

RESEARCH ARTICLES

High heterogeneity in methods used for the laboratory confirmation of pertussis diagnosis among European countries, 2010: integration of epidemiological and laboratory surveillance must include standardisation of methodologies and quality assurance

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Despite extensive childhood immunisation, pertussis remains one of the world's leading causes of vaccine-preventable deaths. The current methods used for laboratory diagnosis of pertussis include bacterial culture, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) serology. We conducted a questionnaire survey to identify variations in the laboratory methods and protocols used among participating countries included in the European surveillance network for vaccine-preventable diseases (EUVAC.NET). In February 2010, we performed the survey using a web-based questionnaire and sent it to the country experts of 25 European Union countries, and two European Economic Area (EEA) countries, Norway and Iceland. The questionnaire consisted of 37 questions which covered both general information on surveillance methods and detailed laboratory methods used. A descriptive analysis was performed. Questionnaires were answered by all 27 contacted countries. Nineteen countries had pertussis reference laboratories at the national level; their functions varied from performing diagnosis to providing technical advice for routine microbiology laboratories. Culture, PCR and serology were used in 17, 18 and 20 countries, respectively. For PCR, nine laboratories used insertion sequence IS481 as the target gene, which is present in multiple copies in the *Bordetella pertussis* genome and thus has a greater sensitivity over single copy targets, but has been proved not to be specific for *B. pertussis*. Antibodies directed against pertussis toxin (PT) are specific for *B. pertussis* infections. For ELISA serology, only 13 countries' laboratories used purified PT as coating antigen and 10 included World Health Organization (WHO) or Food and Drug Administration

(FDA) reference sera in their tests. This present survey shows that methods used for laboratory confirmation of pertussis differ widely among European countries and that there is a great heterogeneity of the reference laboratories and functions. To evaluate the effects of different pertussis immunisation programmes in Europe, standardisation and harmonisation of the laboratory methods are needed.

Introduction

Bordetella pertussis is an exclusive human pathogen which causes whooping cough or pertussis. Before the introduction of childhood vaccination, pertussis was a major cause of infant deaths in the world including Europe [1-4]. However, despite the extensive vaccination, pertussis has remained endemic [1-4]. The disease has resurged in the last decade and remains the least controlled of vaccine-preventable disease worldwide [5-13].

Surveillance of pertussis in European countries

Within Europe, the reported incidences vary widely. In 2010, the highest rate (97/100,000) was reported in Norway and zero cases were reported from Cyprus, Iceland and Luxemburg [14]. Data collected by the European surveillance network for vaccine-preventable diseases (EUVAC.NET) from 28 European countries conducting surveillance on whole population showed a stable number of pertussis cases in the period 2003-10, and an increase in incidence in adolescents [14]. In France, where pertussis surveillance at whole-population level is complemented by surveillance in infants below the age of six months in selected hospitals, a national incidence of 276/100,000 in 0-2 month-old

infants was extrapolated for the period 1996–2005 [7]. This epidemiological picture underlines the need for both better surveillance and control of the disease and careful interpretation of the surveillance data.

Surveillance of 47 diseases and two health conditions is mandatory in the European Union (EU) and European Economic Area/European Free Trade Association (EEA/EFTA) countries and EU case definitions should be used for reporting [15]. Pertussis is included among those diseases [16]. The case definition includes clinical, epidemiological and laboratory criteria. However, laboratory procedures and completeness of reporting may differ between countries and through time, and therefore direct comparability of laboratory-confirmed or clinically-diagnosed cases across Europe, and between years cannot be assumed. Laboratory confirmation is always warranted when there is a clinical suspicion of pertussis, because atypical symptoms often occur in infants, vaccinated adolescents and adults. Furthermore, co-infections with other microbial pathogens have been reported [17,18], and no clinician can differentiate symptoms caused by *B. pertussis* and other *Bordetella* species such as *B. parapertussis* [1].

Laboratory methods to diagnose pertussis in European countries

At present, the laboratory methods available to diagnose pertussis include bacterial culture, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) serology.

Culture is the basic method for the diagnosis of pertussis. The specimen collection for the bacterial testing is a critical part of the diagnosis. Because *B. pertussis* binds to the ciliated epithelial cells of the human upper respiratory tract, that are found in the nasopharynx, culture specimens should be taken from the posterior nasopharynx, either by nasopharyngeal (NP) swabs or aspiration. Calcium alginate, dacron and rayon swabs can be used. Because *B. pertussis* is a fragile bacterium, NP swabs or aspirates should be sent to the laboratory within four hours of collection, at room temperature. The swab or the tip of the catheter can also be placed in Reagan–Lowe transport medium. The other critical part for a successful diagnosis based on culture is an accurate identification of bacterial species.

Polymerase Chain Reaction (PCR) has proved to be more sensitive and faster than culture. Its advantages over culture include detecting bacterial nucleic acid fragments from both dead and viable bacteria. Specimens for PCR should be taken from the posterior nasopharynx by NP swab or aspiration. Dacron or rayon swabs are recommended, whereas swabs made of cotton or calcium alginate are not suitable. Insertion sequence IS₄₈₁, pertussis toxin promoter region (*ptxA-Pr*) and porin gene can be used as amplification targets in PCR for *B. pertussis*. Since porin can be found in other bacteria, a positive PCR result is not specific for *B. pertussis*. The most frequently used target gene is

IS₄₈₁ because of its high copy number in the genome of *B. pertussis*. However, the IS₄₈₁-based PCR is not able to differentiate *B. pertussis* from *B. holmesii* and *B. bronchiseptica* [19,20]. Compared to IS₄₈₁ PCR, the *ptxA-Pr* based PCR is found to be specific for *B. pertussis* but is less sensitive due to its single copy number in the genome of *B. pertussis*. A positive result for both IS₄₈₁ and *ptxA-Pr* based PCRs can be considered as a definite *B. pertussis* infection.

In terms of serological tests, those detecting IgG antibodies to purified pertussis toxin (PT) are the most specific for *B. Pertussis*, so PT is recommended as a coating antigen in both in house ELISA and commercial kits [21–23].

Both culture and PCR are suitable diagnosis methods during the early stage of the disease (i.e., < 3 weeks of onset), making them more suitable for children and infants with severe disease [21]. Enzyme-linked immunosorbent assay serology has shown to be useful for the late stage of disease (i.e., > 3 weeks of onset), especially in older children and adults, who may seek healthcare treatment later due to a milder clinical presentation, and for whom the higher maturity of the acquired immune system allows more reliable ELISA results [21]. It is known that many factors can affect specificity and sensitivity of these methods [24]. In many laboratories, PCR and ELISA serology used are usually validated in-house and therefore results are not comparable across laboratories. There is also considerable variation in criteria necessary for validation. Methods to identify the bacteria in bacterial culture can also differ between laboratories.

Aims of the study

EUVAC.NET was a European surveillance network for vaccine preventable diseases, based at the Statens Serum Institut, Copenhagen, Denmark. From September 2011, the coordination of the activities was transferred to the European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden. EUVAC.NET activities included epidemiological surveillance of pertussis and the development of laboratory-based surveillance of pertussis. This study was part of the latter activity. The aims were to identify the availability of pertussis reference laboratories established in respective EU and EEA/EFTA countries and the functions of these laboratories, and to identify and describe methods used for laboratory confirmation of pertussis.

Methods

Design of the survey

A questionnaire was designed by the European *Bordetella* expert (EUpertstrain) group in collaboration with the EUVAC.NET hub. The EUpertstrain group consists of representatives of the *Bordetella* reference laboratories in their respective EU countries [21].

TABLE 1

Presence or absence of pertussis reference laboratories in EU and EEA/EFTA countries, 2010 (n=27)

Reference laboratory	Number of countries	Countries
Absent	8	Cyprus, Estonia, Greece, Iceland, Ireland, Lithuania, Malta and Poland
Present	19	Austria, Belgium, Bulgaria, Czech Republic, Denmark, England, Finland, France, Germany, Hungary, Italy, Latvia, Luxembourg, Netherlands, Norway, Romania, Slovakia, Slovenia and Sweden

EEA: European Economic Area; EFTA: European Free Trade Association; EU: European Union.

Countries participating in the survey

As part of the EUVAC.NET activities to develop laboratory-based surveillance in EU member states, a group of laboratory experts on pertussis was included in the network in 2010. In this manuscript this group is referred to as pertussis country experts. The experts were appointed by national health authorities as requested by the ECDC. As of February 2010, 25 EU countries and two EEA countries, Norway and Iceland, had identified one respective expert. Bulgaria, Cyprus and Latvia identified two experts. All pertussis country experts were invited and agreed to respond to the questionnaire.

Data collection and analysis

The web-based questionnaire consisted of 37 specific questions. The questionnaire covered general information and asked about the existence of a national reference laboratory for pertussis and its function. Questions on detailed laboratory methods used for the diagnosis of pertussis were also included. Of the 37 questions, 25 required single answer, nine required multiple answers and three required description. Data was analysed in a descriptive way.

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Results

All of the contacted countries responded (27/27), such that the response rate to the survey was 100%.

Reference laboratories and their functions at national level

Nineteen of 27 countries stated to have pertussis reference laboratories at the national level, whereas eight countries did not (Table 1).

An inventory of a reference laboratory's functions was not available at the time the survey was undertaken, accordingly we asked the countries to list the functions in a descriptive manner. Thereafter, the reference functions were categorised as following: diagnosis, bacterial typing, surveillance and technical advice for routine microbiology laboratories (Table 2). Fourteen of 19 countries' laboratories had responsibility for diagnosis, seven for surveillance and 11 for technical advice. Only eight reference laboratories performed bacterial typing, an important method to monitor emerging *B. pertussis* strains as well as to compare vaccine antigens to bacterial antigens in circulating isolates. Of the 19 reference laboratories, twelve laboratories had two functions, three laboratories had three functions, and the laboratory for England had all four functions. Of the 12 laboratories having at least two functions, only three had both functions for diagnosis and bacterial typing.

Estimated number of laboratories performing pertussis diagnostics

Among countries, the number of estimated laboratories performing pertussis diagnostics per country varied a lot. Two countries (Hungary and Luxembourg)

TABLE 2

Functions of the pertussis reference laboratories, in the countries (n=19) where a pertussis reference laboratory is present, EU and EEA/EFTA countries, 2010

Functions of the pertussis reference laboratories	Number of countries	Countries
Diagnosis	14	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, England, Germany, Hungary, Latvia, Luxembourg, Netherlands, Romania and Slovakia
Bacterial typing	8	Austria, Belgium, England, Finland, France, Netherlands, Norway and Sweden
Surveillance	7	Denmark, England, Hungary, Italy, Latvia, Norway and Romania
Technical advice	11	Czech Republic, England, Finland, France, Germany, Hungary, Italy, Norway, Romania, Slovakia and Sweden

EEA: European Economic Area; EFTA: European Free Trade Association; EU: European Union.

had only one respective laboratory performing pertussis diagnostics. Thirteen countries (Austria, Belgium, Bulgaria, Cyprus, Denmark, Finland, Greece, Iceland, Ireland, Latvia, Lithuania, Malta and Slovenia) had less than 10; four countries (Estonia, Norway, Slovakia and Sweden) had 10 to 30; three countries (Czech Republic, Italy and the Netherlands) had from 30 to 100; and three countries (England, France and Germany) had more than 100. The number of estimated laboratories was not known in Romania and Poland.

Laboratory methods for diagnosis of pertussis

When the laboratory methods for diagnosis of pertussis were surveyed, 17 countries had laboratories performing culture, 18 PCR and 20 ELISA (Table 3).

Culture

In the reference laboratories of 17 countries, culture was performed for diagnosis (Table 3). In 10 countries both NP aspirates and swabs were accepted as specimens by laboratories, in six countries (Czech Republic, Latvia, Lithuania, Malta, Romania and Slovenia) only swabs were accepted, and in one country (Greece) only aspirates. A dacron swab for sampling was the most common type. Although cotton wool swabs are not recommended, these were utilised in three countries. The common media used for culture were Regan–Lowe and Bordet–Gengou (either medium in 7 countries and

both in 2). For bacterial identification, specific methods were used in 12 countries and PCR was performed in five (Austria, Bulgaria, Luxembourg, Slovakia and Slovenia). In Greece, only gram stain was performed for bacterial identification and in Romania only biochemical characters were analysed (e.g. oxidase and urease).

Polymerase chain reaction

According to our survey, 18 countries had laboratories using PCR (Table 3). Twelve countries had laboratories using real-time PCR, whereas five (Belgium, Bulgaria, Denmark, Finland and Hungary) had laboratories using block-based PCR. In Estonia both types of PCR were in use. The most common instrument used for real-time PCR was the LightCycler (Roche). The preferred sample type for PCR was a NP swab in four countries, NP aspirate in two countries, or both in 11 countries. The following NP swabs were used: dacron in seven countries, rayon in four countries and nylon (copan) in two countries. Solubilisation of the samples before deoxyribonucleic acid (DNA) extraction was applied in 12 countries. For DNA extraction, a commercial kit was used in 17 countries and a respective in house preparation in one (Denmark). Among commercial DNA extraction kits, the Qiagen kit was used in 11 countries and other kits (AmpliSens, Argene, Biomerieux, Chemagen and Roche) were used in six countries. Of the 18 countries where PCR was employed, 15 had laboratories using extraction control (water or PBS) alongside the real sample to check for contamination. Laboratories in Czech Republic, Iceland and Italy did not have any such controls.

TABLE 3

Methods to laboratory confirm a pertussis case in EU and EEA/EFTA countries, 2010 (n=27)

Method to confirm a pertussis case	Number of countries	Countries
Culture	17	Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Malta, Romania, Slovakia, Slovenia
PCR	18	Austria, Belgium, Bulgaria, Czech Republic, Denmark, England, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Luxembourg, Slovakia, Slovenia
ELISA ^a	20	Austria, Belgium, Czech Republic, Cyprus, Denmark, England, Estonia, Finland, France, Germany, Greece, Hungary, Latvia, Lithuania, Netherlands, Norway, Poland, Romania, Slovakia, Slovenia

ELISA: enzyme-linked immunosorbent assay; EEA: European Economic Area; EFTA: European Free Trade Association; EU: European Union; PCR: polymerase chain reaction.

^a In Italy, ELISA was implemented for diagnosis after the questionnaire survey was done; and in Sweden, ELISA is used for seroepidemiology studies.

Of the target genes used in *B. pertussis* PCR, IS₄₈₁ was used in 14 of 18 countries' laboratories (Table 4). The PCR targeting IS₄₈₁ was the sole assay in eight countries' laboratories while six countries had laboratories using this PCR in combination with a PCR targeting the *ptxA-Pr*. The laboratories in Bulgaria and Luxembourg had *ptxA-Pr* and porin gene as targets, respectively. Ten countries' laboratories used internal probes to confirm the amplified PCR products. For ten countries, the PCR reaction had a volume of 20 µl, for four (Belgium, Estonia, France and Ireland) 25 µl, for one (Finland) 50 µl, and for three (Czech Republic, Denmark and Germany) other reaction volumes not indicated above. In all of the 18 countries' laboratories both positive and negative controls in each PCR run were included. However, only in nine countries was an extraction control done, and in seven, an internal amplification control, to check for the presence of inhibitors in the extracted DNA.

Of the 18 countries whose laboratories performed PCR for detection of *B. pertussis*, 16 also did PCR for detection of *B. parapertussis*. Insertion sequence IS₁₀₀₁ was used in laboratories in 13 countries, either as sole assay in nine countries, or in combination with *ptxA-Pr* in France and Germany, to confirm *B. parapertussis*.

Enzyme-linked immunosorbent assay serology

TABLE 4

Targeted genes by polymerase chain reaction for diagnosis of pertussis, EU and EEA/EFTA countries, 2010 (n=27)

Country	Polymerase chain reaction target genes ^a			
	IS481	PtxA-Pr	IS481 and ptxA-Pr	Porin
Austria	Yes	No	No	No
Belgium	Yes	No	No	No
Bulgaria	No	Yes	No	No
Cyprus	–	–	–	–
Czech Republic	No	No	Yes	No
Denmark	Yes	No	No	No
England	No	No	Yes	No
Estonia	ND	ND	ND	ND
Finland	Yes	No	No	No
France	No	No	Yes	No
Germany	No	No	Yes	No
Greece	Yes	No	No	No
Hungary	No	No	Yes	No
Iceland	ND	ND	ND	ND
Ireland	No	No	Yes	No
Italy	Yes	No	No	No
Latvia	–	–	–	–
Lithuania	–	–	–	–
Luxembourg	No	No	No	Yes
Malta	–	–	–	–
Netherlands	–	–	–	–
Norway	–	–	–	–
Poland	–	–	–	–
Romania	–	–	–	–
Slovakia	Yes	No	No	No
Slovenia	Yes	No	No	No
Sweden	–	–	–	–
Total	8	1	6	1

EEA: European Economic Area; EFTA: European Free Trade Association; EU: European Union; ND: not defined; ptxA-Pr: pertussis toxin promoter.

In the table, 'yes' indicates 'used', 'no' indicates 'not used' and '–' indicates 'not performed'.

^a In a single assay, which can be either a reaction targeting one gene, or a reaction targeting two genes simultaneously.

Altogether, ELISA serology was performed for diagnosis in the laboratories of 20 countries (Table 3). Of these, 13 used single serum testing and 11 paired serology. In two countries (Denmark and Romania), laboratories performed paired serology only if the first sample indicated no evidence of pertussis infection. In thirteen countries, laboratories used purified PT as coating antigen in ELISA, in three countries commercial kits were used, in two (Finland and Greece) whole-cell bacteria, in one (Slovenia) filamentous haemagglutinin (FHA), and in one (Czech Republic) the coating antigen was not defined (Table 5). Only in six countries did laboratories use the World Health Organization (WHO) international reference sera [25] and in four the Food

and Drug Administration (FDA) reference sera (Table 5) [26]. In each run of the ELISA, laboratories in 12 countries had both in-house positive and negative control sera included, in three countries (Belgium, Czech Republic and Germany) only in-house positive control sera were present, in one country (Poland) only buffer, and in three countries (Estonia, Norway and Slovenia) controls were not specified.

For the antibody class measured in ELISA, 19 countries' laboratories tested for IgG, 17 for IgA and 12 for IgM. The ELISA units of the test serum calculated were based on: (i) comparison of the response curve of the test serum to that of the reference sera in laboratories

TABLE 5

Coating antigens and standard sera used in ELISA for diagnosis of pertussis, EU and EEA/EFTA countries, 2010 (n=27)

Country	ELISA					
	Coating antigen			Standard sera		
	PT	Kit ^a	Others ^b	WHO	FDA	Others ^c
Austria	No	Yes	No	No	No	Yes
Belgium	Yes ^d	No	No	Yes	No	No
Bulgaria	–	–	–	–	–	–
Cyprus	Yes ^d	No	No	Yes	No	No
Czech Republic	No	No	Yes	No	No	Yes
Denmark	Yes	No	No	Yes	No	No
England	Yes	No	No	Yes	No	No
Estonia	No	Yes	No	No	No	Yes
Finland	No	No	Yes	No	No	Yes
France	Yes	No	No	Yes	No	No
Germany	Yes ^e	No	No	Yes	No	No
Greece	No	No	Yes	No	No	Yes
Hungary	Yes	No	No	No	Yes	No
Iceland	–	–	–	–	–	–
Ireland	–	–	–	–	–	–
Italy	–	–	–	–	–	–
Latvia	Yes	No	No	No	Yes	No
Lithuania	Yes	No	No	No	Yes	No
Luxembourg	–	–	–	–	–	–
Malta	–	–	–	–	–	–
Netherlands	Yes ^f	No	No	No	Yes	No
Norway	No	Yes	No	No	No	Yes
Poland	Yes	No	No	No	No	Yes
Romania	Yes	No	No	No	No	Yes
Slovakia	Yes	No	No	No	No	Yes
Slovenia	No	No	Yes	No	No	Yes
Sweden	–	–	–	–	–	–
Total	13	3	4	6	4	10

ELISA: enzyme-linked immunosorbent assay; EEA: European Economic Area; EFTA: European Free Trade Association; EU: European Union; FDA: Food and Drug Administration; FHA: filamentous haemagglutinin; PT: pertussis toxin; prn: pertactin; WHO: World Health Organization.

In the table, 'yes' indicates 'used', 'no' indicates 'not used' and '–' indicates 'not performed'.

^a For kits the coating antigen is not specified.

^b Includes FHA, pertactin, whole bacteria or not defined.

^c Includes in-house controls or not defined.

^d Both PT and FHA are used.

^e Both PT and prn are used.

^f For IgG: PT is used; for IgA: whole bacteria is used.

of eight countries, (ii) comparison of the absorbance of the test serum to that of the in-house positive human sera in laboratories of two countries, (iii) comparison of the absorbance of the test serum to that of a response curve of the in-house positive human sera in laboratories of two countries, or (iv) other alternatives in laboratories of eight countries. The cut-off values used to define recent pertussis infection were >100 IU/ml for IgG-anti PT (referred to the WHO international reference sera) in France, Lithuania and Romania; >50 IU/ml for IgG-anti PT and >12 IU/ml for IgA-anti PT in Austria, Cyprus and Latvia; and other criteria in 14 countries.

Discussion

We performed a survey among 27 EUVAC.NET participating countries and found a significant variation in the procedures used to confirm *B. pertussis* infection.

In this study, the participants were the country experts for pertussis appointed by the health authorities of the respective countries, and therefore the answers most likely reflect the situation of pertussis diagnosis in their countries. However, only up to two experts per country were included in this network, with one expert for most countries (24 of 27) and therefore the answers were probably related to the laboratory of his/her affiliation. In larger countries or in countries where pertussis diagnosis is performed by more than one laboratory, this might have contributed to a less accurate description.

Having and sustaining a reference laboratory is a critical part of laboratory-based surveillance and quality control. In this present survey, we found that only 19 countries had pertussis reference laboratories at the national level. Routine primary diagnosis was found to be the main function among the reference laboratories. Only eight reference laboratories performed bacterial typing. Bacterial typing is perhaps the most specific and important function of the reference laboratories. Indeed, marked changes have been found in the *B. pertussis* population and differences have been observed between vaccine strains and circulating isolates [3,4]. It is important to monitor emerging *B. pertussis* strains. This is especially important for Europe since almost all European countries have changed from whole cell vaccines to acellular vaccines. Acellular vaccines contain only one to five antigens. Variations between vaccine strains and current circulating isolates have been found in four of the five antigens [3,4]. Further, a new, more virulent *B. pertussis* lineage (designated P3 lineage) has been recently described and has spread worldwide [27]. The P3 lineage now predominates in many European countries and its emergence was found to be associated with increased notifications in the Netherlands. Moreover, in France, where the surveillance of clinical isolates has been performed since 1990 and where acellular vaccines have been introduced since 1998 regular increased isolation of *B. pertussis* without expression of vaccine components is observed since 2006 [28]. *B. pertussis*

isolate without expression of pertactin (Prn) was also reported in Italy [29]. This observation demonstrates the importance of microbial surveillance in order to follow the effectiveness of the pertussis vaccines used in the field. It is then of high importance to monitor the expression of vaccine antigens in currently circulating isolates. Another noteworthy phenomenon is the increased reporting of pertussis-like disease caused by other *Bordetella* species such as *B. holmesii* [30,31]. This is important because *B. holmesii* can cause false positivity in IS481-based PCR most commonly used for detection of *B. pertussis*. Therefore, the capacity by a reference laboratory to perform bacterial typing remains essential to monitor emerging isolates or species, and to inform and guide vaccine development and vaccination policies.

It is difficult to evaluate what functions each reference laboratory should have. Ideally, however, a national reference laboratory should be capable to carry out bacterial typing, diagnosis, surveillance and provide and disseminate technical advice. The technical advice should also include training of personnel who perform routine diagnosis in clinical microbiology laboratories, making data and laboratory diagnostic criteria comparable at the national level. In an ECDC published report on 'Core functions of microbiology reference laboratories for communicable diseases', the core functions were identified as: (i) reference diagnostics, (ii) reference material resources, (iii) scientific advice, (iv) collaboration and research, (v) monitoring, alert and response [32]. These functions are partially overlapping and elaborate further on the functions identified in our survey. Our assessment offered the opportunity to confirm that there is need to disseminate information with the functions suggested and implement them across Europe. This will require a coordinated approach and both technical and political commitment.

Culture has been the basic tool for the diagnosis of pertussis, although PCR and ELISA serology are the main diagnostic methods today. In this present study, throat swab is still in use for culture in one country, and non-specific methods are used for bacterial identification in two countries. It should be kept in mind that bacterial culture is important not only for diagnosis but also for continuous monitoring of emerging *B. pertussis* antigenic variants and of antimicrobial resistant strains [33,34]. Therefore, performing bacterial cultures in diagnostic laboratories should be encouraged.

In this study, a wide variation was observed in methods and protocols for PCR. Guidelines for *B. pertussis* PCR methods are needed across Europe to ensure accurate diagnosis of pertussis as well as other *Bordetella* infections.

The development of ELISA serology in the early 1980s allowed a new understanding of pertussis epidemiology. In vaccinated older children, adolescents and adults, pertussis is a rather common infection and is

usually not suspected before they have had cough for several weeks [1,2]. Culture and PCR are then often negative but many of the patients can be diagnosed by single-point ELISA serology. Indeed, of the 6,876 laboratory-confirmed cases in Finland from 1999 to 2006, 82% were diagnosed by serology and 18% by culture and PCR [35]. Most of the young patients, less than two years of age, were diagnosed by culture and PCR, whereas the older patients were more often diagnosed by serology. This increasing use of serology testing is likely, at least in part, to have influenced reported increases in pertussis in adolescents and adults: previously these cases were not being confirmed. In Norway, a total of 49,052 pertussis cases were notified from March 1996 to October 2010 [36]. About 80,000 to 90,000 pertussis tests were performed each year, resulting in about 5% positivity rate. Serology was frequently used throughout the entire time period and about 65–70% of the reported cases were diagnosed by serology. Some of the serology tests were in young children who had recently been vaccinated, thereby potentially leading to false positive diagnoses. Moreover, serological diagnostic cut-offs used were not standardised among counties, nor were they consistent through time. All of the facts mentioned above may contribute to the high incidence reported in Norway. For countries with low incidence rates, factors may include the level of awareness of the disease in clinicians and/or lack of laboratory diagnostic tests.

Because serological tests detecting IgGs to purified PT are the most specific for *B. pertussis*, PT is recommended as a coating antigen in ELISA [21]. In the present study, only 13 of the 20 countries' laboratories performing serology used PT as the coating antigen. When the performance of 11 ELISA kits commercially available in Europe was recently compared by a German group [22], the study clearly shows that kits with purified PT as an antigen should be used and IgG antibodies to PT should be measured.

For the serological diagnosis, a significant increase in anti-pertussis antibodies between the paired sera would be the most reliable method. However, many patients do not consult a physician until they have had symptoms for several weeks, and the first serum is often taken too late to detect a significant increase in a second sample. Therefore, the single-point ELISA serology is commonly used and in clinical practice, one-point serology is a diagnostic tool with interpretation difficulties due to lack of standardisation. In this present study, more than 60% of countries' laboratories performing serology used single serum testing. However, only 32% of the laboratories included the WHO international reference sera in their ELISA [25]. Obviously, the cut-off values used to define recent pertussis infection in many of these laboratories were not based on the WHO international reference sera. Furthermore, a number of these laboratories still measured anti-pertussis IgA and IgM antibodies, which have been proved to be less specific and sensitive [1,2,22].

The number of laboratories performing pertussis diagnostics varied among the countries. About half of the countries reported less than 10 laboratories performing pertussis diagnosis, whereas three countries had even more than 100 such laboratories. Since about half of countries have a small number of pertussis diagnostic laboratories, it might be possible to standardise the laboratory methods by means of organising training workshops among these countries first.

Clearly, small countries, in terms of population number, might not be able to offer all diagnostic services. Since infants may have severe and life-threatening illness due to pertussis, the order of importance for surveillance should be infants, children and adults. As recommended by the European *Bordetella* expert group EUpertstrain [21], PCR and/or culture should be performed in neonates and infants. Therefore, the diagnostic service with rapid real-time PCR should be considered.

This present survey clearly demonstrates that the methods and protocols used for laboratory confirmation largely differ among European countries and that there is a need for standardisation and harmonisation of the laboratory methods in Europe. Furthermore, surveillance reporting laboratory-confirmed cases via a European case definition will be much more valuable if laboratory methods are comparable. The survey highlighted that there is a need to implement and organise the functions of the European National reference laboratories. After the present survey, we organised two external quality assurance (EQA) studies to assess performance of the in-house PCR and ELISA for diagnosis of pertussis used in these reference laboratories within the EU [23,37]. Data from the two EQA studies confirmed the results obtained from this questionnaire survey. Since it is a big challenge for an EU-wide standardisation and harmonisation of laboratory methods for diagnosis of pertussis, the following steps should be considered: (i) to establish consensus protocols for both PCR and serology; (ii) to set up a reference laboratory or functions in each country and do standardisation first in the reference laboratories; and (iii) to have reference laboratories in each country in turn conduct standardisation among diagnostic laboratories. The EUpertstrain group consists of 12 pertussis reference and research laboratories within 10 European countries (see appendix). Because the third step is critical, it is important to set up some European pertussis reference centres, e.g. among the EUpertstrain group. The reference centres may help the national reference laboratories across Europe to organise regular workshops and carry out EQA survey at national level.

This present survey shows that the methods used for laboratory confirmation of pertussis differ widely among European countries and that there is a great heterogeneity of the reference laboratories and in their functions. To evaluate the effects of different pertussis

immunisation programmes in Europe, coordinated activities for laboratory-based surveillance are needed for the European diagnostic laboratories. The activities should include standardisation of real-time PCR methods for detection of the genus *Bordetella* (in particular *B. pertussis*), standardisation of ELISA methods for determination of IgG anti-PT antibodies, and regular EQA studies for the diagnostic methods. Besides being important from the infectious disease surveillance perspective, standardisation and harmonisation of methods would be beneficial for the clinical diagnosis in terms of both specificity and sensitivity. In addition, long-term molecular surveillance of *B. pertussis* circulating isolates across Europe is needed.

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