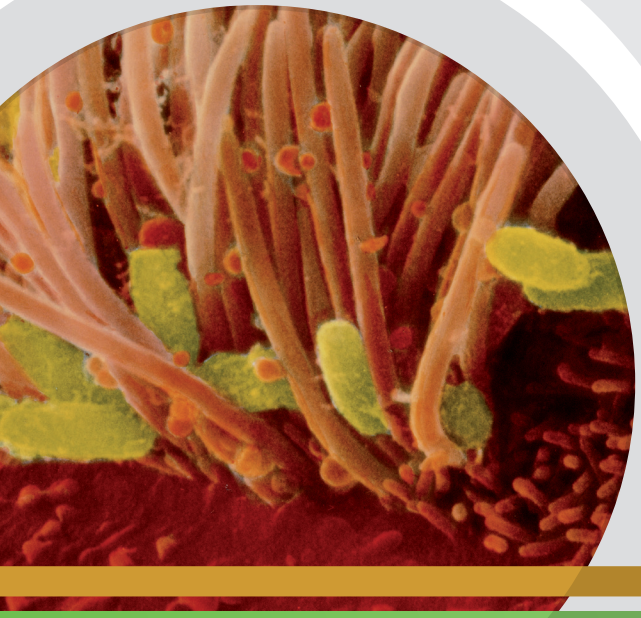

TECHNICAL REPORT

External quality assurance scheme on PCR for *Bordetella pertussis*, 2012

On behalf of EUpert-labnet network

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ECDC TECHNICAL REPORT

External quality assurance scheme on PCR for *Bordetella pertussis,* 2012

On behalf of the EUpert-labnet network

This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Dr Adoracion Navarro Torné and produced by Dr Norman Fry, Health Protection Agency (London, UK), Dr Tine Dalby, Statens Serum Institut (Copenhagen, Denmark) and Dr Qiushui He, National Institute for Health and Welfare (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).

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Contents

List of tables

Abbreviations

CFU	Colony forming unit(s)
Cq	Crossing threshold
DSN	Dedicated surveillance network
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EQA	External quality assurance
EU	European Union
EUpert-labnet	Consortium of European pertussis experts funded by ECDC for this current programme
EUVAC.NET	A former European surveillance network for selected vaccine-preventable diseases hosted at the Statens Serum Institut, Denmark
HPA	Health Protection Agency (UK)
IS	Insertion sequence element
NIBSC	National Institute for Biological Standards and Control (UK)
PCR	Polymerase chain reaction
QCMD	Quality Control for Molecular Diagnostics (Scotland)
qPCR	Quantitative (real-time) PCR
RSIL	Respiratory and Systemic Infection Laboratory (of the HPA, London)
THL	Terveyden ja Hyvinvoinnin Laitos / National Institute for Health and Welfare (Finland)

Executive summary

Twenty-one laboratories from 21 EU/EEA countries participated in the first external quality assurance scheme for *Bordetella pertussis* PCR by ECDC on behalf of the EUpert-labnet network. The panel included dilutions of purified genomic DNA from *B. pertussis* at three concentrations designated 'high', 'medium' and 'low'. Duplicate samples of the *B. pertussis* 'medium' dilution were included to test the reproducibility of the PCR assays. Genomic DNA from other *Bordetella* species — *B. parapertussis*, *B. holmesii*, *B. bronchiseptica* — were included in the panel, together with *Haemophilus influenzae*. Two 'blank' samples, i.e. with no added DNA, were also included to check for potential contamination.

Of 22 datasets (one laboratory submitted two datasets) all (100%) reported the 'high' concentration as positive for *B. pertussis* and 21/22 also reported the 'medium' concentration duplicate samples as *B. pertussis* positive.

Only 15 out of 22 reported the 'low' concentration as positive for *B. pertussis* or *Bordetella* spp.

Real-time *Bordetella* PCR assays (both in-house and commercial) demonstrated greater sensitivity than conventional PCR assays as demonstrated by 85% (11/13) of those using qPCR reporting the 'low' concentration positive for *B. pertussis* or *Bordetella* spp. compared with 57% (4/7) of those using conventional PCR.

None of the laboratories reported *Bordetella* DNA in the two blank samples, demonstrating good molecular laboratory practice.

The most common targets for *B. pertussis* and *B. parapertussis* were IS*481* and IS*1001*, respectively. However, these targets are not completely specific for these species. Therefore IS*481* positive-only results should be reported as probable *B. pertussis* (*B. pertussis* / *B. holmesii* / *B. bronchiseptica*) and IS*1001* positive-only results should be reported as probable *B. parapertussis* (*B. parapertussis* / *B. bronchiseptica*).

Only laboratories using specific *B. holmesii* assays (e.g. targeting *recA*) could correctly differentiate this species from other *Bordetella* species including *B. pertussis*.

Several commercial kits are available for the detection of *B. pertussis* and/or *B. parapertussis*; however, care should be taken in the interpretation of these results as indicated above.

An internal process control to check for the presence of PCR inhibitors is recommended to avoid false-negative reporting.

This report presents the results of the first external quality assurance (EQA) scheme for *Bordetella pertussis* PCR funded by the European Centre for Disease Prevention and Control (ECDC). The EQA study was conducted between February and March 2012.

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. Within 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.'[1]

External quality assurance (EQA) is part of quality management systems and evaluates performance of laboratories, by an outside agency on material that is supplied specially for the purpose. ECDC's disease-specific networks organise a series of EQA for EU/EEA countries. In some specific networks 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 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 external quality assurance schemes include:

- assessment of the general standard of performance;
- assessment of the effects of analytical procedures (method principle, instruments, reagents, calibration);
- evaluation of individual laboratory performance;
- identification of problem areas;
- providing continuing education;
- identification of training needs.

Pertussis (whooping cough) is an acute bacterial infection usually caused by *Bordetella pertussis*, which can affect people of all ages. A similar illness is caused by *Bordetella parapertussis*, but this is not affected by current pertussis vaccines. Infants are the most vulnerable group with the highest rates of morbidity and mortality, whilst older children and adults usually display milder symptoms. Increases in both awareness of pertussis infections and reported numbers of cases in many countries have highlighted the need for good laboratory tests for the detection, identification and characterisation of clinical infections caused by *B. pertussis* and other *Bordetella* species.

The utility of PCR in the laboratory confirmation of *B. pertussis* infection is now well established and can provide improved sensitivity over culture [1,2]. In addition, improvements in both nucleic acid extraction and real-time PCR (qPCR) [3,4] technologies can now provide more rapid turnaround times [2,5].

The absence of existing EQA programmes for *B. pertussis* PCR in European diagnostic laboratories was reported in 2005 by Muyldermans, et al [2]. These authors described results obtained with two proficiency panels, sent to six and nine European Laboratories, respectively, containing: (i) a series of dilutions of three previously characterised *B. pertussis* clinical isolates and two negative controls; and (ii) a series of dilutions of reference strains of *B. pertussis*, *B. holmesii*, *B. hinzii*, and *B. bronchiseptica*, as well as negative controls. Results from the first panel revealed no false positives by six laboratories (seven datasets) and limits of detection of the three *B. pertussis* strains varied from 4 to 4 000, 9 to 9 000, and 3 to 30 000 CFU/ml. Results from nine laboratories for the second panel were as follows: one laboratory reported a positive result for one of the negative controls and also found a *B. parapertussis*-positive sample to be positive for *B. pertussis*. Eight laboratories using IS*481*-based assays, reported positive results for the samples containing *B. holmesii* and *B. bronchiseptica*. One laboratory using an assay targeting the pertactin gene designed to be specific for *B. pertussis* detected *B. pertussis* with 100% specificity illustrating the critical nature of the choice of the target gene for specific identification in *B. pertussis* PCR assays.

Since the above study, two commercial EQA programmes for *B. pertussis* PCR became available from Quality Control for Molecular Diagnostics (QCMD), Glasgow, Scotland and INSTAND e.V. Düsseldorf, Germany.

An ECDC-funded EQA distribution for the 'Evaluation and standardisation of real-time PCR for detection of *B. pertussis*' was first distributed by the Statens Serum Institut (Copenhagen, Denmark), under the auspices of the EUVAC.NET (ECDC Grant ECD.2042 Work area 4) in January 2011. A panel of eight samples comprising one negative, and seven containing *Bordetella* DNA (five with *B. pertussis* DNA in varying concentrations, one with *B. parapertussis* DNA and one with *B. holmesii* DNA). Twenty-four laboratories from 19 European countries participated in this EQA. *Bordetella parapertussis* was misidentified by three laboratories and *B. holmesii* was misidentified by 15 laboratories as either *B. pertussis* or *B. parapertussis*. Real-time PCR demonstrated greater

[1] Regulation (EC) No 851/2004 of the European Parliament and of the Council of 21 April 2004 establishing a European centre for disease prevention and control. OJ L 142, 30.4.2004, p. 1. Article 5(3).

[2] 2002/253/EC: Commission Decision of 19 March 2002 laying down case definitions for reporting communicable diseases to the Community network under Decision No 2119/98/EC of the European Parliament and of the Council (notified under document number C(2002) 1043).

sensitivity than traditional block-based PCR and two laboratories reported the negative sample as weakly positive for *B. pertussis.* These findings were presented by Dalby, et al [6] at the 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 31 March – 3 April 2012.

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 was 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 (Turku, Finland).

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

2. Introduction

Pertussis remains endemic worldwide and is an important public health issue. There has been a marked increase in reported pertussis cases in many countries despite high vaccination coverage. Laboratory diagnosis of pertussis is important for treatment, prevention and surveillance. It is noteworthy that there is wide variation in the reporting of laboratory-confirmed pertussis cases globally as well as within Europe and one of the reasons for this may be the differences in the methodologies used for diagnosis.

External quality assurance is important to ensure accurate diagnosis and good laboratory performance. This EQA programme builds upon the previous collaborative studies on qPCR organised by the EUVAC.NET and EUpertstrain consortia.

The current programme is designed to develop and standardise diagnostic techniques for confirmation of pertussis infection in individuals and in outbreak situations. Through EQA schemes, the performance of the national reference laboratories from each EU/EEA Member State can be independently assessed. Furthermore, through the scheme, recommendations for improvements to methodologies and areas for further training can be identified.

The specific aims of this *Bordetella* PCR EQA were:

- to evaluate sensitivity, specificity and reproducibility of current assays to detect *B. pertussis* DNA;
- to evaluate the ability of assays to differentiate between *B. pertussis* and other *Bordetella* species associated with respiratory clinical infections, namely *B. parapertussis*, *B. holmesii* and *B. bronchiseptica*;
- to assess good laboratory practice of the PCR technique by checking for evidence of contamination;
- to assess differences in interpretation and reporting of *Bordetella* PCR results;
- to identify training needs;
- to assist the establishment of 'best practice' in current assays, interpretation and reporting.

3. Materials and methods

3.1. Organisation

The first EUpert-labnet *B. pertussis* PCR EQA was organised by the UK's Health Protection Agency, Colindale (London) intended for National Reference Laboratories in EU Member States, Iceland, Liechtenstein and Norway. Invitations were initially sent to ECDC-designated pertussis laboratory experts in November 2011 (Annex 1). In total, 21 laboratories participated, 20 of which were in EU countries and one from Norway (Annex 2).

3.2. Selection of panel

Clinical isolates of *B. pertussis*, *B. parapertussis*, *B. holmesii*, *B. bronchiseptica* and *Haemophilus influenzae* were selected from the culture collection at the HPA's Respiratory and Systemic Infection Laboratory (see Table 1). Purified genomic DNA was prepared from bacterial pellets using the Nucleon BACC2 DNA extraction kit (GE Healthcare Life Sciences) including an RNase A treatment to prevent confounding of quantification. The concentration of DNA was determined at A_{260} using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The panel was designed to include dilutions of purified genomic DNA from *B. pertussis* at three concentrations 'high', 2pg/µl; 'medium', 0.2pg/µl and 'low', 0.02pg/µl. Duplicate samples of the *B. pertussis* 0.2pg/µl dilution were included to test reproducibility. The DNA concentration of all other *Bordetella* species included in the panel (*B. parapertussis*, *B. holmesii*, *B. bronchiseptica* and *Haemophilus influenzae*) was 2pg/µl. Two 'blank' samples containing only 10 mM Tris buffer, pH 8.0, i.e. no added DNA, were included to check for potential contamination.

The panel was tested three times prior to dispatch by the sending laboratory.

3.3. Carriage of panels

The panels were prepared, packed according to local regulations, collected by courier on 8 February 2012 from HPA - Colindale, London 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 30 March 2012.

Table 1. Characteristics of the strains used to prepare genomic DNA for inclusion in the first EUpert-labnet *B. pertussis* PCR EQA (February 2012)

HPA reference no.	Organism	Isolation date	Specimen type	Clinical details
H114260371	*Bordetella pertussis* serotype 1,3	Oct 2011	Pernasal swab	Whooping cough
H114560403	*Bordetella parapertussis*	Nov 2011	Pernasal swab	Respiratory infection
H104780607	*Bordetella holmesii*	Nov 2010	Blood culture	Pyrexia
H111580382	*Bordetella bronchiseptica*	Apr 2011	Sputum	Not provided
H120420371	*Haemophilus influenzae* non-capsulated	Jan 2012	Blood culture	Bacteraemia

3.4. Testing

Participants were instructed to treat the panel as 'extracted DNA samples' and test them three times using their usual *B. pertussis* PCR assay and report their qualitative and/or quantitative results. A reporting sheet for the results and a questionnaire to ascertain details of each test were sent to each participant by e-mail as Word (Microsoft) documents to be returned by the deadline.

The main questions concerned each participant's use of PCR for the diagnosis of *Bordetella* infections; the type of assay used (i.e. 'in-house', published or commercial kit); whether real time (qPCR) or traditional block-based thermocycler; which gene targets were used; the types of controls used including internal controls to check for inhibition; the types of clinical samples tested and approximate number tested per year; the method of DNA extraction used; participation in any existing EQA programme for *Bordetella* PCR. Participants were also asked to provide any other comments on the use of *Bordetella* PCR by European laboratories.

The full questionnaire is reproduced in Annex 3.

3.5. Data analysis

The intended results of the submitting laboratory (Table 2) were used as a basis for the scoring.

Participants were expected to:

- detect *B. pertussis* at both the 'high' and 'medium' dilutions;
- differentiate between *B. pertussis* and other *Bordetella* species;
- accurately interpret and report results depending on the targets used and results obtained;
- obtain a 'negative' result (for *Bordetella*) with the two blank samples;
- obtain a 'negative' result (for *Bordetella*) with the sample containing *H. influenzae.*

The 'low' dilution of *B. pertussis* was designed to be challenging.

Qualitative EQA data scoring system

Results were scored with a possible 10 out of 10 (100%). Results were also considered acceptable if *Bordetella* species was reported for samples containing *Bordetella* DNA, but incorrect if the wrong species was reported.

Table 2. Intended results for the first EUpert-labnet *Bordetella pertussis* PCR EQA (February 2012)

Sample number	Qualitative Result		Sample details		
	B.pertussis	*Bordetella spp.*	Strain	Concn.	Vol (µL)
1	Negative	Negative	Tris buffer (10mM, pH 8.0)	0	200
2	Positive	Positive	*Bordetella pertussis* (H114260371)	2pg/µL	200
3	Positive	Positive	*Bordetella pertussis* (H114260371)	0.2pg/µL	200
4	Negative	Positive	*Bordetella holmesii* (H104780607)	2pg/gµL	200
5	Positive	Positive	*Bordetella pertussis* (H114260371)	0.2pg/µL	200
6	Negative	Negative	Tris buffer (10mM, pH 8.0)	0	200
7	Negative	Positive	*Bordetella bronchiseptica* (H111580382)	2pg/µL	200
8	Negative	Positive	*Bordetella parapertussis* (H114560403)	2pg/µL	200
9	Negative	Negative	*Haemophilus influenzae* (H120420371)	2pg/µL	200
10	Positive	Positive	*Bordetella pertussis* (H114260371)	0.02pg/µL	200

4. Results

The intended results are shown in Table 2. All 21 laboratories submitted results and completed questionnaires by the deadline. One laboratory submitted two datasets, thus 22 datasets are presented. The overall results for the 22 datasets from 21 EU/EEA laboratories are shown in Table 3. Results are presented by platform, as conventional (i.e. traditional block-based PCR with amplification products detected by gel electrophoresis, staining with ethidium bromide and image capture under ultraviolet light), or real time (qPCR) using fluorescent detection of amplification products. These categories were further subdivided by use of a commercial kit or an 'in-house' assay. Finally, two laboratories presented data using a combination of methods and these are described as 'other'. These 'other' methods comprise:

- conventional PCR using a commercial kit and in-house PCR and an in-house real-time PCR for *B. pertussis* plus real-time PCR commercial kit and a conventional in-house PCR for *B. parapertussis* (one dataset);
- conventional in-house together with direct amplification and DNA sequencing (one dataset).

A summary of answers from the questionnaire is shown in Table 4.

Bordetella pertussis dilution series

All laboratories (22/22 datasets) detected *B. pertusis* DNA in the 'high' dilution (sample number 2), and all but one (21/22) detected *B. pertusis* in the 'medium' dilution duplicates (sample numbers 3 and 5). However, the one laboratory that scored no. 3 negative for *B. pertussis* PCR also used direct amplification and DNA sequencing of the 16S rDNA and reported *B. pertussis* identified by sequencing of 16S rRNA gene (99.6%). Only 15/22 reported *B. pertussis* (or *Bordetella* spp.) for the 'low' dilution of *B. pertussis* DNA (sample number 10).

Bordetella holmesii

Only 16/22 correctly reported sample number 4 as *B. holmesii* (or *Bordetella* spp.) or negative for *B. pertussis*.

Bordetella parapertussis and *Bordetella bronchiseptica*

All (22/22) correctly reported sample number 8 as *B. parapertussis* (or *Bordetella* spp.), or negative for *B. pertussis* but only 13/22 correctly reported sample number 7 as *B. bronchiseptica* (or *Bordetella* spp.) or negative for *B. pertussis*.

Negative controls

All 22/22 datasets correctly reported negative results (for any *Bordetella* spp.) with sample numbers 1 and 6.

Haemophilus influenzae

Twenty of twenty-two datasets reported negative results (for any *Bordetella* spp.) with sample number 9 containing *Haemophilus influenzae* DNA.

Crossing thresholds

Nine of the 13 laboratories that performed qPCR reported crossing thresholds (Cqs) [3,4] for *B. pertussis* and/or *Bordetella* spp. (see Table 5). Generally, the reproducibility for the duplicate samples (sample numbers 3 and 5) within laboratories was very good (mean values less than 2 Cq apart), apart from one laboratory (using *ptxA*) where the difference was >3.7 Cq. Where available, reproducibility within laboratories was also good for repeated runs on the same sample (i.e. <0.5, <1, <2.3 Cq).

Controls

Only 14/22 laboratories used an internal control (to check for inhibition) and 13/22 used an internal calibrator/positive control.

Table 3 Number of correct qualitative results per panel member and technology type

Sample number	Sample content	Sample concn. (pg/µl)	Total datasets n = 22		PCR													
					Conventional						qPCR						Other	
					Commercial n = 1		In-house n = 4		Both n = 2		Commercial n = 1		In-house n = 11		Both n = 1		n = 2	
			n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
2	*B. pertussis*	2pg/µl	22	100	1	100	4	100	2	100	1	100	11	100	1	100	2	100
3	*B. pertussis*	0.2pg/µl	21	95	1	100	4	100	2	100	1	100	11	100	1	100	1	50
5	*B. pertussis*	0.2pg/µl	22	100	1	100	4	100	2	100	1	100	11	100	1	100	2	100
10	*B. pertussis*	0.02pg/µl	15	68	0	0	4	100	0	0	0	0	10	91	1	100	0	0
8	*B. parapertussis*	2pg/µl	22	100	1*	100	4	100	2	100	1	100	11	100	1	100	2	100
4	*B. holmesii*	2pg/µl	16	73	1*	100	1	25	2	100	0	0	9	82	1	100	2	100
7	*B. bronchiseptica*	2pg/µl	12	55	1*	100	2	50	0	0	0	0	8	73	0	0	1	50
9	*Haemophilus influenzae*	2pg/µl	20	91	1	100	3	75	2	100	1	100	10	91	1	100	2	100
1	Negative		22	100	1	100	4	100	2	100	1	100	11	100	1	100	2	100
6	Negative		22	100	1	100	4	100	2	100	1	100	11	100	1	100	2	100

The shading indicates duplicate samples.

** Kit used, Pneumobacter ACE detection kit (Seegene), claims to detect* Bordetella pertussis *and* Haemophilus influenzae*; gave negative results for* B. holmesii / B. bronchiseptica / B. parapertussis / H. influenzae *(numbers 4, 7, 8, 9) in panel, but scored as 'correct' here as 'not* B. pertussis*'.*

Table 4. Summary of responses to questionnaire

	No. Laboratories	%
1. *B. pertussis* PCR used		
a. routinely	16	76
b. outbreak/ special investigations only	4	19
2. PCR assay		
a. in-house	19	90
b .commercial	8	38
c. both	6	29
3. Assay methodology and platform		
a. Published method? For methods, see [7-21]	16	76
b. Real-time (qPCR)	13	62
c. Platform		
TaqMan (ABI)	3	14
LightCycler (Roche)	10	48
LC 1.0	2	
LC 2.0	7	
LC 480	1	
RotorGene	2	10
d. Conventional PCR		
iCycler (BioRad)	1	
Veriti (ABI)	1	
9700 (ABI)	1	
MasterCycler (Eppendorf)	4	
Thermal Cycler Gradient PCR (TaKaRa)	1	
Mx300SP (Stratagene)	1	
e. Targets used for *B. pertussis*		
Pertussis toxin promoter	11	
Pertussis toxin	2	
IS*481*	20	
Pertactin	0	
Other		
f. Target(s) used for *B. parapertussis*		
IS*1001*	15	
g. Other *Bordetella* spp. targeted?		
B.holmesii	8	
B.bronchiseptica	3	
B.petrii	1	
pan-Bordetella	1	
Other targets used?		
Outer membrane porin	1	
recA	7	
h. Internal control (to check for inhibition)	14	
i. Internal calibrator (positive control /standard curve)	13	
4. Commercial kit	8	
PneumoBacter (Seegene Inc,)	4	
B.pertussis/*para* (TIB-MOLBIOL)	1	
Bordetella pertussis R-gene (Argene)	1	

Bordetella parapertussis R-gene (Argene)	1	
Bordetella R-gene (Argene)	1	
Dia-Bor-020 (Diagenode)	1	
5. Type of clinical specimens analysed		
Respiratory	14	
Pernasal swabs	10	
Nasopharyngeal aspirates	14	
Other		
Naspharyngeal swabs	2	
Bronchial alveolar lavage	2	
Post-mortem samples (e.g. lung)	1	
6. Approximate no. specimens processed per annum		
of 18 laboratories providing figures	Range 5 to 9000 (average, 866; median, 225)	
7. Method of DNA extraction		
Automated	7	
Manual	15	
Both	1	
8. Participation in EQA scheme	14	
QCMD	7	
INSTAND	6 (+1 used to)	
EUVAC.NET *B.pertussis* EQA (2011)	13	
UK NEQAS (every 2 years)	1	
Comments (see below)	6	

Questionnaire comments on use of *Bordetella* PCR by European laboratories

- To help establish DNA contamination procedures
- Specificity problems of IS*481*
- Other commercial kits also validated (see [22])
- Currently using IS*481* as a single target for detection of *B. pertussis*. Well aware of the issue of false positive reactions with *B. holmesii*; in the process of validating another target, the insertion element IS*1001*, in order to distinguish between *B. holmesii* (which is positive for both insertion elements IS*1001* and IS*481*) from *B. pertussis* which is positive for IS*481* but negative for IS*1001*
- Taxonomic genes 16S rDNA, and IS*481* used for differentiation
- Just starting *Bordetella* PCR
- Participation in the EQA will be very useful for our laboratory

Table 5. Crossing thresholds for samples containing *Bordetella* DNA reported by submitting laboratories

Sample number	*Bordetella pertussis*	Laboratory number								
		6	7	8	13	15 (*ptxA*)	16	18	20	21
2	*B. pertussis*	21.96-23.30	24.26 ±0.38	18.4	20.52 ±0.26	27.34	15.21 ±0.17	22.92	22.17	20.36
3	*B. pertussis*	32.58-34.87	34.1	28.1	30.54	38.00	24.33	33.27	31.73	29.82
5	*B. pertussis*	32.59-34.65	33.8 (*ptxA*P)	28.3	30.40	34.13	24.25	33.61	31.58	29.35
10	*B. pertussis*	40.63-43.12		35.2	>35	0	30.77 ±0.39	40.38	35.41	
8	*B. parapertussis*					29.44				
4	*B. holmesii*				26.04 ±0.32	0	22.41 ±0.13			
7	*B. bronchiseptica*					30.12				
Sample number	***Bordetella* spp.**	**Laboratory number**								
		6	7	8	13	15 (IS*481*)	16	18	20	21
2	*B. pertussis*	34.66-35.15	18.90 ±0.08			22.2				
3	*B. pertussis*		28.6±0.14			32.21				
5	*B. pertussis*		28.6±0.13							
10	*B. pertussis*		36.6±1.21 (IS*481*)			40.24 (IS*481*)				34.74 (IS*481*)
8	*B. parapertussis*		23.7±0.15 (IS*1001*)	24.43		0 (IS*481*)		20.73	29.99	25.26
4	*B. holmesii*	27.79-28.86	25.2 (IS*481*) 29.1 (*recA*) 35.9 ±0.21 (IS*1001*)	25.97		28.03		30.56		
7	*B. bronchiseptica*		25.7 (IS*1001*)	30.1		0		22.97	32.63	27.8

*ptx*P: pertussis toxin S1 promoter
ptxA, pertussis toxin S1 gene
recA, gene for protein RecA

5. Discussion

Sensitivity, specificity and reproducibility

All but one laboratory (21/22) detected *B. pertussis* at both the 'high' and 'medium' dilutions, the exception was a laboratory using a block-based assay, just preparing a *B. pertussis* PCR service and participating in a *B. pertussis* PCR EQA for the first time.

The real-time assays demonstrated greater sensitivity than the conventional PCR illustrated by the detection of *B. pertussis/Bordetella* spp. DNA at the 'low' dilution by only three of seven laboratories using conventional PCR versus 10 of 11 deploying qPCR.

The major issue for specificity was one of reporting. It is known that IS*481* is not specific for *B. pertussis* and can be found in *B. holmesii* and some strains of *B. bronchiseptica*, thus IS*481* positive-only results should not be reported as confirmation of *B. pertussis*. Similarly, the insertion element IS*1001* can be found in both *B. parapertussis* and *B. bronchiseptica*, and whilst clinical infection by *B. bronchiseptica* is rare, IS*1001* positive-only results should not be reported as confirmation of *B. parapertussis*. Commercial kits claiming to specifically detect *B. pertussis* and *B. parapertussis* using these targets should also update their interpretation.

Differentiation between *B. pertussis* and other *Bordetella* species associated with respiratory clinical infections, namely *B. parapertussis*, *B. holmesii* and *B. bronchiseptica*

B. pertussis targets

All but two laboratories (20/22) used IS*481* as a target for *B. pertussis*; twelve of these also use a single copy target in addition, 11 use *ptxA* promoter region and one the *ptxA* gene. One laboratory used only *ptxA* gene (in-house conventional PCR) but in combination with a commercial (conventional) kit.

B. parapertussis targets

Fifteen out of 22 laboratories used IS*1001* as a target, 14 of which also used IS*481* for *B. pertussis*.

Other *Bordetella* species targeted

Seven laboratories used the *recA* gene to specifically detect *B. holmesii*. Two laboratories used the *fla* gene (conventional PCR) for detection of *B. bronchiseptica*.

One laboratory targeted pan-*Bordetella*, *B. holmesii*, *B. bronchiseptica* (unpublished data). However, this was one of two laboratories that reported a *Bordetella* spp. result for the sample containing *H. influenzae* DNA.

Thus, several gene targets are required in order to successfully detect and differentiate between *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. holmesii*.

Evidence of good laboratory practice of the PCR technique by checking for evidence of contamination

This was demonstrated by all laboratories evidenced by no false-positive reporting of the two 'negative' samples.

Controls

There is considerable debate about the use of controls in clinical PCRs [23]; an internal control is recommended to avoid false-negative reporting. If one is not readily available a replicate sample tube can be 'spiked' with a known low amount of positive control material and the signal compared with the 'unspiked' one.

Training needs

Many of the participants in this EQA have been providing PCR for *B. pertussis* for a number of years. However, a number of laboratories were considering moving from conventional to real-time and/or introducing a PCR service. In conjunction with ECDC and the EUpert-labnet training activities seven laboratories participated in a workshop on pertussis real-time PCR (April 23–27, 2012, Turku, Finland).

The availability of a particular qPCR platform can dictate which of the existing assays may be used. However, the main difference in the qPCR platforms is the use of two dual-labelled probes per target with the LightCycler 1.0 or 2.0 (Roche) compared with the single hybridisation probes per target typically used with the LightCycler 480 (Roche), TaqMan (ABI) and RotorGene (Qiagen).

Establishment of best practice in current assays, interpretation and reporting

In conjunction with ECDC and the EUpert-labnet activities a document providing guidelines and protocols for the use of real-time PCR in laboratory diagnosis of human infection with *Bordetella pertussis* or *Bordetella parapertussis* has been produced.

Limitations

The strains included were clinical isolates and the whole genome sequence for these was not available.

Although RNase treatment should have allowed accurate DNA quantification using the Nanodrop at A_{260}, greater accuracy can be achieved using a Fluorimeter, e.g. Qubit (Qiagen).

The samples included in the panel were purified high molecular size genomic DNA and more realistic 'simulated' samples could in addition include, for example, human DNA.

6. Recommendations

Results which are PCR positive, 'IS*481* only', should be considered as probable *B. pertussis* as this insertion sequence element can also be found in *B. holmesii* and some strains of *B. bronchiseptica*. Reports to date indicate that *B. holmesii* may be more likely as a potential confounder for *B. pertussis* in older age groups [20].

Results which are PCR positive, 'IS*1001* only', should be considered as probable *B. parapertussis* as this insertion sequence element can also be found in some strains of *B. bronchiseptica*. Although *B. bronchiseptica* infection is rare in humans, in certain patient groups such as cystic fibrosis patients, it may be more likely.

An internal process control to check for the presence of PCR inhibitors is recommended to avoid false-negative reporting.

Commercial kits for the detection of *B. pertussis* and/or *B. parapertussis* are available, some of which have been assessed [22]. However, care should be used in the interpretation of these results, as indicated above.

Overall, the results of the 2012 EQA scheme are quite satisfactory and present an improvement compared to the former edition. Nonetheless, the EQA exercises perform a crucial role and are extremely useful to participants and to ECDC in order to improve and validate the data collected from the Member States. The support of the EUpert-labnet network is valued by the participants as reflected in the high level of participation, provision and receipt of training, and in the harmonisation of methods and protocols for diagnostics and characterisation of *Bordetella* spp.

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Annex 1. Invitation letter to participants for the *Bordetella pertussis* PCR external quality assurance programme

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14 November 2011

Dear Colleagues,

Re: Invitation to participate in a *Bordetella pertussis* PCR External Quality Assurance program

You will have received this letter because we believe you are the officially designated ECDC laboratory expert for pertussis in your country. If you are not the designated laboratory expert for pertussis please could you let us and Zaibun Nisa [at ECDC] know, thank you.

Following the award of the Framework Contract No ECDC/2011/013 to a consortium of European pertussis experts of nine countries (Denmark, Finland, France, Germany, Italy, Netherlands, Norway, Sweden and UK), we are responsible for Workpackage 2: EQA scheme for the laboratory diagnostics and molecular typing of pertusiss.

Subject to the finalisation of subcontract arrangements with the coordinator of this project National Institute for Health and Welfare THL, Finland, we intend to distribute a panel of genomic DNA extracts (ready for PCR) for testing by PCR with the same procedure you would normally use in your laboratory for testing clinical samples for *Bordetella pertussis*. The panel will also included DNA from at least one other *Bordetella* species and non-*Bordetella* species.

We plan to send this *B. pertussis* PCR EQA panel in February 2012.

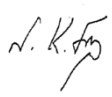
If you are interested in participating in this EQA please confirm your willingness to participate by emailing Norman Fry (by November 30, 2011) and let us know the consignee's details, full address, telephone and fax number and e-mail address.

An EQA collaborative study to assess current laboratory performance of pertussis serology will be arranged in mid-2012. Further details of this program will follow in due course

Should you need further information, do not hesitate to contact us.

Many thanks for your attention

With best regards



Norman K Fry, PhD HPA – RSIL, Colindale

Dorothy Xing, PhD HPA – NIBSC, Potters Bar

Kevin Markey, PhD HPA – NIBSC, Potters Bar

Annex 2. List of participants

Country	Contact person	Laboratory /Institution
Belgium	Denis Pierard, Oriane Soetens, Fedoua Echahidi	Laboratory of Microbiology, UZ Brussel Hospital
Bulgaria	Stefan Panaiotov, Nadia Brankova	Department of Microbiology, National Center of Infectious and Parasitic Diseases
Czech Republic	Jana Zavadilova	National Reference Laboratory for Pertussis and Diphtheria, National Institute of Public health
Denmark	Jørgen Jensen Tine Dalby, Marianne Gam	Department of Microbiological Surveillance and Research, Statens Serum Institut
Estonia	Laura Kunder	Health Board Laboratory for Communicable Diseases
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Germany	Marion Riffelmann, Carl Heinz Wirsing von König, Nicole Kennerknecht	Labor Medizin Krefeld MVZ, HELIOS Klinikum Krefeld
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Hungary	Ildiko Paluska	Legionella - *Bordetella* Laboratory, Hungarian National Center for Epidemiology
Ireland	Juanita Grogan, Catriona Logan	Molecular Laboratory, Microbiology Department, Our Lady's Children's Hospital, Crumlin
Italy	Paola Stefanelli	Istituto Superiore di Sanità
Latvia	Diana Dusacka	Infectology Center of Latvia
Lithuania	Algirdas Griškevicius	Molecular Biology Testing Subdivision, National Public Health Surveillance Laboratory
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Slovenia	Metka Paragi, Tamara Kastrin	Department of Medical Microbiology
United Kingdom	Norman Fry	Respiratory and Systemic Infection Laboratory, Health Protection Agency

Annex 3. The EUpert-labnet *Bordetella pertussis* PCR EQA questionnaire

1.		Do you use PCR for the diagnosis of *Bordetella* infections?	
	a.	Routinely	Yes/No
	b.	Outbreaks/special investigations only	Yes/No
2.		If yes do you use an 'in-house' or a commercial assay or both?	
	a.	In-house:	Yes/No
	b.	Commercial:	Yes/No
	c.	Both	Yes/No
3.		If you use an in-house assay:	
	a.	Is the method you follow published?	Yes/No
		(If published please give reference):	
	b.	Real-time?	Yes/No
	c.	If real-time; which platform?	
		Taqman (TaqMan)	Yes/No
		Model	
		LightCycler (Roche)	Yes/No
		Model	
		RotorGene (Qiagen)	Yes/No
		Model	
		Other (please state):	
	d.	If not real-time, which thermocycler block do you use?	
		Please state manufacturer and model:	
	e.	Which target(s) do you use for *Bordetella pertussis*?	
		Pertussis toxin promoter	Yes/No
		Pertussis toxin	Yes/No
		IS*481*	Yes/No
		Pertactin	Yes/No
		Other (please state):	
	f.	Which target(s) do you use for *Bordetella parapertussis*?	
		IS*1001*	Yes/No
		Other (please state):	
	g.	Do you target any other *Bordetella* spp. apart from *B.pertussis* and *B.parapertussis* in your assays?	Yes/No
		If so which *Bordetella* species and which targets do use you?:	
	h.	Do you use Internal Controls to check for inhibition?	Yes/No
		(if yes please give details):	
	i.	Do you use an internal calibrator (positive control/standard curve)?	Yes/No
		Please give details:	

4.	Do you use a commercial kit	Yes/No
	If yes please give details of Kit (please state):	
	Manufacturer (please state):	
	Platform (please state):	
5.	What kind of clinical samples do you analyse?	
	Respiratory samples	Yes/No
	Pernasal swabs	Yes/No
	Nasopharyngeal aspirates	Yes/No
	Other (please state):	
6.	Approximately how many clinical samples do you process for *Bordetella* PCR per year?	
7.	What method of DNA extraction do you use?	
	Manual or automated?	
	Which kit?	
	Which manufacturer?	
8.	Do you regularly take part in an External Quality Assessment Program for *Bordetella* PCR?	Yes/No
	If yes which one?	
	(please give details):	

Any other comments on the use of *Bordetella* PCR by European laboratories:

Annex 4. The EUpert-labnet *Bordetella pertussis* PCR EQA result submission form

#	Qualitative Result		Quantitative Result				Comments (e.g. inhibition, etc.)
			Crossing Point (Cq values)		Calculated Amount (pg or GU) or Concentration (pg/µl or GU/ml), of *Bordetella* DNA**		
	B. pertussis (Pos/Neg/NT*)	*Bordetella spp.* (Pos/Neg/NT*)	*B. pertussis*	*Bordetella*			
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

* Pos = Positive (detected); Neg = Negative (not detected); NT = Not Tested. Cq = crossing threshold (for real-time PCR)
** Calculated from standard curve (real-time PCR).