participating laboratories were asked to quantify concentrations of anti-PT IgG using their own routine diagnostic enzyme-linked immunosorbent assays (ELISA) or multiplex immunoassays (MIA). Raw data were also returned to NIBSC for secondary analysis.

Of the 17 participating laboratories, one did not return results. Fifteen of the 16 laboratories that returned data used only one diagnostic method (either an in-house ELISA, an in-house MIA or a commercial ELISA kit) to determine the anti-PT IgG concentrations of the sera panel. One laboratory submitted the results obtained using all three methods (an in-house ELISA, an in-house MIA and a commercial kit). A total of 57 data sets from individual assays were collected for 18 assay methods.

Overall, there is a trend towards more laboratories using commercial kits for the serodiagnosis of pertussis. It also appears that the recommendations made in previous ECDC studies are being followed, as all kits used purified pertussis toxin (PT) as coating antigen and reference sera that are calibrated in IU/ml.

This technical report recommends that only purified PT is used in in-house ELISAs, commercial kits or in-house MIAs for serological testing of anti-PT IgG in human sera. Also, a reference serum should be included in each assay and it should be calibrated in IU/ml using one of the international standards.

1. Introduction

Pertussis (whooping cough) is a highly contagious acute respiratory infection most commonly caused by the bacterium *B. pertussis*. A similar illness is caused by *B. parapertussis*, but this is unaffected by current pertussis vaccines. Despite being a vaccine-preventable disease, pertussis remains endemic worldwide and is an important public health issue, typically showing cyclical outbreak periods every three to five years. Infants, particularly those who are unimmunised, are most vulnerable to pertussis infection. Older children and adults can display milder symptoms, but are responsible for transmission. In recent years, there has been a marked increase in the number of cases in areas with high vaccination coverage, such as the EU and North America [1-4].

Pertussis is a notifiable disease in EU/EEA countries, although there are differences in reporting systems between countries. The key prevention strategy for pertussis is high immunisation coverage starting early in life with an effective vaccine. The rationale for pertussis surveillance is the monitoring of the impact of national immunisation strategies, including the identification of high-risk groups and detection and investigation of clusters and outbreaks.

ECDC has coordinated pertussis surveillance at the European level since 2011, when EUVAC.NET was transferred to ECDC. EUVAC.NET was a European surveillance network for selected vaccine-preventable diseases, such as pertussis, hosted at the Staten Serum Institute (SSI) in Denmark.

The mapping and development of assessment of laboratory performance for pertussis was included in EUVAC.NET tasks in 2009 and was contracted to the National Institute for Health and Welfare (THL) based in Turku, Finland. From 2011 to 2014, pertussis laboratory activities continued to be coordinated by ECDC through a framework service contract with THL and ERLNPert-Net was created. From 2015 to 2022 (2015–2019 and 2019–2022), second and third framework service contracts between ECDC and the University of Turku were signed and the activities of the ERLNPert-Net continued.

EQA schemes are an important part of quality management systems. They use an external agency to evaluate performance of laboratory assays on material that is supplied specifically for the purpose. ECDC's disease networks organise a series of EQAs for EU/EEA countries.

The aim of ECDC EQAs is to identify areas for improvement in laboratory diagnostic capacities relevant to the surveillance of the 52 communicable diseases listed in Decision No. 2119/98/EC 2 [5] and to ensure comparability of results between laboratories from all EU/EEA countries.

The main aims of this specific ECDC *B. pertussis* serology EQA scheme were to:

- assess the ability of laboratories to quantify and distinguish concentrations of anti-PT IgG in a panel of serum samples with different concentration of anti-PT IgG,
- assess the possibility of setting up assay validity criteria for standardising serological tests using the World Health Organization (WHO) International Standard or Reference Reagent sera,
- assess the general standard of performance and ensure comparability of results between laboratories from all EU/EEA countries,
- assess the effectiveness of analytical procedures (method principle, instruments, reagents, calibration),
- evaluate individual laboratory performance,
- identify problem areas,
- provide continuing education, and
- identify training needs.

Laboratory diagnosis of pertussis infection is important for surveillance, treatment and prevention. There have been wide variations in the reporting of laboratory-confirmed pertussis cases globally [6] and one of the reasons could be differences in the methods used for diagnosis. This highlights the importance of harmonisation and standardisation of diagnostic methods in successful laboratory-based surveillance systems.

In 2011, the members of the EUpertstrain group published the following recommendations for serological diagnosis of pertussis: i) the use of non-detoxified purified PT only as coating antigen [7] and ii) data points should cover a broad linear range and express results quantitatively in international units per millilitre (IU/ml) [7]. These recommendations were also endorsed in the ECDC *Guidance and Protocol for the serological diagnosis of human infection with* Bordetella pertussis [8].

Serological analysis by ELISA has been widely used to evaluate antibody responses to pertussis vaccination and to diagnose infection [6]. ECDC has organised three previous pertussis serology EQAs [6,9,10]. The first of these EQAs took place from July to October 2010, as part of the EUVAC.NET contract with ECDC (Pertussis Work Area 4), and assessed laboratory performance of serological assays for pertussis, compared in-house references that were being used and identified any needs for standardisation of serological assays [10]. The second was undertaken from July to October 2012, as part of the ECDC contract for the coordination of activities for laboratory surveillance of whooping cough in EU/EEA countries (ECDC/2011/013) [6]. The third was organised from February to April 2016, as part of the ECDC contract (ECDC/FWC/2015/009) to continuously improve the serological diagnosis of pertussis among EU/EEA Member States [9]. These three EQAs have been thoroughly

reviewed elsewhere [11]. These studies have found that the number of laboratories using only the recommended purified PT as coating antigen has increased through the subsequent EQA rounds from 65% to 92%. There has also been an increase in the use of reference sera calibrated in IU from 59% to 92%. Manufacturers increasingly followed the recommendations and, overall, the three EQAs led to greater harmonisation in methods among different laboratories, showing a significant improvement of the ELISA methods used for serodiagnosis of pertussis [11].

As part of the current contract with ECDC (ECDC/2019/023) and to continuously improve the serological diagnosis of pertussis among EU/EEA Member States, an EQA for pertussis serology was organised from April to September 2020. Details of the countries that took part in this EQA are given in the Annex.

This report presents the results of the EQA scheme for *B. pertussis* serology, the analysis of laboratories' performance, and recommendations.

2. Study design and methods

2.1. Organisation

The ERLNPert-Net *B. pertussis* serology EQA was organised by the NIBSC and was intended for national reference laboratories in EU/EEA Member States. All national reference laboratories in Europe that are currently performing serological assays for diagnosis of pertussis infection by measuring serum antibody to PT were invited to participate in the EQA in February 2020. A total of 31 laboratories in 30 countries were invited (two laboratories in Belgium); of these, 17 laboratories agreed to participate in this EQA (16 in EU countries and one in Norway (Table 1A)). There was no response from six laboratories. The majority of those that declined cited lack of resources due to the COVID-19 pandemic. One laboratory that accepted the invitation to participate was unable to return results for the same reason.

Throughout this report, participating laboratories and assay results have been anonymised. Separate experiments have been numbered sequentially within laboratories.

2.2. Selection of sera panel

The same freeze-dried human sera samples that were used in the 2016 pertussis serology EQA [9] and are available from NIBSC were used to prepare the sera panel for this study (NIBSC code 18/146). The sequence in which the panel was organised was rearranged and the samples were given new codes. Also, one sample was duplicated. Details of the panel are given in Table 1.

Table 1. Panel of human sera used in the EQA scheme for *Bordetella pertussis* serology, 2020

Sera panel code	Sample number in NIBSC sera panel (NIBSC code 18/146)	Estimated mean IgG-PT in pooled sample (IU/ml)*	NIBSC filling number after freeze drying
Sample A	Sample E	80	14/190
Sample B	Sample F	106**	06/144
Sample C	Sample D	62	14/188
Sample D	Sample B	24	SS-366
Sample E	Sample G	131	14/192
Sample F	Sample C	43	14/186
Sample G	Sample A	<2	SS-369
Sample H	Sample E	80	14/190
Reference serum	Pertussis Antiserum (Human) 1st WHO Reference Reagent (106 IU/ml anti-PT IgG)		06/142

* *Based on results of previous EQA [9]*
** *Based on results from WHO collaborative study [12]*

2.3. Sample shipment

Each participating laboratory received the panel of eight samples of human serum coded by letters and an ampoule of 06/142 (WHO Reference Reagent) (Table 1).

The panels were prepared, packed according to local regulations and collected by international courier on 2 June 2020 from NIBSC and were then dispatched to the participating laboratories (Table 1A). All packages were received in a timely manner, providing laboratories with sufficient time to meet the initial deadline of 30 June 2020. However, the study took place during the COVID-19 pandemic and many participants had to prioritise pandemic-related work; therefore, some results were returned long after the deadline and the remainder of the study timeline was updated accordingly.

2.4. Assay methods

To achieve the primary aim of the study, each participating laboratory was asked to determine the concentrations of anti-PT-IgG in each sample of the panel of sera using their routine serological methods (i.e. in-house or commercial ELISA or in-house MIA assays).

Laboratories that performed in-house ELISA and MIA 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.

2.4.1. Antiserum preparations

The following recommendations were provided to the participants:

- Ampoules of lyophilised sera should be stored at -20°C.
- Reconstituted samples should be divided into aliquots and stored at -20°C.
- The aliquots should be used only once and freeze-thawing was not recommended.
- An initial dilution at approximately 1/100 of the reconstituted sera samples, followed by 1/2 dilutions, would be suitable.

Information on the volumes to use for reconstituting each sera sample were also included in the shipment package.

In addition, individual laboratories were encouraged to perform a pilot study to choose the suitable dilution for the samples under their own experimental conditions.

2.5. Study design

Participants were asked to complete the assay sheets (describing in detail their assay procedure/conditions) and return them to NIBSC, together with the raw data for each assay.

2.5.1. For laboratories using their own in-house ELISA and MIA

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 eight serum preparations (reference serum and seven testing sera). Participants were also asked to include their in-house reference serum (IHR) in all the assays. 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 or three replicates per assay, and preferably each dilution curve would include at least four doses in the linear region. All preparations were to be included in each assay.

Laboratories were also advised to use their own methodology, reagents and calculation methods, to include their in-house references and controls, and to use assay runs that met their internal validity criteria.

2.5.2. For laboratories using commercial ELISA kits

The participating laboratories were asked to perform a minimum of three independent assays on the panel of eight serum preparations provided in this study, using the commercial ELISA kit that is routinely used in their laboratory. Participants were asked to include the reference serum provided in the kit in all of the assays. The three independent assays were to be run on three different days. In each assay, dilution curves for each preparation were to have at least two replicates per assay and preferably each dilution curve would include at least four doses in the linear region.

Laboratories were also advised to use the methodology and validity criteria recommended by the kit manufacturer.

2.6. Statistical methods

The participating laboratories submitted the raw data for each assay to NIBSC for analysis to ensure, as much as possible, consistent calculation of results and to allow inter-laboratory comparison. All analyses were performed using the European Directorate for the Quality of Medicines' CombiStats software (Version 6.1). For all assays, the data for each test preparation were analysed separately against the reference preparation.

Parallel line analysis of log-transformed assay response against log dose was performed, selecting a linear section of the dose-response curve. Linearity was assessed visually, and parallelism was assessed by looking at the ratio of slopes between the test and reference samples. Samples were considered non-parallel if the ratio of slopes fell outside the range of 0.80 to 1.25.

All laboratory mean potency estimates shown are weighted or semi-weighted geometric mean (GM) estimates calculated according to the methods described in Chapter 5.3 of the *European Pharmacopoeia* [13]. Semi-weighted means have been used where significant heterogeneity of assay estimates was detected ($p<0.05$). Overall mean potencies and confidence intervals are calculated as unweighted GM values. Variability is expressed as a percentage using a geometric coefficient of variation (GCV) (GCV = {10s-1}x100% where s is the standard deviation of the $\log_{10}$ transformed potency estimates).

3. Results

3.1. Summary of data returned

Of the 17 laboratories that received samples, 16 completed questionnaires and submitted results. Laboratory 5 failed to return results due to a lack of resources during the COVID-19 pandemic. All other participants performed either an in-house or commercial ELISA or MIA, with the exception of Laboratory 12, which used both an in-house and a commercial ELISA, as well as an MIA. Thus, data from a total of 18 assay methods were returned for analysis. Three laboratories used their own in-house ELISA methods, 13 laboratories used commercial kits from six different manufacturers, and two laboratories used MIAs. The different kit manufacturers are referred to as Manufacturer A to F. All of the laboratories that performed in-house ELISAs and MIAs also submitted the raw data from each assay, allowing for recalculation at NIBSC using a common statistical analysis. Of the 13 laboratories that performed assays using commercial ELISA kits, four performed a range of dilutions and returned the raw data so that common analysis could be performed by NIBSC.

3.2. Estimates of anti-pertussis toxin IgG concentrations in serum samples

Table 2 presents the statistical analysis of data supplied by participants using in-house ELISA and MIA methods that could be analysed using parallel line analysis at NIBSC. As expected, Sample G was found to be outside the response range of the reference or under detection limits for the majority of the assays and, therefore, has been omitted.

For the in-house ELISA/MIA assays (N = 5), the GM for Sample A was 82.45 IU/ml, for Sample B was 107.54 IU/ml, for Sample C was 56.84 IU/ml, for Sample D was 21.74 IU/ml, for Sample E was 131.60 IU/ml, for Sample F was 40.36 IU/ml and for Sample H was 73.23 IU/ml.

Table 3 shows the results of the common analysis performed by NIBSC using parallel line analysis for the commercial kits from four laboratories that performed a range of dilutions. It also shows participants' own calculated results from the remaining nine laboratories that performed single point assays and were therefore not suitable for parallel line analysis. Overall, all assays correctly identified Sample G as the negative control, as indicated by the fact that this sample gave a non-parallel response (<5 IU/ml).

The majority of kits generated results with the expected values. However, the kit from Manufacturer B (used by Laboratories 2 and 10) generated results approximately twice the expected value of most of the samples. Therefore, the GMs for each sample are presented with these participants both included and excluded from the calculations. With Laboratories 2 and 10 excluded, slightly lower GM estimates were found for commercial kit assays (N = 11) (Sample A: 78.44 IU/ml, Sample B: 98.77 IU/ml, Sample C: 61.96 IU/ml, Sample D: 22.59 IU/ml, Sample E: 128.88 IU/ml, Sample F: 42.88 IU/ml and Sample H: 76.76 IU/ml). With Laboratories 2 and 10 included, the GMs for each sample are much higher (data not shown).

The overall GM of WHO Reference Reagent (Sample B), was found to be 107 IU/ml for in-house methods and 99 IU/ml for commercial kits.

The GCVs for the laboratories using in-house ELISA or MIA assays were 20-28% for the different samples (Table 2). For the laboratories using commercial kits, the inter-laboratory GCVs ranged from 12-51% for all participants (Table 3). When Laboratories 2 and 10 are removed, the range is 11-24%. Histograms of the GM for all sera samples (except Sample G (negative)) from each replicate calculated at NIBSC using parallel line analysis against the WHO Reference Reagent (06/142) are presented in Figure 1. Figure 2 shows the histograms of the GMs for the same samples from the participants' own calculated results.

Table 2. Results of in-house ELISA and MIA assays calculated relative to the WHO Reference Reagent at NIBSC* or reported by participants

Lab	Source of Ag**	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F	Sample G	Sample H
4	In-house	97.32	121.64	97.38	34.76	131.49	55.72	RR	74.66
		104.96	122.90	54.73	20.72	111.94	29.88	RR	72.15
		64.63	95.22	49.85	11.71	151.80	33.03	RR	82.44
	GM	**101.32**	**112.58**	**52.41**	**20.78**	**129.90**	**38.62**	**–**	**74.15**
6	Glaxo-Smith-Kline	70.98	87.97	47.95	24.59	105.72	42.59	RR	54.12
		54.97	73.63	40.11	16.47	85.66	31.64	RR	49.51
		65.55	84.01	50.30	21.63	100.35	34.84	RR	57.63
	GM	**63.70**	**81.69**	**47.79**	**20.56**	**97.95**	**35.79**	**–**	**54.56**
11	In-house	105.27	102.81	76.39	24.61	172.32	54.11	RR	93.00
		110.27	117.77	78.94	27.19	179.94	52.48	RR	97.68
		101.56	125.89	79.32	23.57	182.92	50.46	RR	95.61
	GM	**106.61**	**111.51**	**77.22**	**26.17**	**177.74**	**52.18**	**–**	**96.03**
12a***	List Biological	77.55	145.89	54.39	24.57	133.38	42.47	RR	76.11
		53.18	170.86	41.80	17.21	115.88	26.96	RR	60.42
		64.96	99.17	44.31	22.33	119.31	36.66	RR	67.63
	GM	**64.58**	**134.94**	**46.67**	**21.16**	**120.47**	**34.78**	**–**	**67.77**
12b***	List Biological	91.74	100.54	71.95	NR	165.13	45.42	–	84.04
		68.11	110.33	NR	NR	133.46	NR	–	76.01
		82.77	90.50	63.84	20.52	146.30	42.55	–	87.85
	GM	**85.73**	**103.92**	**65.73**	**20.52**	**144.86**	**42.67**	**–**	**79.99**
GM of lab GMs		82.45	107.54	56.84	21.74	131.60	40.36	–	73.23
95% limits		(60.97-111.49)	(85.85-134.71)	(43.35-74.53)	(19.10-24.75)	(100.06-173.07)	(32.92-49.47)	–	(56.52-94.88)
GCV		28%	20%	24%	11%	25%	18%	–	23%

GCV: geometric coefficient of variation; GM: geometric mean; RR: outside the response range for the reference.
** See Section 2.6. Statistical methods.*
*** All participants used purified PT as coating antigen.*
**** Laboratory 12 provided two sets of results using ELISA and MIA. The respective results are therefore labelled as 12a and 12b.*

Table 3A. Results of commercial kit assays calculated relative to the WHO Reference Reagent at NIBSC* (where possible) or reported by participants

Lab	Kit manufacturer	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F	Sample G	Sample H	Comments
1	Manufacturer A	81.66	87.72	NP	28.13	107.13	40.67	RR	81.19	_
		81.76	96.20	54.88	28.10	131.67	41.96	RR	79.51	
		82.83	87.62	53.80	28.37	124.13	38.83	RR	82.91	
	GM	**81.96**	**90.93**	**54.37**	**28.20**	**121.53**	**40.52**	ND	**81.03**	
2	Manufacturer B	190.50	283.33	135.45	30.41	>350	74.92	<1	248.22	Participant reported results
		213.43	317.09	159.61	32.57	>350	73.80	<1	248.30	
		271.40	335.97	147.94	32.11	>350	86.84	<1	240.18	
	GM	**222.63**	**311.35**	**147.33**	**31.68**	**>350**	**78.30**	**<1**	**245.53**	
3	Manufacturer C	85.50	90.50	68.00	21.00	110.50	43.50	<5	79.50	Participant reported results
		84.50	96.50	71.00	24.00	136.50	49.50	<5	66.00	
		78.50	90.50	68.00	24.00	123.00	47.50	<5	69.00	
	GM	**82.77**	**92.46**	**68.99**	**22.96**	**122.88**	**46.77**	**<5**	**71.27**	
7	Manufacturer A	65.77	93.13	55.79	19.22	99.55	26.43	RR	72.28	_
		70.88	86.10	58.55	20.90	122.75	38.42	RR	63.55	
		71.88	107.09	61.86	23.51	143.97	42.20	RR	86.44	
	GM	**67.81**	**93.89**	**57.00**	**20.96**	**121.83**	**36.89**	ND	**73.36**	
8	Manufacturer C	77.72	99.74	58.64	20.09	122.71	37.86	RR	59.73	_
		74.99	96.07	56.45	21.74	123.71	39.30	RR	63.47	
		75.39	99.95	55.99	20.57	126.82	37.08	RR	64.58	
		68.72	ND	52.29	ND	ND	35.68	ND	63.17	
		77.16	ND	60.67	ND	ND	40.91	ND	71.75	
	GM	**74.91**	**99.67**	**56.72**	**20.73**	**124.11**	**38.12**	ND	**64.46**	
9	Manufacturer D	87.7	107	76.4	23.6	123	55.5	<10.0	90	Participant reported results
		87.6	105	73.7	22.9	121	53.5	<10.0	89.4	
		91.2	108	74.3	23.6	122	54	<10.0	93.6	
	GM	**88.82**	**106.66**	**74.79**	**23.36**	**122.00**	**54.33**	**<10**	**90.98**	
10	Manufacturer B	192.5	279.0	111.0	21.0	>350	83.5	<1	188	_
		142.5	205.0	117.0	28.0	>350	78.0	<1	149	
		161.5	269.0	139.0	32.0	>350	76.0	1.0	201.5	
	GM	**164.24**	**248.72**	**121.76**	**26.60**	**>350**	**79.10**	**1.00**	**178.05**	
12c	Manufacturer A	63.7	86.9	51.2	20	107.3	36.8	1.4	61.9	Participant reported results
		64.2	87.2	49.1	23	110	36.7	2.3	64.6	
		69.56	100.28	48.8	18.2	103.5	35.1	0.5	63.8	
	GM	**65.77**	**91.25**	**49.69**	**20.31**	**106.90**	**36.19**	**1.17**	**63.42**	
13	Manufacturer A	56.84	109.03	63.87	24.77	148.59	36.95	0.4	82.72	Participant reported results
		54.38	110.57	65.85	26.73	149.55	39.99	1.66	87.45	
		61.12	106.21	58.99	23.04	129.54	36.91	ND	72.5	
	GM	**57.38**	**108.59**	**62.84**	**24.80**	**142.25**	**37.92**	**0.81**	**80.64**	
14	Manufacturer E	124	127	77	14	150	58	1	95	Participant reported results
		122	131	82	17	148	53	1	99	
		127	129	76	20	153	58	1	97	
	GM	**124.32**	**128.99**	**78.29**	**16.82**	**150.32**	**56.28**	**1.00**	**96.99**	
15	Manufacturer C	89.4	103.2	76	32.5	147.6	45.1	<5	86.8	Participant reported results
		90.75	114.05	78.1	28.9	160.25	58.55	<5	88.35	
		89.43	99.13	73.20	24.83	133.33	54.60	<5	79.87	
	GM	**89.86**	**105.28**	**75.74**	**28.57**	**146.65**	**52.44**	**<5**	**84.92**	
16	Manufacturer F	84.73	95.85	56.03	18.37	147.50	40.68	RR	77.95	ND
		95.92	110.44	69.96	22.05	159.40	47.34	RR	90.75	
		82.47	121.13	59.24	NP	155.85	38.70	RR	74.98	
	GM	**85.23**	**100.89**	**59.54**	**19.33**	**151.40**	**40.80**		**77.32**	

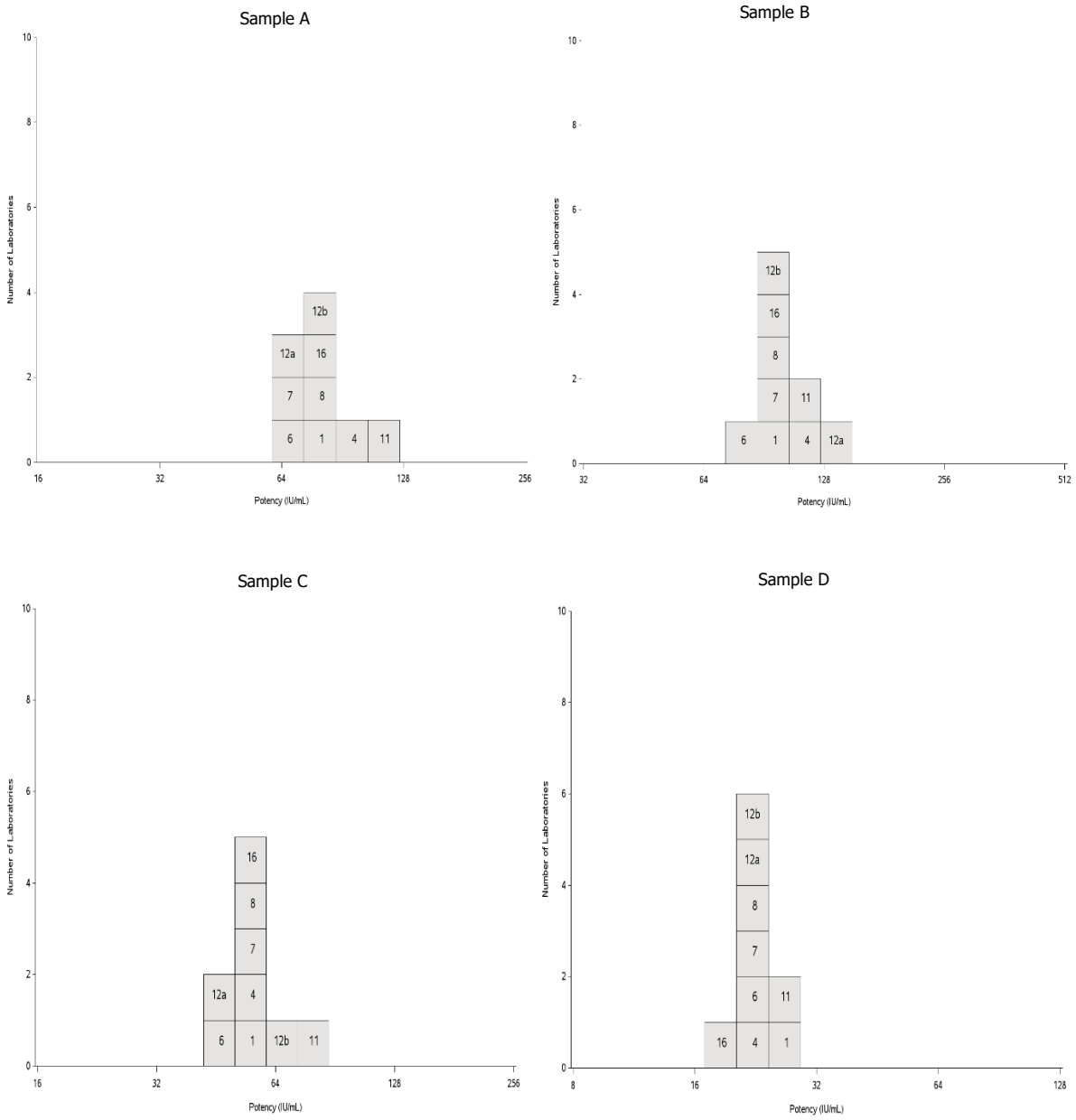
GM: geometric mean; ND: not determined; NP: non-parallel at 1% level; RR: outside the response range for the reference.
** See Section 2.6. Statistical methods.*

Table 3B. Results of commercial kit assays calculated relative to the WHO Reference Reagent at NIBSC* (where possible) or reported by participants

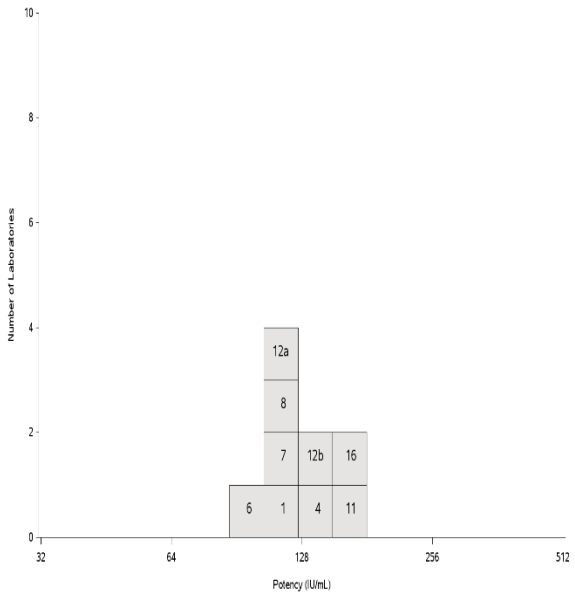
Lab	Kit manufacturer	Sample A	Sample B	Sample C	Sample D	Sample E	Sample F	Sample G	Sample H	Comments
17	Manufacturer A	61.7	97.2	47.3	24.3	129	39.5	2.75	62.6	Participant reported results
		57.8	93.9	50.5	25.2	105	37.9	2.67	70.8	
		69.8	49.2	57.9	26.7	117	35.8	2.86	69	
	GM	**62.91**	**76.58**	**51.71**	**25.38**	**116.59**	**37.70**	**2.76**	**67.37**	
GM of lab GMs		89.97	115.83	69.77	23.47	128.88	47.08	–	89.56	–
95% limits		(70.97-114.05)	(90.41-148.39)	(57.27-84.99)	(21.07-26.16)	(119.23-139.32)	(39.88-55.58)	–	(70.23-114.20)	–
GCV		48%	51%	39%	20%	12%	32%	–	50%	–
Excluding results from Labs 2 and 10										
GM of lab GMs		78.44	98.77	61.96	22.59	128.88	42.88	–	76.76	–
95% limits		(67.93-90.58)	(90.35-107.96)	(55.64-69.01)	(20.25-25.19)	(119.23-139.32)	(38.32-47.99)	–	(69.96-84.22)	–
GCV		24%	14%	17%	18%	12%	18%	–	15%	–

GCV: geometric coefficient of variation; GM: geometric mean; NP: non-parallel at 1% level.
** See Section 2.6. Statistical methods.*
All kits used purified PT.

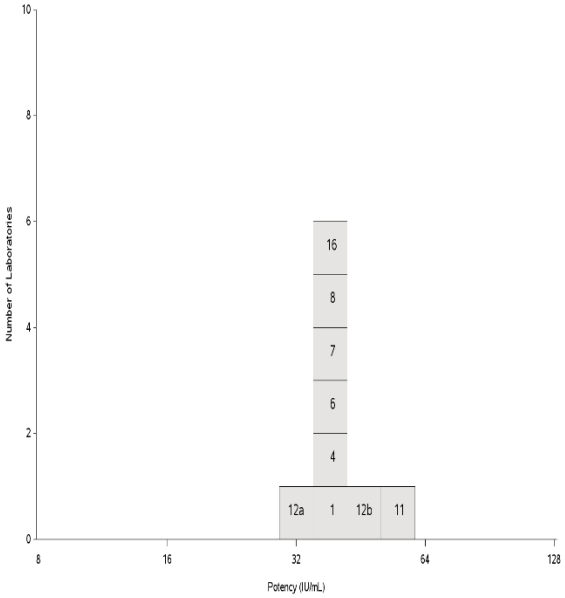
Figure 1. Histograms of geometric mean estimates (IU/ml) for each participant's results for all sera samples (except G) calculated relative to the WHO Reference Reagent at NIBSC



Sample E



Sample F



Sample H

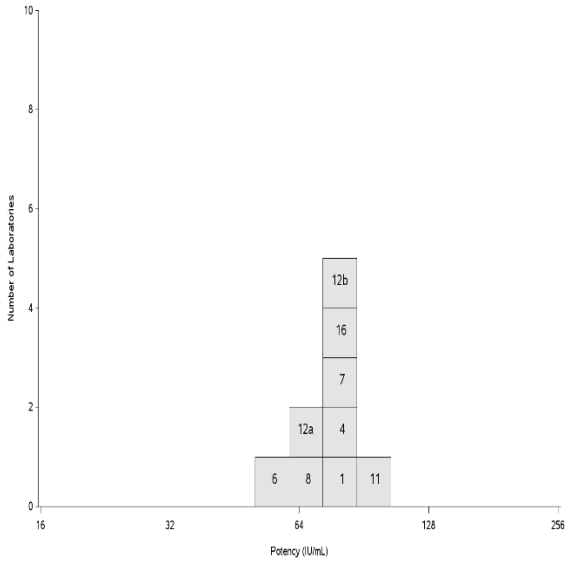
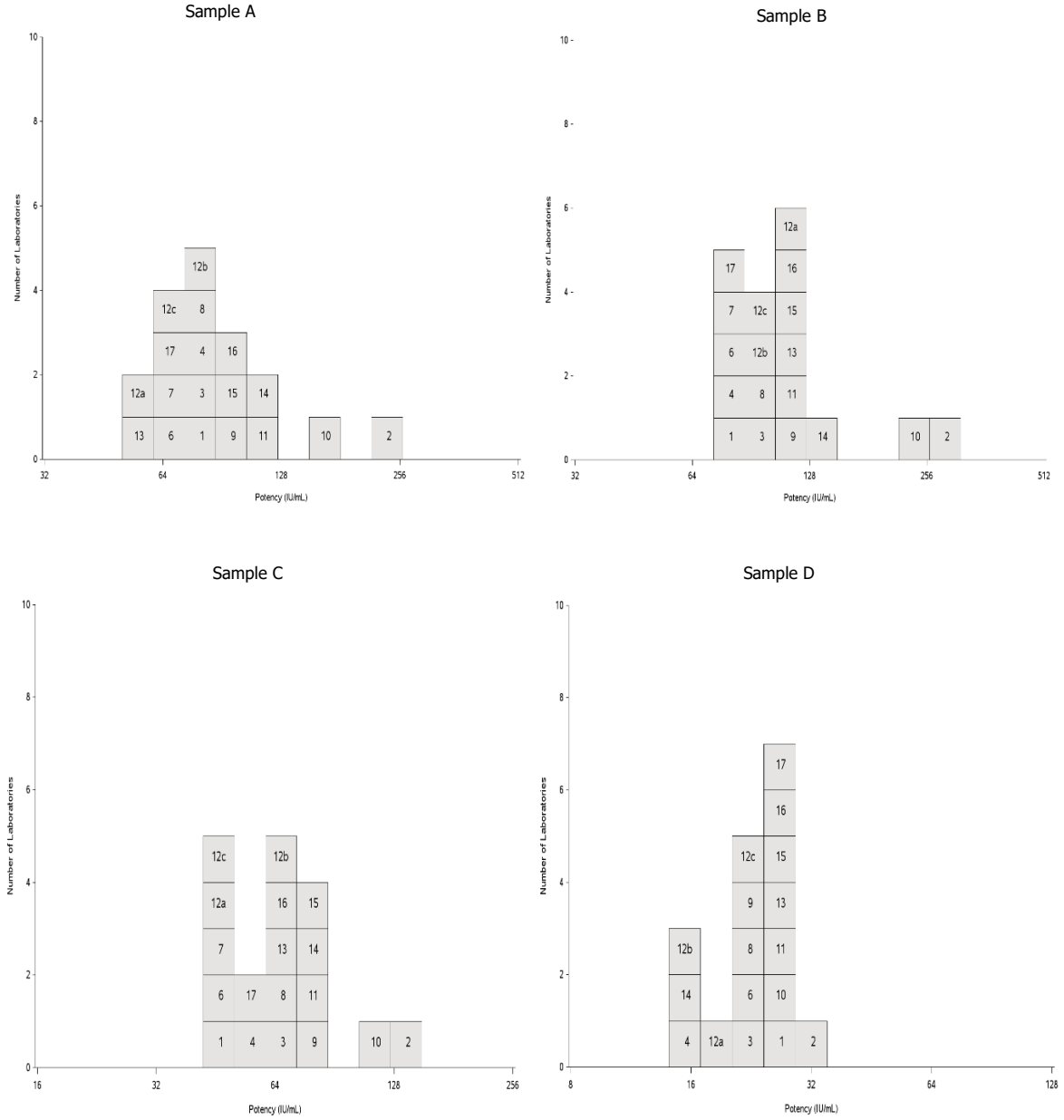
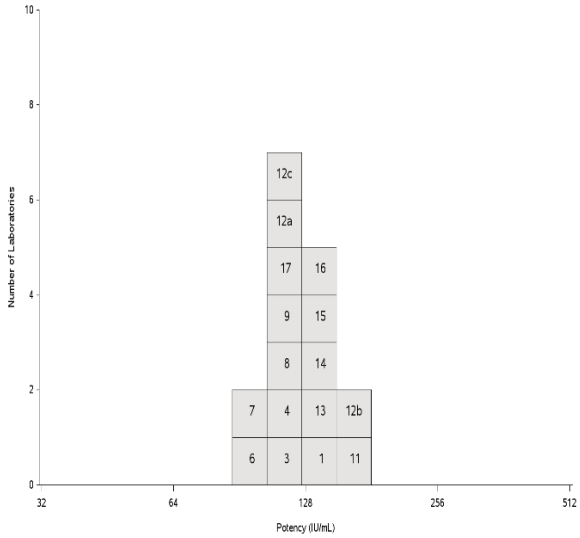


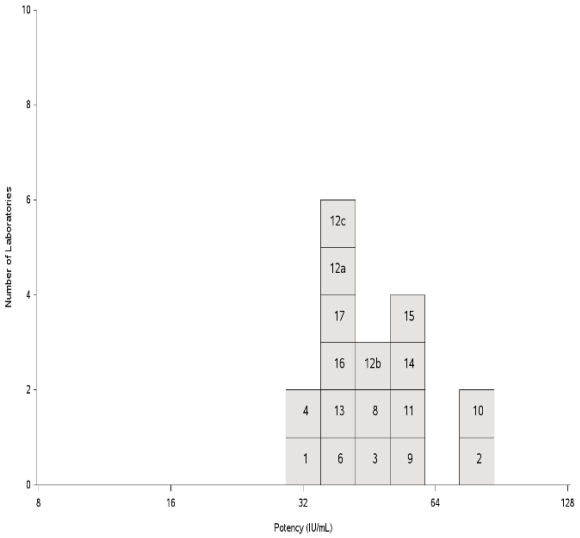
Figure 2. Histograms of geometric mean estimates (IU/ml) for each participant's results for all sera samples (except G) calculated relative to routine reference sera



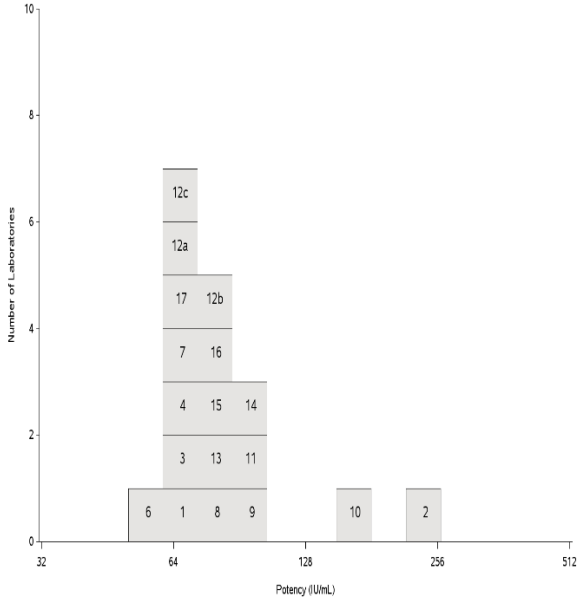
Sample E



Sample F



Sample H



3.3. Ranking of sera samples in increasing IgG concentrations

Another aim of this EQA was to assess the ability of the participants to quantify the anti-PT IgG concentration of blinded sera samples and to rank them by increasing concentrations. Table 4 shows the sera samples ranked in order of increasing anti-PT IgG concentrations, based on both results calculated at NIBSC using parallel line analysis (where applicable) and final concentrations determined by the participants using their routine in-house methods. Of the 18 data sets returned, 17 were ranked in the expected order using the participant's reported in-house results, those calculated at NIBSC or both. However, participants' in-house results did not correspond to the NIBSC analysis of the same data in three instances (Laboratories 1, 4 and 12a) and the associated rankings were therefore not in the expected order. In the results generated by Laboratory 13, Sample C was higher than expected.

Table 4. Sera samples ranked in order of increasing anti-PT IgG concentrations, NIBSC analysis (where applicable) and participants' in-house results

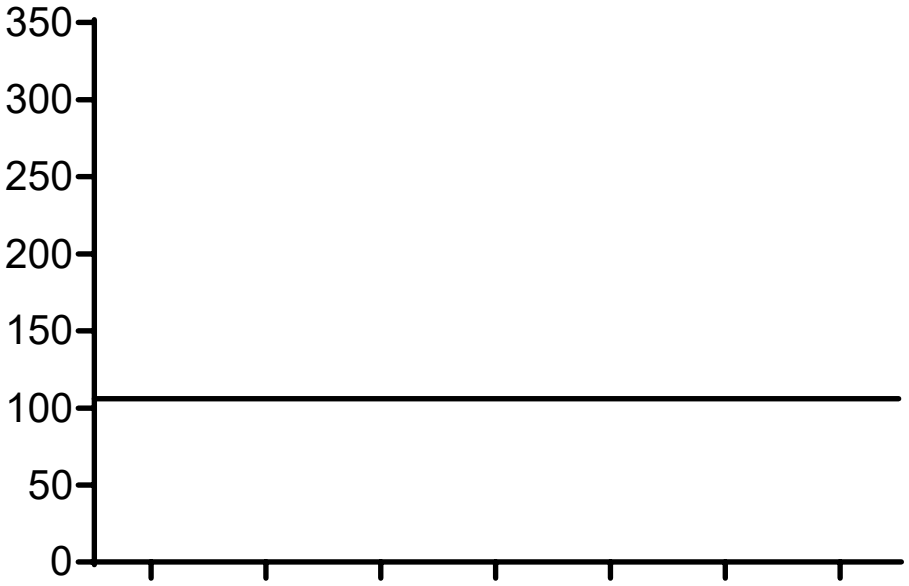
Laboratory	Method of calculation	Ranking by increasing anti-PT IgG concentration							
		1	2	3	4	5	5	6	7
Expected rank order		**G**	**D**	**F**	**C**	**H/A**	**A/H**	**B**	**E**
1	NIBSC analysis	G	D	F	C	H	A	B	E
1	Participant's in-house results	–	D	F	C	H	B	A	E
2	Participant's in-house results*	G	D	F	C	A	H	B	E
3	Participant's in-house results**	G	D	F	C	H	A	B	E
4	NIBSC analysis	G	D	F	C	H	A	B	E
4	Participant's in-house results	G	D	F	C	H	B	A	E
5	Did not return results	–	–	–	–	–	–	–	–
6	NIBSC analysis and participant's in-house results	G	D	F	C	H	A	B	E
7	NIBSC analysis***	G	D	F	C	A	H	B	E
8	NIBSC analysis and participant's in-house results	G	D	F	C	H	A	B	E
9	Participant's in-house results**	G	D	F	C	A	H	B	E
10	Participant's in-house results**	G	D	F	C	A	H	B	E
11	NIBSC analysis and participant's in-house results	G	D	F	C	H	A	B	E
12a	NIBSC analysis	G	D	F	C	A	H	E	B
12a	Participant's in-house results	G	D	F	C	A	H	B	E
12b	NIBSC analysis and participant's in-house	G	D	F	C	H	A	B	E
12c	Participant's in-house results*	G	D	F	C	H	A	B	E
13	Participant's in-house results*	G	D	F	A	C	H	B	E
14	Participant's in-house results*	G	D	F	C	H	A	B	E
15	Participant's in-house results*	G	D	F	C	H	A	B	E
16	NIBSC analysis and participant's in-house results	G	D	F	C	H	A	B	E
17	Participant's in-house results*	G	D	F	C	A	H	B	E

** Raw data not suitable for parallel line analysis.*
*** Raw data not returned.*
**** Participant's in-house results returned samples D, F and G as negative and are not included here.*
Rankings or results that deviated from what was expected are highlighted in red.

3.4. Comparison of values obtained for the WHO reference sample, Sample B

In this study, Sample B was a batch of freeze-dried human serum (NIBSC code: 06/144) that was prepared (as much as possible) in the same way as the current WHO Reference Reagent Pertussis Antiserum (Human) 1st Reference Reagent (NIBSC code: 06/142). It was prepared from the same pool of serum, but on two separate days, and was blinded in this EQA. A previous collaborative study calibrated the anti-PT IgG for this standard at 106 IU/ml when the current 1st Reference Reagent was being established [12]. Figure 3 compares the different ELISA and MIA methods that returned raw data that could be analysed at NIBSC, based on the type of method used and the results calculated by the participants when the model could not be used. The means for the in-house methods and the majority of kits were close to the expected value of 106 IU/ml. The most notable exception was the kit produced by Manufacturer B and used by Laboratories 2 and 10. The mean for this kit was over twice the expected value at 282 IU/ml. The mean values for the remaining test methods ranged from 93 IU/ml to 129 IU/ml.

Figure 3. Box plots of the quantitative results of anti PT-IgG ELISAs for Sample B calculated against the Reference Reagent 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 indicate outliers and the solid line represents the mean.

4. Discussion

ELISA tests for measuring antibodies to pertussis antigens have been widely employed in acellular vaccine clinical trials and in sero-epidemiological studies [1,14]. They have also become more popular for diagnostic purposes [10]. While in-house ELISA methods have been used for years, recently, the number of diagnostic laboratories using different commercial kits has increased [6]. The ability of national reference laboratories from EU/EEA Member States to correctly perform laboratory serodiagnostic tests for pertussis is the main focus of this EQA. The scheme helped to identify methodologies in need of improvement and areas for training, including where particular laboratories could improve their methods, procedures and global performance.

To achieve the primary aim of the EQA, participating laboratories evaluated eight serum samples to determine concentrations of anti-PT IgG against a reference sera preparation using immunoassays. Through this exercise, it was possible to ascertain how well the assays currently being used by different national pertussis reference laboratories in Europe could differentiate the sera samples and quantify the concentrations of anti-PT IgG.

Of the 16 participating laboratories, 13 used commercial kits for their serological diagnosis of pertussis. All laboratories were able to distinguish the negative sample G (estimated concentration of IgG-PT <2 IU/ml) from the other samples with higher anti-PT IgG concentrations. The majority of participants obtained results close to the expected values for the remaining samples in the panel and 17 (94%) of the 18 data sets returned ranked the samples in the expected order (Table 4) using the participants' reported results, those calculated at NIBSC or both. However, there were three instances where the participants' results did not correspond with those calculated at NIBSC and the rankings were different. One sample containing 80 IU/ml anti-PT IgG was duplicated in the panel as samples A and H. For the in-house methods, the GM for sample A was 82.45 IU/ml, but for sample H the value was lower at 73.23 IU/ml. There was greater consistency in the results obtained using kits, with the GM of sample A being 88.85 IU/ml and sample H being 89.87 IU/ml for all participants and 77.29 IU/ml and 77.08 IU/ml with Laboratories 2 and 10 removed. It should be noted that this EQA study was conducted during the COVID-19 pandemic and that many of the participating laboratories could have been involved in the diagnosis of SARS-CoV-2 infection in their respective countries, thus reducing their laboratory capacity for other diagnostic work.

In EQAs undertaken in 2016 and 2012, 23 (88%) of the 26 participating laboratories and 19 (91%) of 21 participating laboratories, respectively, ranked the samples in the expected order. The results of these and the present EQA indicate satisfactory performance of these assays during the past decade.

In the 2012 EQA, 10 (45%) participating laboratories used in-house ELISA or MIA methods and 12 (55%) used commercial kits, while 8 (31%) used in-house methods and 18 (69%) used commercial kits in 2016. The growing trend in the use of commercial kits in diagnostic laboratories continued in this EQA, with 13 (81%) participating laboratories using them in this study. In the previous EQAs, participants used kits from six manufacturers. Six commercial kits were also employed in the present study, but the kits from two manufacturers (Manufacturers B and E) were used for the first time in this study. Previous studies have indicated that commercial kits coated with a mixture of antigens and not just purified PT have insufficient power to distinguish samples with high and low anti-PT IgG concentrations, and were found to be less accurate than methods that used purified PT [6,8,9,15,16]. In this EQA, all participants used purified PT as the coating antigen.

Despite having purified PT only as coating antigen and including reference sera calibrated in IU/ml against the WHO standards, the kit produced by Manufacturer B generated results that were approximately twice the expected values. The only sample that was close to the expected value was the low titre, non-negative sample D. This kit was used by Laboratories 2 and 10, and both generated consistently high results (Table 3, Figures 2 and 3). This kit was not used in previous studies. This overestimation of the anti-PT IgG concentration in samples may result in false positive diagnoses and effect surveillance data. The kit produced by Manufacturer E and used by Laboratory 14 was also seen for the first time in this study. While coated with PT, it also generated results that were higher than expected. Although the in-house results from Laboratory 14 were closer to the expected values than those from Laboratories 2 and 10, the GM for Sample B was 129 IU/ml, which is 22% higher than the expected value of 106 IU/ml (Figure 3). The reasons why these two kits produced higher than expected results is unknown.

Five laboratories used kits from Manufacturer A (Laboratories 1, 7, 12c, 13 and 17). The results obtained were consistent between users, with the GMs slightly lower than the overall GMs. The results were approximately 10-15% below the expected values. For sample B, the mean of all assays using this kit was 93 IU/ml, compared to the expected value of 106 IU/ml (Figure 3).

The kit produced by Manufacturer C was used by three laboratories (Laboratories 3, 8 and 15). Overall, the results obtained from each participant were close to the expected values. Laboratories that used this kit obtained a range of 92 IU/ml (Laboratory 3) to 105 IU/ml (Laboratory 15) for sample B, indicating the overall accuracy of the kit.

The kits produced by Manufacturer D and Manufacturer F were each used by a single participant (Laboratories 9 and 16, respectively). Both generated results close to the expected values. The calculated results for sample B were 107 IU/ml and 109 IU/ml, respectively, indicating the accuracy of these kits.

In general, the increasing use of commercial kits for routine diagnosis of pertussis makes accurate diagnosis more challenging, as the quality of some kits may not be good enough and could produce false positive results (as shown in this EQA study), which may result in inaccurate surveillance data. It is unclear how many different ELISA kits are available for serological diagnosis of pertussis in Europe and how these kits are being used in local diagnostic laboratories.

In the present study, all assay methods (100%) followed the recommendations and used purified PT only as the coating antigen. In the 2016 EQA, 24 (92%) of 26 assay methods used purified PT as coating antigen, while two used a mixture of PT and filamentous haemagglutinin (FHA) [9]. This was an increase from the previous EQA, in which 78% of the methods used purified PT as the coating antigen [6]. An EQA from 2010 found that 13 (65%) of 20 participants used purified PT, while the remaining labs used PT plus FHA (n = 5), PT plus FHA with LPS (n = 1), or whole cell lysate (n = 1) [10]. Taken together, these studies indicate that an increasing number of laboratories are following the ECDC recommendations [8].

Five different sources of purified PT were used for in-house ELISAs and MIAs in the present study (Table 2), and the results were independent of these sources. There was a difference in the variability of in-house methods compared to commercial kits. The GCV for in-house methods ranged from 11-28%, while the range for commercial kits was 12–71% (Table 3). The high GCV in the kit assays was also due to unexpectedly high readings from two kits (Manufacturers B and E); when the results from these kits were removed, the GCV ranged between 11–17%.

Pertussis Antiserum (Human) 1st WHO Reference Reagent (06/142) has been assigned unitage of 106 IU/ampoule of anti-PT IgG in an international collaborative study [12]. Of the 18 assay methods returned, 13 also reported calculated values for the 1st WHO Reference Reagent using routine methods and references, of which two were in-house methods. The overall GM of these in-house methods was 98 IU/ml. The range of values obtained was 84–106 IU/ml. This may suggest that in-house references used by these laboratories are reasonably calibrated in terms of the 1st WHO International Standard. The results from the 11 laboratories that used commercial kits (all of which calculated the concentration of 06/142 in IU/ml) were found to range from 65 IU/ml to >350 IU/ml. The overall GM for kits was 117 IU/ml. However, this range and GM includes results from Laboratories 2 and 10; when these are removed, the GM is 100 IU/ml. In the present study, all reference sera in the kits were calibrated in IU/ml using the WHO International Standard (06/140), suggesting that standardisation of commercial ELISA kits for pertussis diagnosis is progressing. Since many routine laboratories perform serological diagnosis using commercial kits, guidelines on how to evaluate commercial kits may be useful.

5. Conclusions

Overall, the present study indicated that the recommendations to use only purified PT as coating antigen and to include reference sera calibrated in IU/ml are being followed. The overall accuracy of sample scoring and the fact that all laboratories detected the negative preparation indicate good laboratory practice and methods. However, there was variability among both kits and in-house methods, which highlights the ongoing need for EQA studies in the future. A continued increase in the number of laboratories using kits has been observed; however, it is important that they utilise high-quality purified PT as coating antigen and include reference sera calibrated in IU/ml. It may also be beneficial for diagnostic laboratories to have access to more detailed guidelines on how to evaluate commercial kits. Regular EQAs are also valuable in the context of numerous and new manufacturers. Finally, the current EQA was performed in 2020, during the COVID-19 pandemic. Nonetheless, 16 of the 17 participants were able to return results, indicating that they have the capacity to continue testing for *B. pertussis* infection during trying circumstances.

6. Recommendations

The present study indicates that there is a further increasing trend towards the use of commercial kits for serodiagnosis of pertussis. Based on the results of this study, laboratories currently using or considering switching to using commercial kits should consider a number of factors. Firstly, the ELISA plates in kits should be coated with purified PT only and reference serum should be calibrated in IU/ml using one of the international standards. The kits should also be CE-marked to conform with appropriate regulations.

In the future, more laboratories may use MIA methods, as antibodies against multiple antigens can be analysed in a shorter timeframe and with less sample sera than with conventional ELISA. However, the recommendations for MIAs remain the same as those for ELISAs in relation to coating antigen and reference sera.

The results of the present EQA were more satisfactory than those of the previous studies [6,9], indicating the benefits of regular EQA studies. It is difficult to set a fixed timeframe for when EQAs should be performed, but they should be considered when changes are made to routine tests, such as switching from in-house ELISA to using commercial kits or implementing an MIA. The serum panel used in the present study is available from NIBSC (code: 18/146) and can be used to determine the performance of new or existing assay methods, as reagents can degrade over time or due to repeated freeze-thaw cycles. A large number of anti-PT IgG

concentrations are used as cut-off values to determine the presence of recent infection [7]. It may also be useful for the pertussis diagnostic community to decide on uniform cut-off values in IU/ml.

Overall, the present EQA study found that using a range of dilutions and comparing sera samples to the Pertussis Antiserum (human) 1st WHO Reference Reagent (06/142) on plates only coated with purified pertussis toxin (PT) facilitated accurate determination of anti-PT IgG concentrations in IU/ml. This corresponds to observations made previously. Therefore, the previous recommendation should be reinforced that only purified PT is to be used in immunoassays, whether for in-house ELISA, commercial kits or MIAs for serological testing of anti-PT IgG in human sera. Also, a reference serum should be included in each assay and it should be calibrated in IU/ml using one of the international standards.

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Annex

Table 1A. List of EQA participants

Country	Contact person	Laboratory/Institution
Belgium	Isabelle Desombere	National Reference Centres for *Bordetella pertussis*, Sciensano
Croatia	Selma Bošnjak	Bacteriological serology section, Department of Microbiology, Croatian Institute of Public Health
Czechia	Jana Zavadilova	National Reference Laboratory for Pertussis and Diphtheria, National Institute of Public Health
Denmark	Tine Dalby Charlotte Sværke Jørgensen	Infectious Disease Preparedness, Statens Serum Institut
Estonia	Liidia Dotsenko	Laboratory of Communicable Diseases, Health Board, Tallinn
Finland	Qiushui He Alex-Mikael Barkoff	University of Turku, Institute of Biomedicine, Center for Infections and Immunity
Hungary	Ildiko Paluska Ferencz	National Public Health Center, Department of Bacteriology Mycology and Parasitology
Ireland	Adele Habington	Microbiology Department, Children's Health Ireland at Crumlin
Italy	Giorgio Fedele Paola Stefanelli	Department of Infectious Diseases, Istituto Superiore di Sanità
Latvia	Sanita Kuzmane	Riga East University Hospital, Laboratory service, Laboratory "Latvian Centre of Infectious Diseases" (National Microbiology Reference Laboratory)
Netherlands	Pieter van Gageldonk Gerco den Hartog	Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM)
Norway	Tove Karin Herstad Audun Aase	Norwegian Institute of Public Health
Portugal	Paula Palminha Raquel Neves	National Institute of Health Doutor, Ricardo Jorge, Department of Infectious Diseases
Romania	Cristina Oprea	"Cantacuzino" National Institute for Medical Military Research - Development
Slovakia	Lucia Maďarová	Regional Authority of Public Health Banská Bystrica
Slovenia	Tamara Kastrin	National Laboratory of Health, Environment and Food, Department for Public Health Microbiology
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