



TECHNICAL REPORT

External quality assurance scheme for *Bordetella* identification and *B. pertussis* typing

as part of the EUpert-Labnet surveillance network

ECDC TECHNICAL REPORT

**External quality assurance scheme for
Bordetella identification and *B. pertussis*
typing 2013**

As part of the EUpert-Labnet network



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Abbreviations

DSN	Dedicated surveillance networks
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EQA	External Quality Assurance
ELISA	Enzyme-linked immunosorbent assay
EUpert-Labnet	Consortium of European pertussis experts funded by ECDC for this programme
HPA	Health Protection Agency (UK)
IS	Insertion sequence element
MAb(s)	Monoclonal antibody (antibodies)
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MAST	Multiple Antigen Sequence Typing
MLVA	Multiple-Locus Variable-Number Tandem Repeat Analysis
MRHA	Medicines and Healthcare Products Regulatory Agency
NCTC	National Collection of Type Cultures (UK)
NIBSC	National Institute for Biological Standards and Control (UK)
PCR	Polymerase chain reaction
PFGE	Pulsed-Field Gel Electrophoresis
PHE	Public Health England
qPCR	Quantitative (real-time) PCR
RIVM	National Institute for Public Health and the Environment, Ministry of Health, Welfare and Sport (Bilthoven, The Netherlands)
RSIL	Respiratory and Systemic Infection Laboratory (of the HPA, London)
RVPBRU	Respiratory and Vaccine Preventable Bacteria Reference Unit (of PHE, London)
SOP	Standard Operating Procedure
SSU rDNA	small subunit ribosomal DNA/ 16S rDNA
THL	Terveyden ja Hyvinvoinnin Laitos / National Institute for Health and Welfare (Finland)

Executive summary

Main findings

- Sixteen laboratories from 16 EU/EEA countries participated in the external quality assurance (EQA) scheme for *Bordetella* identification and *B. pertussis* typing by ECDC as part of the EUpert-Labnet network. The panel included *Haemophilus influenzae*, *Bordetella pertussis*, *B. parapertussis*, *B. bronchiseptica*, and *B. holmesii*. Four *B. pertussis* strains belonging to three different serotypes (1, 2; 1, 3 and 1, 2, 3) were included. Two epidemiologically and genotypically related *B. pertussis* strains were included to test the ability of the participant's laboratory to score related and unrelated isolates. The EQA was divided into three sections: identification, serotyping of *B. pertussis* and genotyping of *B. pertussis*. For serotyping two reagents (supplied by NIBSC) were provided, code 06/124 WHO International Standard Monoclonal Antibody for Serotyping *B. pertussis* Fimbrial Antigen 2 (1st WHO IS) and 06/128 WHO International Standard Monoclonal Antibody for Serotyping *B. pertussis* Fimbrial Antigen 3 (1st WHO IS).
- All laboratories correctly identified the four *Bordetella pertussis* isolates. Fifteen of the 16 laboratories correctly identified *Bordetella bronchiseptica* to genus level and 13 identified to species level. Fifteen of the 16 laboratories correctly identified *Bordetella parapertussis* and *Bordetella holmesii* correctly to genus level and 14 to species level. Identification methods comprised traditional phenotypic techniques, MALDI-TOF, genotypic methods (DNA sequencing and/or specific PCRs) or a combination.
- Only 7/16 laboratories correctly identified the serotype of the four *B. pertussis* strains using the monoclonal reagents. This low score was due to a combination of reported auto-agglutination using the slide agglutination protocol and inexperience with this technique.
- There was a wide variation in genotyping methods used for *B. pertussis* typing. Seven laboratories performed genotyping, four used PFGE, two MLVA and six MAST. One laboratory used qPCR to identify the MAST types. PFGE successfully determined the correct epidemiological relatedness between the four *B. pertussis* isolates.
- National reference laboratories should be able to identify all clinically important species of *Bordetella* to the level of species including *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. holmesii*.
- National reference laboratories should also be able to perform fimbrial serotyping and genotyping of *B. pertussis* isolates.
- An update of the recommendations on standard methods for epidemiological typing of *B. pertussis* isolates is required.
- EQAs should be organized regularly for *Bordetella* identification and *B. pertussis* typing in order to obtain comparable data and sustained surveillance data in Europe.
- Hospital laboratories in EU/EEA countries should be encouraged to isolate putative *Bordetella* species from clinical cases and refer to National reference laboratories for confirmation and further typing.

This report presents the results of the first external quality assurance (EQA) scheme for *Bordetella* identification and typing funded by the European Centre for Disease Prevention and Control (ECDC). The EQA study was conducted between February and May 2013.

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' (Article 5.3, EC 851/2004).

External quality assurance (EQA), a part of quality management systems (QMS), evaluates performance of laboratories by an outside agency on material that is supplied especially for this purpose. ECDC organises a series of EQAs for EU/EEA countries. Some non-EU/EEA countries are also involved in the EQA activities. 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 ('state of the art')
- Assessment of the effects of analytical procedures (method principle, instruments, reagents, calibration)
- Evaluation of individual laboratory performance
- Identification and justification of problem areas
- Providing continuing education
- Identification of needs for training activities.

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 methods for the detection, identification and characterisation of clinical infections caused by *B. pertussis* and other *Bordetella* species.

Bordetella pertussis and *B. parapertussis* have traditionally been identified by standard phenotypic methods based on nutritional characteristics and agglutination with polyclonal rabbit antisera containing antibodies directed against the somatic lipopolysaccharide (LPS), or O-antigens. Both of these species are included as part of the general bacteriology scheme in the United Kingdom National External Quality Assessment Service (UK NEQAS) for Microbiology. Increasingly MALDI-TOF is also used to identify bacteria (including *Bordetella* species) in hospital microbiology laboratories and the use of PCR for direct detection of *B. pertussis* and *B. parapertussis* in clinical specimens may have led to an overall decrease in attempts to isolate these species. Sanger DNA sequencing of the small subunit ribosomal DNA (SSU rDNA), the outer membrane protein A gene (*ompA*) or *RisA* response regulator-encoding gene (*risA*) have also been used to differentiate *Bordetella* species, but these methods are incapable of distinguishing between *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* as each of these gene sequences is identical in all three species.

The serological typing of freshly isolated strains of *B. pertussis* was first described in 1953 [1], and subsequently confirmed by other workers [2-5]. All strains possess a common antigen (antigen 1), but may also possess one or more of a series of type-specific antigens (antigens 2, 3, 4, 5, 6). Originally these antigens were all referred to as agglutinogens, subsequently it was demonstrated that antigens 2 and 3 were fimbrial antigens. These three major antigens are now referred to as agglutininogen 1, fimbrial antigen 2 (Fim2) and fimbrial antigen 3 (Fim3) respectively.

Determining the fimbrial type (expression) of circulating strains of *B. pertussis* is recommended as a simple method of detecting changes in populations. In the host fimbrial antibodies are considered to have a protective role and Fim2 and Fim3 are included in some acellular pertussis vaccines. Agglutininogen and fimbrial typing can be achieved using commercially available polyclonal antisera (e.g. National Institute for Biological Standards and Control (NIBSC)). Subsequently, sera containing monoclonal antibodies against epitopes of Fim2 and Fim3 were developed and these are now also available from NIBSC (see Table 2).

Various genotyping methods have been applied to *B. pertussis* including pulsed-field gel electrophoresis (PFGE) and Sanger DNA sequencing of genes for pertactin (*prn*) and pertussis toxin S1 subunit (*ptxA*). Additional gene targets have also been used, including those for the pertussis toxin promoter (*ptxP*), tracheal colonisation factor (*tcfA*), fimbrial subunit 2 (*fim2*), and fimbrial subunit 3 (*fim3*) [6,7]. Methods for Multiple-Locus Variable-Number Tandem Repeat Analysis (MLVA) [9] and single nucleotide polymorphism (SNP)-based typing [10] have also been described.

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 Workpackage 2: EQAs for laboratory diagnostics and molecular typing of pertussis. Workpackage 2 comprises EQAs for: PCR, serology, *Bordetella* identification and typing. This report details the third of these EQAs for *Bordetella* identification and *B. pertussis* typing.

2. Introduction

Pertussis remains endemic worldwide and is an important public health issue. Despite high vaccination coverage there has been a marked increase in reported pertussis cases in several countries. Correct identification of *B. pertussis* (and other *Bordetella* species) together with epidemiological typing by phenotypic and genotypic methods are important for treatment, prevention and surveillance.

Some of the variation in the reporting of laboratory-confirmed pertussis cases globally, as well as within Europe, may be attributed to be the differences in methodologies. Agreed methods for epidemiological typing would also assist in tracking changes in *B. pertussis* populations and such guidance has been described [6].

External Quality Assurance is important for accurate identification and to assess laboratory performance. The current programme is designed to develop and standardise techniques for the identification and epidemiological typing of pertussis infection in individuals and in outbreak situations. EQA schemes enable the performance of the national reference laboratories from each EU/EEA Member States to be independently assessed. Furthermore, through the scheme, recommendations can be made for improvements to methodologies and areas identified for further training.

The specific aims of this *Bordetella* identification and *B. pertussis* typing EQA were:

- To evaluate the ability of participants to identify *B. pertussis* and *B. parapertussis*
- To evaluate the ability of participants to identify other clinically relevant *Bordetella* species (i.e., *B. holmesii* and *B. bronchiseptica*) and differentiate these from *B. pertussis* and *B. parapertussis*
- To evaluate the ability of participants to identify serotype *B. pertussis* isolates
- To evaluate the ability of participants to identify genotype *B. pertussis* isolates
- To assess differences in interpretation and reporting of *Bordetella* identification and typing results
- To identify training needs
- To assist with the establishment of 'best practice' in current assays, interpretation and reporting.

3. Materials and methods

3.1 Organisation

The EUpert-Labnet *Bordetella* identification and typing EQA, which was organised by the UK Health Protection Agency's Respiratory and Systemic Infection Laboratory (HPA - RSIL) Colindale, UK, was intended for national reference laboratories in EU Member States and non EU countries (Iceland, Liechtenstein and Norway).

Invitations were initially sent to ECDC-designated pertussis laboratory experts in November 2012. In total, 16 laboratories participated, 15 of which were in EU countries together with one from Norway.

3.2 Selection of the panel

Clinical isolates of *B. pertussis*, *B. parapertussis*, *B. holmesii*, *B. bronchiseptica*, and *Haemophilus influenzae* were selected from the HPA - RSIL culture collection (see Table 1). The panel was designed to include *B. pertussis* belonging to the serotypes 1, 2; 1, 3 and 1,2,3. Two of the *B. pertussis* were previously demonstrated to be genotypically indistinguishable and were included to assess the genotypic methods used. The panel was tested prior to dispatch by the dispatching laboratory.

3.3 Carriage of panels

The panels were prepared, packed according to local regulations and collected by courier on 8 February 2013 from HPA – RSIL, Colindale, London and dispatched to the 16 laboratories. All packages were received in a timely manner.

Table 1. Characteristics of the strains in the EUpert-Labnet *Bordetella* identification and *B. pertussis* typing EQA, including eight test strains and two control strains (February 2013)

HPA reference no.	Organism	Isolation date	Specimen type	Details
	<i>Haemophilus influenzae</i>			Recent clinical isolate
H121320680	<i>Bordetella pertussis</i> serotype 1,3	March 2012	Pernasal swab	School outbreak
H121120873	<i>Bordetella pertussis</i> serotype 1,2	March 2012	Pernasal swab	School outbreak
H121340485	<i>Bordetella pertussis</i> serotype 1,3	March 2012	Pernasal swab	School outbreak
H121560437	<i>Bordetella pertussis</i> serotype 1,2,3	April 2012	Pernasal swab	Infant - outpatient
H125040726	<i>Bordetella parapertussis</i>	December 2012	Bronchial lavage	23-month old - inpatient
H124960782	<i>Bordetella bronchiseptica</i>	November 2012	Sputum	34-year old - inpatient
H124340832	<i>Bordetella holmesii</i>	October 2012	Blood	14-year old - inpatient
Reference no.	Organism			
S1	<i>Bordetella pertussis</i> serotype 1,2			Control strain for serotyping
S3	<i>Bordetella pertussis</i> serotype 1,3			Control strain for serotyping

Table 2. Monoclonal antibody reagents for serotyping *B. pertussis* supplied freeze-dried in ampoules with panel

NIBSC code	Description
06/124	WHO International Standard Monoclonal Antibody for Serotyping <i>Bordetella pertussis</i> Fimbrial Antigen 2 (1st WHO IS)
06/128	WHO International Standard Monoclonal Antibody for Serotyping <i>Bordetella pertussis</i> Fimbrial Antigen 3 (1st WHO IS)

3.4 Testing

The EQA was designed in three parts, identification, serotyping and genotyping. Participants were instructed to perform identification to genus level for all isolates using their in-house standard methods (e.g. phenotypic/genotypic/MALDI-TOF.) For each isolate identified belonging to the *Bordetella* genus, participants were asked to further identify to the species level using their standard methods. For each *B. pertussis* isolate identified participants were asked to perform serotyping with the WHO anti-Fim2 and anti-Fim3 monoclonal antibodies supplied. Finally, participants were asked to perform genotyping of the *B. pertussis* isolates using any of the following techniques available in their laboratory:

- Pulsed-Field Gel Electrophoresis (PFGE) using the supplied Standard Operating Procedure (SOP) used at the Pertussis Reference Laboratory of National Institute for Health and Welfare, Turku, Finland (or other method)
- Multi-locus Variable Number of Tandem Repeats Analysis (MLVA) using SOP by the National Institute for Public Health and the Environment, Ministry of Health, Welfare and Sport (RIVM)
- Multiple Antigen Sequence Typing (MAST) DNA sequencing of gene regions for: pertactin (*prn*), the A or S1 subunit of pertussis toxin (*ptxA*), pertussis toxin promoter (*ptxP*), tracheal colonisation factor (*tcfA*), fimbrial subunit 2 (*fim2*) and fimbrial subunit 3 (*fim3*) using the attached SOP by RIVM or other genotyping methods (e.g. qPCR, melting curves) to characterise these genes.

A reporting sheet for the results and a questionnaire to ascertain details of methods used was sent to each participant by e-mail (MS Word document) to be returned by the deadline.

The main questions concerned each participant's routine methods for the identification of *Bordetella pertussis* and other *Bordetella* species; the approximate number of isolates tested per year; laboratory methods used (i.e. cultural, molecular, MALDI-TOF); whether serotyping and/or genotyping of *B. pertussis* is performed; what methods of genotyping (PFGE, MLVA, MAST) and/or other methods.

3.5 Data analysis

The intended results of the submitting laboratory (Table 3) were used as a basis for the scoring.

Participants were expected to:

- distinguish the non-*Bordetella* species (*Haemophilus influenzae*) from the *Bordetella* species
- identify the *Bordetella* species to species level
- perform serotyping of the *B. pertussis* isolates
- perform genotyping of the *B. pertussis* isolates
- accurately interpret and report results depending on the methods used and results obtained.

Table 3. Intended results for the EUpert-Labnet *Bordetella* identification and *B. pertussis* typing EQA (February 2013)

EQA study code no.	Organism	Serotype	Genotype
1	<i>Haemophilus influenzae</i>		
2	<i>Bordetella pertussis</i>	1,3	<i>prn2, ptxA1, ptxP3, tcfA2, fim2-1, fim3-2</i> , MLVA type 27
3	<i>Bordetella pertussis</i>	1,2	<i>prn2, ptxA1, ptxP3, tcfA2, fim2-1, fim3-1</i> , MLVA type 27
4	<i>Bordetella pertussis</i>	1,3	<i>prn2, ptxA1, ptxP3, tcfA2, fim2-1, fim3-2</i> , MLVA type 27
5	<i>Bordetella pertussis</i>	1,2,3	<i>prn2, ptxA1, ptxP3, tcfA2, fim2-1, fim3-1</i> , MLVA type 27
6	<i>Bordetella parapertussis</i>		
7	<i>Bordetella bronchiseptica</i>		
8	<i>Bordetella holmesii</i>		

4. Results

The intended results are shown in Table 3. The EQA consisted of three sections: all 16 laboratories (100%) performed Part 1, identification; 14 of 16 (88%) performed Part 2, serotyping and seven of 16 (44%) performed Part 3, genotyping.

Section 1 – Identification

All laboratories identified the *Bordetella pertussis* isolates successfully using traditional phenotypic techniques, MALDI-TOF, genotypic methods or a combination.

Eleven of 16 laboratories correctly identified the *Haemophilus influenzae* to genus level and six of them identified it to species level. Four laboratories scored this strain as negative/no growth due to lack of growth on *Bordetella* media. One laboratory misidentified the *Haemophilus influenzae* as *B. pertussis*. Fifteen of the 16 laboratories correctly identified *B. parapertussis* and one laboratory misidentified this strain as *Achromobacter*. Fifteen of the 16 laboratories correctly identified *Bordetella bronchiseptica* to genus level and 13 of these identified it to species level. One laboratory reported this as negative. All laboratories (16/16) reported the *Bordetella holmesii* correctly to genus level and 14 of them identified it to species level.

Fifteen of the 16 laboratories used cultural characteristics to identify the strains and two used no other methods. Seven laboratories used MALDI-TOF, five in combination with cultural characteristics. Eight laboratories used specific PCRs in combination with culture and one with MALDI-TOF. Four laboratories used DNA sequencing with one or two other techniques.

Phenotypic characteristics used by participants included growth on various media, colonial appearance, slide agglutination *B. pertussis* and *B. parapertussis* antisera with two polyclonal antibodies (rabbit antisera), for example from REMEL (ref: ZM11 and ZM10) or BD (cat. no. 223091 and 223101) respectively, and biochemical reactions (see Table 4).

Table 4. Phenotypic characteristics used by EUpert-Labnet participants for identification of EQA panel

Characteristics	No. laboratories (out of 16)
Cultural characteristics	14
Growth on media	
• Regan Lowe/charcoal blood agar (plus cephalixin)	6
• Bordet Gengou agar	4
• (Columbia) blood agar	4
• Chocolate agar	3
• MacConkey/lactose agar	3
• Nutrient agar	1
Colonial appearance	3
Pigment expression	1
Slide agglutination	
<i>B. pertussis</i> / <i>B. parapertussis</i> polyvalent antiserum	8
Microscopy	5
Gram stain	5
Motility	1
Biochemical characteristics	9
Catalase	2
Citrate	1
Nitrate	1
Non-carbohydrate acidification	1
Oxidase	8
Urea/urease	5
Voges-Proskauer test	1
API 20 / API 20NE	1/2

MALDI (Bruker) acceptance criteria were given as a score of > 2.0 (or having more than 0.2 difference with other species); score and consistency were used as criteria for identification, as described by the manufacturer. MALDI (Biomérieux) criteria were presented as scored to 99.9% confidence value. Some laboratories also completed identification by cultural characteristics. Results from MALDI-TOF were interpreted in the context of growth/biochemical identification to come to an overall conclusion (presence/absence of growth on blood agar and MacConkey agar, urease and oxidase).

Specific PCRs

Eight laboratories used specific PCRs to identify the *Bordetella* strains; all eight used IS481, four of eight in combination with *ptxP* for the identification of *B. pertussis*. Seven laboratories used IS1001, one used IS1002 and three used specific assays targeting *B. holmesii* (*recA*) and *B. bronchiseptica* (*fla*).

DNA sequencing

Four laboratories used DNA sequencing to identify the *Bordetella* strains; all four used SSU rDNA sequencing, one in combination with *ompA* and *risA* for identification of non-*B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. One laboratory also sequenced the IS481, IS1001, IS1002 and *ptxP* and used MALDI-TOF for confirmation.

Sample no.	Organism	Participants results								Total correct (genus and species)		
			n	%		n	%		n	%		
1	<i>Haemophilus influenzae</i>	<i>Haemophilus</i>	11/16 (genus correct)	69	No growth/negative	4/16	25	<i>Bordetella</i>	1/16	6	6/16	38
		<i>H. influenzae</i>	6/16 (species correct)	38				<i>pertussis</i>				
2	<i>Bordetella pertussis</i>	<i>B. pertussis</i>	15/15 (genus and species correct)								15/15*	100
3	<i>Bordetella pertussis</i>	<i>B. pertussis</i>	16/16								16/16	100
4	<i>Bordetella pertussis</i>	<i>B. pertussis</i>	16/16								16/16	100
5	<i>Bordetella pertussis</i>	<i>B. pertussis</i>	16/16								16/16	100
6	<i>Bordetella parapertussis</i>	<i>B. parapertussis</i>	15/16					Acromobacter	1/16	6	15/16	94
7	<i>Bordetella bronchiseptica</i>	<i>Bordetella</i>	15/16 (genus correct)	94	Negative	1/16	6				13/16	81
		<i>B. bronchiseptica</i>	13/16 (species correct)	81								
8	<i>Bordetella holmesii</i>	<i>B. holmesii</i>	16/16 (genus correct)	100							14/16	88
		<i>B. holmesii</i>	14/16 (species correct)	88								

* One laboratory was sent two slopes of EQA sample no. 3 (and not sample no. 2) in error.

Table 6. Summary of responses to questionnaire

		No. laboratories	%
Participation in each of three EQA sections			
I. Identification of <i>Bordetella</i>		16	
II. Serotyping of <i>Bordetella pertussis</i>		14	
III. Genotyping of <i>Bordetella pertussis</i>		7	
Identification of <i>Bordetella pertussis</i>			
1. Routine identification of clinical isolates of:			
a. <i>Bordetella pertussis</i>		15	94
b. Other <i>Bordetella</i> species		12	75
Average number of isolates received per year:			
a. <i>Bordetella pertussis</i>	<50	12	
	100-250	3	
	NA	1	
b. Other <i>Bordetella</i> species	<50	10	
	0/NA	6	
2. Laboratory methods used for identification			
a. Cultural characteristics		14	
b. DNA sequencing		4	
SSU rDNA		3	
<i>ompA/risA</i>		1	
IS481/IS1001		1	
c. Specific PCRs		8	
Targets:			
IS481		8	
<i>ptxP</i>		5	
IS1001		7	
IS1002		2	
Flagellin		2	
<i>recA</i>		2	
MALDI-TOF		6	
Manufacturer			
Bruker	5		
Biomerieux, Vitek MS	1		
Not stated	1		
Serotyping of <i>Bordetella pertussis</i>			
Using monoclonal antibodies (supplied)		14/16	
Using in-house method		3/16	
Genotyping of <i>Bordetella pertussis</i>			
Pulsed Field Gel Electrophoresis		4/16	
Multiple-Locus Variable Number Tandem Repeat Analysis		2/16	
Multiple Antigen Sequence Typing		7/16	

NA = not applicable; MS= Mass spectrometry

Section 2 - Serotyping results

Using monoclonal antibodies (supplied)

Over half of the laboratories (8/14) reported observations of autoagglutination/agglutination with test strains, and/or in PBS/saline-making, difficulties with the scoring of the agglutination reaction. This was also seen by 2/14 laboratories in the Fim2 and Fim3 control strains S1 and S3 (Table 7). A protocol for slide agglutination was included for the serotyping (Appendix), however two laboratories used an ELISA protocol, both of which scored correct results for all four *B. pertussis* strains (4/4).

Participants used a range of dilutions from 1:10 to 1:50 for the slide agglutination method to 1:2000 for the ELISA method. One laboratory (no. 9) also submitted data from incubation of *B. pertussis* on two different media, Bordet Gengou and Charcoal/Regan Lowe. Incubation conditions for both media were 24 hours at 35°C. Results from both media were similar. With Bordet Gengou, agglutination was reported with both 06/124 and 06/128 for all four test strains, giving a result of serotype (st) 2,3, but agglutination with saline was also reported. Laboratory no. 9 also reported results from its standard in-house assay which also uses the WHO MABs (antisera 04/154 and 04/156) on Bordet Gengou with the following results (EQA no. 2, st 2,3; EQA no.3, st 2,3, EQA no.4, st 3; EQA no.5 st 2) – i.e. one of four strains was correctly identified, however agglutination with saline was also seen on all the test strains.

Two laboratories (no.9 and no.16) reported the use of other antisera for serotyping *B. pertussis*. Laboratory no. 9 used antisera from Becton Dickinson (BD) which resulted in EQA no.2, st 3; EQA no.3, st 2; EQA no.4, st 3 and EQA no.5, st 2 which would score 3/4 correct.

Laboratory no.16 also reported results used polyclonal antisera which scored 4/4 and was in agreement with that found using the monoclonal antisera.

Table 7. Results of *Bordetella pertussis* serotyping using supplied monoclonal reagents

EQA No.	<i>B.p</i> st	Laboratory no.																		
		1		2		3	4		5		6		7							
		124	128	124	128	124	128	124	128	124	128	124	128	124	128	124	128	124	128	
Dilution		1:2000		NS		1:30		1:2000		1:50	1:40	NS	NS	1:10*	1:20	1:50	1:10*	1:20	1:50	
2	1,3	-	+++	-	+++	+	++	-	+	++	++	-	++	+	+	-	+++	+++	++	
3	1,2	++	-	-	-	+++	-	+	-	++	++	+	-	++	+	+	-	-	-	
4	1,3	-	++	-	+++	+++	+++	-	+	-	++	-	++	+	+	-	+++	++	++	
5	1,2,3	+	+	+++	++	++	-	+	+	++	-	+++	+	+++	+++	+++	++	++	-	
S1	1,2	+++	-	++	-	+++	-	+	-	++	-	+++	-	+++	+++	+++	+	+	-	
S3	1,3	-	+++	-	+	-	++	-	+	-	++	-	+	+	+	+	+++	+++	++	
		ELISA				ELISA														
EQA No.	<i>B.p</i> st	Laboratory no.																		
		9		10		11				12				14		15		16		
		124	128	124	128	124	128	124	128	User scored result	124	128	124	128	124	128	124	128	124	128
Dilution		NS		1:50	1:50	1:10	1:50	1:10	1:50	Fim3	1:10	1:50	1:10	1:50	1:10	1:10	1:30	1:30	1:10	1:10
2	1,3	2,3*	-	+++	-	-	-	+	Fim3	ND	ND	ND	ND	++	+++	++	+++	-	+	
3	1,2	2*	++	-	+	+	-	-	Fim2	+++//++	+	+++//+	+/-	++	-	+	+	++	-	
4	1,3	2,3*	-	+++	-	-	+	+	Fim3	++	-	++	-	++	+++	+++	+++	+++	-	+
5	1,2,3	2,3*	+++	++	+	+	+	+	Fim3 Fim2?	+++	+	++		+++	+++	+++	-	++	+	
S1	1,2	2*	+++	-	+				Fim2	+++	+	-		+++	-	+++	-	+	-	
S3	1,3	3	-	+++	-			+	Fim3	+		+++	+	-	+++	-	+++	-	+	

B.p st = *Bordetella pertussis* serotype; 124, NIBSC code 06/124 WHO International Standard Monoclonal Antibody for Serotyping *Bordetella pertussis* Fimbrial Antigen 2 (1st WHO International Standard); 128, NIBSC code 06/128 WHO International Standard Monoclonal Antibody for Serotyping *Bordetella pertussis* Fimbrial Antigen 3 (1st WHO International Standard).

*Laboratory no. 7 shows results from three dilutions of MABs, results used for scoring are indicated in red, others are included for information purposes.

NS = Not stated, ND = Not done – this laboratory was sent two slopes of EQA Sample 3 (and not Sample 2) in error.

Section 3 – Genotyping

Seven laboratories performed genotyping, four by PFGE, two by MLVA and six by MAST. One laboratory used qPCR to identify the MAST types. Participants were asked to provide gel images and conclusions on the relatedness/similarity/differences of the *B. pertussis* isolates.

PFGE

Laboratory no.2

PFGE method used: according to the protocol provided [11,12] using Low Range PFG Marker (Cat. No.N0350S, New England Biolabs).

Analysis of the PFGE profiles using BioNumerics software. Similarity was calculated using the Dice coefficient with a band matching tolerance of 1%. Clustering was performed using UPGMA. Strain EQA no.2 and EQA no.4 show an identical profile. The other two strains show a different profile. The profile of strain 3 shows more similarity to the profile of strains 2 and 4 (93%) than to the profile of strain 5. The profile of strain 5 shows the least similarity to the others (89.2%).

Laboratory no.4

PFGE method used: recommended by EUpert-Labnet (*Xba*I)

Controls strains: Finnish strain PRCP306 (BpSR23) was used as internal control in each run. The relatedness was 95% between EQA2 or EQA4 and EQA3. The relatedness of the above-mentioned three strains was 80% to EQA5.

Strain study code no.	PFGE type	Comment
EQA 1	not Bordetella	
2	BpSR5	
3	BpSR3	
4	BpSR5	
5	Undefined profile	The profile identified was not found in our dataset. However it was very similar to BpFINR12 or BpSR7.
6	BppFINR2	There were only four PFGE profiles identified based on PFGE dataset of <i>B. paraptussis</i> . The most prevalent PFGE profile of <i>B.pp</i> was BppFINR2 [13].
7	Bordetella	
8	<i>Bordetella holmesii</i>	

Laboratory no.6

PFGE method used: as per method provided.

Control strains/markers: *B. pertussis* Tahoma NC13251 control strain and Low Range PFG Marker from New England Biolabs (48.5 kb lambda concatemers). Under run conditions provided in protocol, 48.5 kbp band ran off the end of the gel. All four strains show similar banding patterns in the lower molecular weight region (bottom half of gel), with band difference only observed at higher molecular weights. Strains 3 and 5 clustered together (cluster 1) and strains 2 and 4 clustered together (cluster 2). Cluster 1 strains differed from cluster 2 strains by ≥ 4 bands. Three band differences were observed between strain 3 and strain 5 from cluster 1. Strains 2 and 4 from cluster 2 displayed similar banding patterns, with only band intensity differentiating the strains visually.

Laboratory no. 14

PFGE method used: recommended by EUpert-labnet (*Xba*I) used by THL Finland

Controls strains/markers: Tohama, PRCB 413, PRCB309, FR287,B902, 18323, 1772, B935, PRCB9, PRCB729, PRCB 730, PRCB731, PRCB687, PRCB588, Low range PFG Marker New England BioLabs, Cat. no. N0350S.

Strain study code no.	PFGE type ¹	PFGE type ² (THL)	Comment
EQA2	PRCB731	BpSR5	Position tolerance 1%
EQA3	PRCB729	BpSR3	Position tolerance 1%
EQA4	PRCB731	BpSR5	Position tolerance 1%
EQA5	95% relatedness PRCB729 and PRCB413 (90% relatedness PRCB309)	95% relatedness BpSR3 and BpFINR21 (90% relatedness BpSR7)	Position tolerance 1%

¹ PFGE type reported by participating laboratory.

² PFGE type reported by THL following analysis of submitted image files. For explanation and relatedness of different PFGE profiles, see recent publication by EUpertstrain group [8].

MLVA

Two laboratories (Laboratories 6 and 9) performed MLVA and scored 100%.

Laboratory no. 6

MLVA method used: as per method provided

Controls strains/markers: *B. pertussis* Tohama NC13251 control strain.

All isolates identified as MLVA type 27.

Laboratory no. 9

MLVA method used: as per protocol provided.

Controls strains/markers: RIVM *B. pertussis* control strain (MLVA 27).

All isolates identified as MLVA type 27.

MAST

All six laboratories reported genotypes of pertactin (*prn*), pertussis toxin S1 subunit (*ptxA*), and pertussis toxin promoter (*ptxP*). Five also reported alleles of tracheal colonisation factor (*tcfA*), fimbrial antigen 2 and 3 (*fim2*, *fim3*) as per protocol. One laboratory (no. 5) reported the pertussis toxin S1 subunit allele as *ptxA2* where the intended result was *ptxA1*. All other alleles were scored correctly by all laboratories.

5. Discussion

Identification of *B. pertussis* and *B. parapertussis*

All laboratories successfully identified the four *B. pertussis* strains, and all but one successfully identified the *B. parapertussis* strain. Misidentification of *B. parapertussis* was due to a reported lack of PCR products, biochemical inactivity and 100% SSU rDNA similarity with *Achromobacter*.

Identification of *B. holmesii* and *B. bronchiseptica* and differentiation from *B. pertussis* and *B. parapertussis*

Most laboratories (14/16) successfully identified *B. holmesii* to species level. Two of 16 identified to the level of genus only.

Serotyping of *B. pertussis* isolates

Many laboratories reported auto-agglutination using the slide agglutination protocol. However, it is apparent that serotyping is not routinely performed in all reference laboratories, hence many laboratories lacked experience with this technique. Satisfactory results are possible with the slide agglutination technique, but experience is required. More consistent results may be achieved using the ELISA protocol but this is more appropriate for processing larger numbers of strains at one time.

Genotyping of *B. pertussis* isolates

PFGE

Despite the limitations of detailed PFGE control strains and analysis criteria, all four laboratories reported PFGE profiles of the two epidemiologically-related strains as indistinguishable/belonging to the same cluster and distinct from the other unrelated strains.

MLVA

The MLVA types were checked prior to dispatch by the sending laboratory using the method previously described [8], adapted for analysis using a CEQ8000 genetic analysis system (Beckman Coulter, High Wycombe, United Kingdom) [14]. Only two laboratories performed MLVA, both using the ABI platform and both achieved 100% correct results.

Interpretation and reporting of *Bordetella* identification and typing results

The majority of laboratories readily identified the *Bordetella* strains to genus and species level. However, not all fully identified the *B. holmesii* and *B. bronchiseptica*. There were differences in the degree of identification of the non-*Bordetella* strain (*Haemophilus influenzae*) from negative/no growth to correct identification to species level.

Difficulties were experienced interpreting the Fim serotyping with the monoclonal antisera. Interpretation criteria of the PFGE profiles were not provided, leading to variation in reporting of these data.

Identification of training needs

Agreed protocols and criteria for identification are required. Training needs were identified, particularly with respect to serotyping.

Establishment of best practice in current assays, interpretation and reporting

In conjunction with ECDC and the EUpert-Labnet activities, a document will be produced describing best practices including *Bordetella* identification and *B. pertussis* typing.

Limitations

The strains included were clinical isolates, and although the whole genome sequence (WGS) for three of the *B. pertussis* strains (EQA no.3, 4, 5) had been previously determined, these data were not available for all strains/species.

Serotyping

The ELISA protocol for the MAbs was not supplied to participants.

PFGE

The *B. pertussis* strains were not analysed by PFGE prior to dispatch. Reference strains and analysis criteria were not provided.

MLVA

The four *B. pertussis* strains were all the same type, MLVA 27. This was due to selection of recent isolates and the fact that this isolate was the predominant type in Europe. Although arguably MLVA is less discriminatory than PFGE, it can be performed directly on clinical extracts [15].

MAST

A list of accession numbers of known alleles was not provided.

Overall, the results of the *Bordetella* identification and genotyping sections of this EQA scheme are good. However, the serotyping results were unsatisfactory, most likely due to both inexperience with the slide agglutination method and failure to provide an ELISA method protocol.

These EQA exercises perform a crucial role and are extremely useful to participants and to ECDC for the validation of data collected from the Member States, to improve comparability and to detect changes in trends. . The support of the EUpert-Labnet network is appreciated by the participants, as reflected in the level of interest and the willingness to deploy agreed methodologies for typing and characterization of *Bordetella* spp.

6. Recommendations

National reference laboratories should be able to identify all clinically important species of *Bordetella* to the level of species including *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. holmesii*. Agreed identification criteria for the various phenotypic and genotypic methodologies for each species should be produced.

National reference laboratories should be able to perform fimbrial serotyping of *B. pertussis* isolates.

National reference laboratories should be able to perform genotyping of *B. pertussis* isolates. However, there is a need to update the recommendations to standardise epidemiological typing methods for *B. pertussis* isolates.

Hospital laboratories in EU/EEA countries should be encouraged to isolate putative *Bordetella* species from clinical cases and refer to national reference laboratories for confirmation and further typing.

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Annex 1. The EUpert-Labnet *Bordetella* identification and *B. pertussis* EQA instructions, questionnaire and reply form

Framework Contract No ECDC/2011/013 Work package 2

Bordetella EQA panel - February 2013

Purpose

- Identification of *Bordetella*
- Serotyping of *Bordetella pertussis*
- Genotyping of *Bordetella pertussis*

Panel composition

- A panel of eight (8) coded strains (Study Code No.1 to 8) on charcoal agar slopes
- Two control strains of *Bordetella pertussis* for serotyping (S1 and S3) with yellow labels
- Monoclonal antibodies
 - NIBSC code 06/124 WHO International Standard Monoclonal Antibody for Serotyping *Bordetella pertussis* Fimbrial Antigen 2 (1st WHO International Standard)
 - NIBSC code 06/128 WHO International Standard Monoclonal Antibody for Serotyping *Bordetella pertussis* Fimbrial Antigen 3 (1st WHO International Standard)

Recommended storage

Bacteria on slopes (Study Code No. 1 to 8, control strains S1 and S3): room temperature.

Recommend that you subculture onto agar plates (e.g. charcoal blood agar) on arrival and store/preserve cultures as you would routinely (e.g., frozen at 80°C).

Monoclonal antibodies

NIBSC code 06/124 and NIBSC code 06/128: +4°C until reconstitution then -20°C (see below)

These ampoules were previously frozen and because of the inherent stability of lyophilized material, have been shipped at ambient temperature. Please see attached data-sheets for each ampoule for full instructions.

The entire ampoule contents should be reconstituted with 1ml of saline, dispensed into suitable aliquots and stored at -20°C until needed. Repeat freeze thawing should be avoided.

Participants are requested to perform one or more of the following:

1. Identification to genus level of Study Code 1 to 8 inclusive and to species level of all *Bordetella* isolates found by your usual methods (e.g., phenotypic/genotypic/MALDI-TOF).
2. Serotyping of all *Bordetella pertussis* isolates identified in (1) using the enclosed monoclonal reagents and your usual standard method.
3. Genotyping of the *B. pertussis* isolates identified in (1) by
 - Pulsed-Field Gel Electrophoresis (PFGE) using attached SOP used at the Pertussis Reference Laboratory of National Institute for Health and Welfare, Turku, Finland (or other method)
 - Multi-locus Variable Number of Tandem Repeats Analysis (MLVA) using attached SOP by RIVM
 - Multiple Antigen Sequence Typing (MAST) DNA sequencing of regions of the genes for:
 - pertactin (*prn*)
 - pertussis toxin (*ptxA*) the A or S1 subunit of pertussis toxin
 - pertussis toxin promoter (*ptxP*)
 - tracheal colonization factor (*tcfA*)
 - fimbrial subunit 2 (*fim2*)
 - fimbrial subunit 3 (*fim3*)
 - using attached SOP by RIVM

or other genotyping methods to characterise these genes.

Deadline for reporting of results

- | | |
|--|-------------|
| 1. Identification of <i>Bordetella</i> | 10 May 2013 |
| 2. Serotyping of <i>Bordetella pertussis</i> | 10 May 2013 |
| 3. Genotyping of <i>Bordetella pertussis</i> | 10 May 2013 |

Bordetella EQA reply form

Please return completed form to: Norman Fry, norman.fry@hpa.org.uk, Fax: +44 (0)20 8205 6528

Person for correspondence:

Address:

Tel:

E-mail:

Please tick all the boxes which apply:

1. Do you agree to participate in this EQA and agree to submit all data as requested? Yes

If the answer is "yes", please give the following information:

2. Which part(s) of this EQA are you performing?

- | | | |
|--|-----|--------------------------|
| a) Identification of <i>Bordetella</i> | Yes | <input type="checkbox"/> |
| b) Serotyping of <i>Bordetella pertussis</i> | Yes | <input type="checkbox"/> |
| c) Genotyping of <i>Bordetella pertussis</i> | Yes | <input type="checkbox"/> |

If you have answered yes to a) Identification of *Bordetella* please answer questions below otherwise leave blank

3. Do you routinely perform identification of clinical isolates of *Bordetella pertussis*

Yes No

other *Bordetella* species

Yes No

4. What is the average number of isolates your laboratory receives per year?*

Bordetella pertussis <50, 100-250, 251-500, 501-750, 751-1000, >1000

other *Bordetella* species <50, 100-250, 251-500, 501-750, 751-1000, >1000

* circle as appropriate

5. Which laboratory methods were used for the identification of his panel?

i) Cultural characteristics Yes No

If Yes please describe

.....
 ii) DNA sequencing of gene(s) Yes No

If Yes which one(s)

16S rRNA gene Yes No

ompA Yes No

risA Yes No

other.....

(please state)

Please give criteria for identification used

e.g. number of bases...../percentage identity to reference sequence..... (GenBank reference number.....)

.....
 iii) Identification using specific PCR(s) Yes No

If Yes please give details, gene targets, references etc.

.....
 iv) MALDI-TOF Yes No

If Yes:

Manufacturer / model.....

Please give criteria for identification used (e.g. score/value/confidence)

.....
 Serotyping of *Bordetella pertussis*

Using monoclonal antibodies supplied Yes No

Using in-house method Yes No

Genotyping of *Bordetella pertussis*

PFGE Yes No

MLVA Yes No

MAST Yes No

Other (please state) Yes No

1. Identification of *Bordetella*

Identification to genus level of Study Code 1 to 8 inclusive and to species level of all *Bordetella* isolates found by your usual laboratory methods.

Please complete table below with description to at least genus level for all strains and also to species level for all *Bordetella*.

Strain	Identification		
	Genus	Species	Comment
Study Code no. 1			
Study Code no. 2			
Study Code no. 3			
Study Code no. 4			
Study Code no. 5			
Study Code no. 6			
Study Code no. 7			
Study Code no. 8			

2. Serotyping of *Bordetella pertussis* using monoclonal antibodies to *B.pertussis* fimbriae type 2 and 3.

Laboratory name

Strain	Monoclonal 06/124 Fimbrial antigen 2	Monoclonal 06/128 Fimbrial antigen 3	Polyclonal antibody Anti-agglutigen 2	Polyclonal antibody Anti-agglutigen 3	Other
Dilution from supplied sample					
Study code no.					
Control strain					
S1					
S3					

Please enter the results as +++, ++, + and – to indicate degree of agglutination. If other antibodies or *B.pertussis* strains e.g. FDA 460, have been used, please give details and serotype.

3a. Genotyping of the *B. pertussis* isolates - PFGE

Please complete table below for all *B.pertussis* strains identified.

Strain Study code no.	PFGE type		Comment

PFGE method used.....

Controls strains / markers.....

Please include gel image if possible and your conclusions about relatedness/similarity/differences of *B.pertussis* isolates.

3b. Genotyping of the *B. pertussis* isolates - MLVA

Please complete table below for all *B.pertussis* strains identified.

Strain Study code no.	Identification		
	MLVA profile	MLVA type	Comment

MLVA method used.....

Controls strains / markers.....

Please state your conclusions about relatedness/similarity/differences of *B.pertussis* isolates.

3c. Genotyping of the *B. pertussis* isolates - MAST

Please complete table below for all *B.pertussis* strains identified.

Strain Study code no.	MAST					
	<i>prn</i>	<i>ptxA</i>	<i>ptxA</i>	<i>tcfA</i>	<i>flm2</i>	<i>flm3</i>

Please state your conclusions about relatedness/similarity/differences of *B.pertussis* isolates.

Annex 2. The EUpert-Labnet *Bordetella* identification and *B. pertussis* typing EQA protocols

Slide agglutination protocol for serotyping *B. pertussis* strains using monoclonal antibodies (NIBSC) provided by NIBSC.

Pulsed-Field Gel Electrophoresis (PFGE) protocol provided by Pertussis Reference Laboratory of National Institute for Health and Welfare, Turku, Finland.

Bordetella EQA

Protocol of slide agglutination for serotyping B. pertussis strains using monoclonal antibodies (NIBSC)

Materials

Charcoal agar plates

Microscope slides e.g. BDH Superfrost

Sterile saline and distilled water

Sterile loops (plastic)

Gilson P10 or P20 with tips

Reference *B. pertussis* strains:- strains of known serotype 2, 3 and 2,3.

Polyclonal (rabbit) freeze dried reference antisera (NIBSC reagents):-

B. pertussis anti -agglutinogen 2 89/598

B. pertussis anti - Agglutinogen 3 89/600

Monoclonal antibodies (mouse):-

04/154 WHO Interim Reference Reagents for anti-*B. pertussis* fimbriae 2

04/156 WHO Interim Reference Reagents for anti-*B. pertussis* fimbriae 3

Each vial contains 500µl of frozen mouse ascitic fluid purified by ammonium sulphate precipitation and adjusted with PBS to 4 mg/ml IgG.

Method

1. Grow reference and test strains at 37°C for 24 - 48 hours on charcoal agar plates
2. Preparation of polyclonal antisera. - for 89/598 and 89/600
Open ampoule and reconstitute the freeze dried material in 0.5ml sterile distilled water
Aliquot this solution into 100µl aliquots and freeze at -20°C until needed .
3. Monoclonal antibodies:-
Thaw the frozen preparations 04/154 and 04/156 , aliquot into 100ul aliquots and freeze at -20°C
Make a 1/50 dilution of this material in saline for immediate use.
4. Slide agglutination:- (Use prewashed clean microscope slides e.g. BDH Superfrost)
 - Label slide (on frosted end with pencil or with permanent marker)
 - Using a Gilson pipette place one 10µl drop of saline and one drop each of anti-ag 2 antibody
 - And anti-ag 3 antibody on the slide. (i.e. 3 drops in a line down the slide)
 - Using a sterile loop remove a tiny portion of growth from the surface of the charcoal agar plate and mix this directly into the drop on the microscope slide. Use three separate mixings into the three separate drops with fresh loops on each slide.
 - Mix the growth and antibody with the loop in each drop and when the slide has all three drops containing bacteria gently rock the slide to continue mixing.
 - Observe the drops for agglutination against a black background , in good light using a suitable lens and record the results.
 - Discard slide after recording the result. The result should be read rapidly before the drops start to dry out.

Results

Agglutination is observed as 'clumping' of the bacteria within the drop.

The saline control – first/top drop should remain a clear smooth suspension, if this shows signs of agglutination the test must be repeated – auto-agglutination can occur.

Record the results together with the strain and antibody information on the assay sheet.

SOP for pulsed-field gel electrophoresis used at the Pertussis Reference laboratory, THL, Turku, Finland

For questions please email alex-mikael.barkoff@thl.fi or qiushui.he@thl.fi

Instruments

CHEF-DR[®] III Pulsed Field Electrophoresis System, Bio-Rad Laboratories

Materials

Plug-molds

- CHEF Disposable Plug Mold, Bio-Rad Laboratories, cat# 170-3713

Wash caps

- Screened caps, Bio-Rad Laboratories, cat# 170-3711

Gel comb

- 15-Well Adjustable-Height Comb 1,5 mm, Bio-Rad Laboratories, cat# 170-4324
- 20-Well Adjustable-Height Comb 1,5 mm, Bio-Rad Laboratories, cat# 170-4322

Wash falcons, water poultice

Buffers

TBE: stock 10x, work solution 1x

TE: stock 10x, work solution 0.5x

EDTA, 0.5 M, pH 8

Reagents

Low Melting (LM)-agarose

- SeaPlaque[®] GTG[®] agarose, 25 g, BioWhittaker Molecular Applications, cat# 50111
- Storage at room temperature (RT)

PFGE-agarose

- Pulsed Field Certified Agarose, 100 g, Bio-Rad Laboratories, cat# 162-0137
- Storage at RT

Proteinase-K

- Proteinase K (fungal), 1 g (>20 U/mg), Invitrogen, cat# 25530-031 diluted in aqua 20 mg/ml, storage in -20 °C.

Sarcosyl

- N-lauroylsarcosine, 100 g, Sigma, cat# L-5125 diluted in aqua (10 %, 100 mg/ml), storage in RT.

Restriction enzyme XbaI

- 3000 U, 20 000 U/ml, New England BioLabs, cat# R0145S. package includes also 10xNEBuffer and 100xBSA
- Storage at -20 °C.

Low range PFG Marker

- 25 µg/ml, gelstick, New England BioLabs, cat# N0350S
- Storage -20 °C.

Ethidiumbromide (EtBr) 10 mg/ml

- Storage at RT.

Time schedule

Freshly made plugs directly to the gel (Mon-Fri).

Day 1: Making of plugs and proteinase-K treatment overnight (O/N)

Day 2: Washing of plugs and restriction O/N

Day 3: Stopping the restriction and starting the gel-run

Day 4: Gel-run on

Day 5: Gel-run ready, gel staining and imaging

Plugs in storage, gel-run done later (I phase: two days, II phase Mon-Thu or Tue-Fri)

I phase (making of plugs)

Day 1: Making of plugs and proteinase-K treatment overnight (O/N)

Day 2: Washing of plugs and storage.

II phase (plugs from storage to gel-run)

Day 1: Restriction O/N

Day 2: stopping of restriction and starting the gel-run

Day 3: Gel-run on

Day 4: Gel-run ready, gel staining and imaging.

Plugs (estimated time around 2hours, start between 13-14)

- First, turn on water poultice (one hour before start) to +55°C
- Prepare LM-agarose (2%)
 - 0.5xTBE (fresh!): 1 ml 10xTBE + 19 ml aqua
 - 200 mg LM-agarose + 10 ml 0.5xTBE (e.g. in 50 ml falcon tube)
 - Melting in microwave oven, Balancing in water poultice/heater +50-55 °C for one hour
 - Can be stored in RT (months) and melted in microwave oven, when next time used. If done like this remember to balance it in +55°C.
- Dilutions from samples (2e9 cfu/ml) in 1xTE-buffer (OD₆₅₀ = 0.66). Strains cultured 2-3 days
- Mark Eppendorf tubes (1 tube/strain) and plug molds (2 mold wells/strain)
- Pipet bacterial suspension to the Eppendorf tubes, 110 µl/tube
- Pipet balanced LM-agarose to Eppendorf tube, 110 µl / tube. Resuspend/mix the bacterial suspension and LM-agarose with pipet and pipet into plug molds (ca. 100 µl/mold well, a little bit over the top)
- Filled plug molds to refrigerator for coagulation, at least for 20 minutes.

Proteinase-K –treatment

- Mark the round bottom (2ml) Eppendorf tubes, 2 tubes/strain
- Lysis buffer

	5 strains = 10 plugs	10 strains = 20 plugs	15 strains = 30 plugs
Sarcosyl, 10 %	1 ml	2 ml	3 ml
EDTA, 0.5 M, pH 8	9 ml	18 ml	27 ml

- Proteinase-K –buffer

	5 strains = 10 plugs	10 strains = 20 plugs	15 strains = 30 plugs
Lysis buffer	9,5 ml	19 ml	28.5 ml
Proteinase-K, 20 mg/ml	500 µl	1 ml	1.5 ml

- Pipette 500 µl of lysis buffer (10 plugs) or 1 ml (20 plugs) or 1,5 ml (30 plugs) away and replace this volume with Proteinase-K (diluted in the freezer)
 - Plugs into Eppendorf tubes (1 plug/tube, 2 tubes/strain) and add Proteinase-K –buffer, 1 ml/tube
 - Plugs into water poultice/heater O/N, +55 °C, if possible poultice with mixing function
 - For the next day washing, make 1x TE-buffer (wash). Put washing buffer also in +55 °C O/N:

	5 strains = 10 plugs	10 strains = 20 plugs	15 strains = 30 plugs
Need 3 x 7.5 ml/plug	225 ml	450 ml	675 ml, make 1000 ml
H ₂ O in volumetric flask	250 ml	500 ml	900 ml
10 x TE-buffer	25 ml	50 ml	100 ml

- 10 x TE-buffer is diluted with aqua.

Washing the plugs (estimated time around 4h)

- If control not included in samples, see further restriction!!!
- Tubes on ice (shaking) for 10 minutes
- Transfer plugs from Eppendorf tubes into 50ml washing falcons (1 plug/falcon tube)
- Wash with 7.5 ml 1xTE-buffer, 3 x 1 h, in shaking (if possible)
 - Note! Pour the washing buffer carefully out of the falcon tube, through the green caps. So that the plugs are not broken/crushed. Green caps can be kept on the tubes during all washes. New washing buffer is pipetted in to the tube through the hole on the green cap
 - 2 first washes with pre-warmed buffer and in +55 °C (water poultice/heater)
 - Third wash in RT
 - Between the washes tubes on ice (shaking) for 10 minutes
- Mark the storage Eppendorf tubes (2 ml, round bottom), 1 tube/strain, 2 plugs/tube***
- Transfer the washed plugs from falcons to Eppendorf tubes (2 ml, round bottom), add 1 ml of 0.5 M EDTA pH8 to all tubes
 - Storage in refrigerator (+4°C)

Restriction and final wash (estimated time around 2h)

- Take the plugs from storage tubes (EDTA-tube in fridge) and put into wash falcon tubes, 1 plug/strain

	1 gel		2 gels	
	15-well comb	20-well comb	15-well comb	20-well comb
Samples + Control	11 + 1	16 + 1	22 + 1	32 + 1
Need 40 ml/tube	480 ml	680 ml	920 ml	1320 ml
H ₂ O in volumetric flask	500 ml	1000 ml	1000 ml	1000 + 500 ml
10 x TE-buffer	50 ml	100 ml	100 ml	100 + 50 ml

- 10 x TE-buffer is diluted with aqua into 1x TE-buffer (wash-buffer)
- Plugs and control in TE-buffer +4 °C about 4 h (shaking or occasionally disturbed).
- ***If you continue directly to restriction after washes, add TE-buffer into wash falcon tubes, 40 ml/tube
 - One hour in +4 °C is enough (shaking or occasionally disturbed)
 - First plug to restriction 1 plug/strain, second plug into storage tube after wash (see above)
- Mark the Eppendorf tubes (2 ml, round bottom)
- Transfer plugs into Eppendorf tubes one by one, as follows:
 - Pour the wash-buffer out of the falcon tube through the green cap
 - Transfer the plug with a loop onto a clean petri dish
 - Cut with surgery knife ca. 1/5 of the plug into Eppendorf tube (2ml, round bottom)
 - Note! If you run two gels, prepare two pieces from the control strain into two different Eppendorf tubes!
- Transfer rest of the plug back into the EDTA-storage tube

Do the restriction mixes in 2 ml Eppendorf tubes

- 15-well comb
 - For one gel: 13 restriction mix (11 samples + control + pipetting reserve 1 rxn)
 - For two gel: two 13 restriction mix (22 samples + 2 controls + pipetting reserve 2 rxn)
- 20-well comb
 - For one gel: two 9 restriction mix (16 samples + control + pipetting reserve 1 rxn)
 - For two gel: three 12 restriction mix (32 samples + 2 controls + pipetting reserve 2 rxn)

Reagents:	1 rxn	9 rxns	12 rxns	13 rxns
Aqua (sterile or commercial)	131 µl	1179 µl	1572 µl	1703 µl
100 x BSA	1,5 µl	13,5 µl	18 µl	19,5 µl
10 x buffer	15 µl	135 µl	180 µl	195 µl
Xba I (20000 U/ml, 50 U/reaction)	2,5 µl	22,5 µl	30 µl	32,5 µl
Total	150 µl	1350 µl	1800 µl	1950 µl

- Pipette restriction mix on the top of plugs in the Eppendorf tubes, 150 µl/tube
 - Rest of the mix can be shared equally to the Eppendorf tubes
- Samples into water poultice/heater, +37 °C (shaking) O/N (16-18 h)
- Next morning, stopping of restriction
 - Pipet 1 ml/tube 0,5 M EDTA pH8 into the Eppendorf tubes on top of the restriction mix
 - In RT (shaking or occasionally disturbed) for 15 minutes
 - Tubes into +4 °C to wait the gel-run.

Gel-run

- Restricted plugs into 0.5 x TBE-buffer
 - Pipet the EDTA away and replace it with 1,3 ml of 0.5 x TBE-buffer
 - Put also ladders (Low Range PFG Marker) into 0,5 x TBE-buffer
 - ca. 1 mm thick pieces, 3 pieces/gel, can be put into same tube
- Tubes in +4 °C (shaking or occasionally disturbed) at least for 1 h
 - Preparing the agarose-gel (300 ml Erlenmeyer flask)
- 1.2 g PFGE-agarose + 120 ml 0.5 x TBE (= 1% PFGE agarose)
 - Melting in microwave oven in Erlenmeyer bottle
 - Balancing in water poultice/heater +55°C (glass bottle) at least for 1 h
- Melting of LM-agarose in microwave oven
 - Put also in +55°C at least for 1h
- Build up the gel-mold (gel-tray)
- Attach the comb into the stand and set the plugs onto the bottom part of the gel-comb spikes
 - Set the ladders to the edge and centre of the gel
 - After ladder (centre), the control strain is added (according to your selection)
 - Samples are added according to the run-list
 - Pipet the extra buffer away around the plugs
 - Plugs are fixed to the comb by pipetting a few drops of LM-agarose on the top of the plug
 - Let coagulate for 5 minutes
- Fix the comb+stand into the gel-mold (gel-tray), so that the bottom edge does not touch the bottom plate of the gel-mold
- Pour the balanced PFGE-agarose carefully into the gel-mold (gel-tray). Do not shake the bottle containing balanced gel when taking it from +55°C (water poultice/heater)
 - Let coagulate at least for 40 min
- Make 0.5 x TBE-run-buffer in 2 l and 1 l volumetric flasks (ca. 2.2 l/run-chamber is needed)
 - 2 l = 100 ml 10 x TBE + aqua
 - 1 l = 50 ml 10 x TBE + aqua
- Preparation of the PFGE-machinery
 - Pour the run-buffer into the run-chamber until the holes (close to the backwall) are covered (and a little bit over).
 - Start the PFGE-machinery
 - PFGE-machine ("Power" –button in the machine)
 - Cooler (bottom shelf)
- Cool down the run-buffer until it reaches the run-temperature
 - Adjust the cooler temperature to +14°C ("Set temp" → "Raise" or "Lower")
 - Turn on the pump ("Pump" –button in PFGE-machine)
 - Do not start the run before run-buffer is cooled down (+14°C, check from the cooler by pressing the "Actual temp" –button)
- Start the run (As late as possible at the afternoon, whereas the run takes 40 h)
 - Take the gel-comb off the gel and clean the bottom plate with hand paper (extra gel away)
 - Put the gel and bottom plate into the run-chamber (samples on to the backside edge) and close the lid
 - Program the run:

	Initial sw. time	Final sw. time	Run time	Volts/cm	Included angle
Block 1	5	6	16	5,5	120°
Block 2	8	35	24	5,5	120°

- Check that run-time in Block 3 is zero!
- Start form "Pause/Start run" –button

Gel staining

- When gel-run is stopped, turn off the pump, PFGE-machine and cooler
- Carefully transfer the gel, with help of the bottom plate, into the staining solution
 - Make always a fresh staining solution
 - 50 µl EtBr (10 mg/ml) + 500 ml aqua
 - Staining 40 min
- Transfer the gel carefully into the rinse water
 - Rinse for 45 min.
- Imaging the gel and save the picture for analysis.

During the staining:

- Empty the run-buffer from run-chamber (PFGE-machine)
- Fill the run-chamber with aqua and turn on the PFGE-machine and pump
 - Rinse the run-chamber at least for 30 minutes (during staining)
 - Change the rinse water once (Remember to turn off PFGE-machine always before you open the lid! Risk for electric shock! Do this during the rinse)
 - At the end, turn off all machines and empty and dry the run-chamber (be careful with the electrodes!)
 - Leave the lid a little bit open when you are ready (e.g. put paper between the lid and machine).