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RAPID COMMUNICATIONS

- Cutaneous infection caused by *Bacillus anthracis* in Larissa, Thessaly, Central Greece, July 2012** 2
by A Stefos, NK Gatselis, A Goudelas, M Mpakarosi, J Papaparaskevas, GN Dalekos, E Petinaki

SURVEILLANCE AND OUTBREAK REPORTS

- Travel-associated Legionnaires' disease in residents from England and Wales travelling to Corfu, Greece, August to October 2011** 5
by R Maini, F Naik, TG Harrison, M Mentasti, G Spala, E Velonakis, C Hadjichristodoulou, B de Jong, A Vatopoulos, N Phin

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** 11
by Q He, AM Barkoff, J Mertsola, S Glismann, S Bacci, on behalf of the European Bordetella expert group (EUpertstrain), the European surveillance network for vaccine-preventable diseases (EUVAC.NET)

LETTERS

- Does the Japanese encephalitis virus (JEV) represent a threat for human health in Europe? Detection of JEV RNA sequences in birds collected in Italy.** 21
by AE Platonov, G Rossi, LS Karan, KO Mironov, L Busani, G Rezza
- Is Japanese encephalitis emerging in Europe?** 23
by H Zeller

Cutaneous infection caused by *Bacillus anthracis* in Larissa, Thessaly, Central Greece, July 2012

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In July 2012, a confirmed case of cutaneous anthrax infection in a stockbreeder in the prefecture of Larissa, Thessaly, Central Greece was reported. The investigation revealed five related deaths in animals (two dogs and three sheep). Control measures have been taken immediately in order to prevent further spread in humans and animals.

On 7 July 2012, a stockbreeder in his early 60s was admitted to the Department of Medicine, University Hospital of Larissa, Greece with high fever up to 39.5 °C accompanied by rigors, malaise and generalised weakness that had been present for the previous six hours. The patient reported the appearance of three pruritic papular lesions on the left forearm five days earlier. He further reported that he had slaughtered and flayed a sheep six days before admission to hospital.

Case description

Upon hospital admission, the patient was febrile, his vital signs were normal, and during the physical examination three painless ulcers on the left forearm with surrounding vesicles and oedema, covered by black eschars were observed (Figure). The left axillary lymph nodes were significantly swollen. No other signs or symptoms were found during the physical examination.

Laboratory results on the day of hospital admission revealed elevation of acute phase response markers (white blood cells: 17,100/μL (range: 4,000-10,000/μL), neutrophils: 14,600/μL (range: 2,400-6,000/μL), C-reactive protein: 3.5 mg/dL (range: 0.5 mg/dL)). A working diagnosis of cutaneous anthrax was established on the basis of the patient's place of residence and typical clinical presentation. Therefore, intravenous treatment with penicillin (24 million units per day) was started immediately [1]. After 10 days of hospitalisation, he was discharged in good health with clinical and laboratory results indicating complete recovery. Although the possibility of inhalation exposure in this case was very unlikely, the precise conditions of the direct contact that took place during flaying are not

known. Therefore, upon discharge from hospital, the patient received amoxicillin (oral dose of 1,500 mg per day) for an additional 45 days as a post-exposure prophylaxis against the potential development of anthrax pneumonitis.

Laboratory investigation

On 8 July, one day after hospitalisation of the patient, biological samples (smears from pustules) were sent to the Department of Microbiology at the Medical School of the University of Thessaly. Microscopic examination of the smears showed the presence of Gram-positive rods, typical for *Bacillus anthracis*. However, bacterial cultures remained negative; this finding could be explained by the fact that, at the time the samples were taken, the patient was already under penicillin treatment at a high dose.

Blood samples were obtained by the local veterinarian from two more sheep that have died in the same herd after 7 July. These two were also inspected and microscopic examination revealed the presence of

FIGURE

Skin lesions due to cutaneous anthrax infection, Larissa, Thessaly, Central Greece, July 2012



Gram-positive rods and bacterial cultures grown for 24 h on 5% blood agar produced grey-white colonies. Preliminary identification was performed using conventional methodology. Briefly, haemolysis detection and motility testing was performed as described previously, using 5% horse blood and trypticase soy broth (Bioprep, BioPa Kerateas, Greece) [2]. Capsular testing was performed using nutrient agar plates supplemented with 0.7% NaHCO₃ (Bioprep), incubated in 5% CO₂ for 24 h, followed by McFadyen methylene blue staining. Genus and species confirmation, as well as detection of the two *B. anthracis* plasmids, pXO1 and pXO2, responsible for the species' pathogenicity, was performed using SYBR Green real-time PCR and the primer pairs BA813F/R, PAG67/68 and CAP57/58, as well as the Genesig *Bacillus anthracis* Real Time PCR kit (PrimerDesign Ltd, Southampton, UK), which is based on TaqMan chemistry [3].

The microorganism isolated from the sheep was identified as *B. anthracis* and carried the two pathogenic plasmids pXO1 and pXO2; the pXO1 plasmid contains the *lef*, *cya* and *pag* genes, which encode the lethal factor, oedema factor and protective antigen, respectively, while the pXO2 plasmid contains the *cap* gene, which encodes the capsule [3].

Epidemiological investigation

The stockbreeder was contaminated after having handled the slaughtered sheep due to direct contact with the infected animal. He had flayed the animal together with his wife and then fed two dogs with the contaminated meat. These dogs died during the next day. After 36 hours, the specific anthrax cutaneous lesions appeared on the exposed area of the stockbreeder's skin. Since 7 July, two more sheep have died in the same herd. No other death occurred in this or other herd in the same village (Tsabournia).

It can be assumed that the stockbreeder's wife was also exposed to the spores of the infected animal. However, she did not present any signs or symptoms of infection and is now under post-exposure prophylactic treatment.

Control measures

The stockbreeder's wife hasn't developed any symptoms during the maximum incubation period of 15 days, but is currently receiving post-exposure prophylaxis. The residents of the village (Tsabournia) have been informed about this case in order to recognise early clinical symptoms of anthrax and they were advised to seek medical treatment immediately if anthrax was suspected. The local health centre and general practitioners are aware of this need for careful monitoring. Special directions have been given to the stockbreeders of Tsabournia regarding the use of protective equipment. The local Veterinary Authority has taken measures for the correct disposal of animal carcasses, including disinfection of contaminated material and

decontamination of the environment. Mass vaccination of 7,000 animals is currently in progress.

Background information

Anthrax is an acute infectious disease caused by a large, spore-forming, toxin-producing bacterium *B. anthracis* [4]. It is the oldest known zoonosis with worldwide distribution and has been known to man for hundreds of years, mostly as an animal disease, typically in agricultural areas [4,5]. The disease is endemic in many countries of the world, particularly in tropical and sub-tropical areas, such as southern Europe, Asia, Africa, North and South America, and Australia [6,7]. It commonly occurs in well defined endemic areas where environmental conditions are particularly favourable for the survival of the spores. In Europe, there is a definite declining trend: The number of reported human cases remained at around 25 cases per year during a ten-year period (1995–2004), and has since decreased even more (2005: 10 cases, 2006: 16 cases, 2007: five cases, 2008: three cases, 2009: 14 cases) [8–12]. In the last four years, several reports of anthrax infections in heroin drug users have been reported in European countries [13–15].

Until 1979, Greece, particularly the northern part of the country, was considered as an enzootic zone for anthrax [6]. Although the number of animal outbreaks between 1970 and 1979 had declined to almost a quarter of that of the previous decade (1960–1969), there were 300 outbreaks a year, mostly involving sheep. During this period, there were 8,475 sheep and 1,675 bovine losses in 3,669 separate outbreaks. During the same period, 482 human anthrax cases occurred in the country and all patients were from rural areas [6]. The highest incidences were observed in the prefectures of Aetoloakarnania, Evros, Ioannina, Larissa, Rodopy and Thessaloniki [6]. Since then, strict control measures have eliminated the disease and only sporadic cases in animals and humans have been reported. According to the epidemiological reports from the European Centre for Disease Prevention and Control (ECDC), only 38 confirmed human cases of anthrax were reported between 1994 and 2010 [8–12]. However, it should be stated that although anthrax is included in the notifiable diseases and every suspected case should be reported to the Hellenic Center for Disease Control and Prevention (HCDCP), there is some degree of underreporting and the low number of reported cases does not allow general conclusions regarding the accurate incidence trend.

Thessaly is a rural region located in Central Greece and includes four prefectures (Karditsa, Larissa, Magnesia, Trikala). The estimated number of goats and sheep in this region is above 2 million. The large majority of them (more than 1 million goats and sheep) are farmed in Larissa prefecture. According to the records of the local Veterinary Authority of Larissa, three outbreaks of anthrax have been reported in Larissa in the past 35 years (in 1978, in 2000, and in 2006) (unpublished

data). All of them occurred in herds kept in two villages (Livadi and Tsaournia) situated at a distance of 35 km from each other in the area of Elassona, Larissa prefecture. Approximately 90 animals were affected in total, and the outbreaks were contained after correct disposal of animal carcasses and vaccination of exposed animals. According to the epidemiological data of the Veterinary Authority, no case of anthrax in animals or humans has ever been declared in the other three prefectures of Thessaly.

In 1978, anthrax infection had been confirmed in animals of three different herds in Tsaournia. However, no human infection has been reported. Vaccination and appropriate control measures have been taken; since then until the incident described here no other anthrax case in animals or in humans has been reported.

Conclusions

From a public health point of view, anthrax is important for Europe as well as for other regions. Infections still occur in Greece and clinicians should be aware of the disease and of the need for immediate management and reporting to the HCDCP [16].

In the management of the case described above, the level of post-prophylactic treatment may be seen as unusual according to the World Health Organization (WHO) recommendations (no post-prophylactic treatment required in a patient previously treated by intravenous penicillin) [1]. Here, post-exposure prophylaxis was nevertheless recommended after hospital discharge because the precise conditions of direct contact which took place during playing were not clearly known [17].

Early recognition of this suspected human case and reporting to the local authorities without delay have led to the prevention of further spread of the disease both in humans and animals.

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Travel-associated Legionnaires' disease in residents from England and Wales travelling to Corfu, Greece, August to October 2011

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Fourteen cases of Legionnaires' disease were confirmed in residents from England and Wales with a history of travel to Corfu, Greece, in the 14 days before symptom onset. These cases were reported to the Health Protection Agency national surveillance scheme for Legionnaires' disease in residents of England and Wales between August and October 2011. In addition, one case in a Greek national and a case of non-pneumonic legionellosis in a resident from Scotland were also reported. Few cases shared the same accommodation site in Corfu during their incubation period. Epidemiological investigations and microbiological analysis of clinical and environmental samples excluded a single source but rather implicated several accommodation sites as sources of sporadic infection. Control measures have since been implemented at these accommodation sites and no further cases have been reported. This incident highlights the value of epidemiological typing and the importance of effective international response to control and prevent legionella infection.

Introduction

Legionnaires' disease (LD) is a notifiable disease across Europe. It presents as pneumonia with a case fatality rate of 10-15% [1]. LD is caused by the inhalation of aerosolised legionellae and early treatment with appropriate antibiotics may reduce the risk of complications [2]. Legionellae are widely distributed in the environment being found in all types of water systems including both natural sources, such as rivers and streams, and man-made systems, such as cooling towers, domestic water systems and spa pools [3-6]. When circumstances allow amplification of the organisms and their dispersal through aerosols to the population, such as in water systems which are not properly designed, installed and/or maintained, then there is the potential for significant numbers of people to be exposed and outbreaks to occur. Susceptible hosts include the elderly, smokers and the immunosuppressed [7].

Prompt investigation of early cases to identify the source and institute control measures is vital to prevent further cases.

Legionella pneumophila urinary antigen testing is frequently used to obtain a rapid clinical diagnosis; however, this test provides very little information about the infecting strain. Increasingly, DNA-sequence-based typing (SBT) is being used to examine isolates of *L. pneumophila* or, more recently, in conjunction with polymerase chain reaction (PCR) directly on clinical samples, to yield highly discriminatory epidemiological typing data which can be used to compare against environmental isolates and hence to more accurately pin-point sources of infection [8,9].

This paper describes the investigation of an unexpected increase in cases from England and Wales with LD associated with travel to Corfu Island, Greece, using SBT analysis of environmental and clinical samples, and the control and prevention measures implemented as a result.

Background

In 2009, the overall rate of LD cases in Greece was 0.13/100,000 population compared with a rate of 1.30/100,000 population in the Netherlands and 2.63/100,000 in Spain [10]. Greece is among the top 10 destinations in Europe that United Kingdom (UK) residents travel to, accounting for about 4% of the 4.7 million UK residents travelling within Europe each year; similar numbers are reported to travel to the Netherlands and Turkey [11]. Since 2000, the average number of LD cases reported to the Health Protection Agency (HPA) national surveillance scheme (for England and Wales) associated with travel to Greece is 10 per year compared with an average of 11 associated with travel to Turkey and two to the Netherlands. Residents of England and Wales have been associated with 10 clusters in Greece since 2000, seven of which

have been at sites on Corfu Island. This compares to 11 clusters identified in Turkey over the same period of time and one cluster identified in the Netherlands [12]. Of these clusters, only three of the 10 clusters in Greece and three of the 11 clusters in Turkey had environmental results reported to the HPA national surveillance scheme. There were no environmental results from the cluster in the Netherlands. As a consequence, no source could be confirmed in any of the clusters in the three countries, highlighting the great difficulty in identifying the source of travel-associated clusters.

On average, five cases of LD associated with travel to Corfu Island are reported annually to the HPA national surveillance scheme. The highest number of cases reported in a year has been nine cases in 2005 and 2006 (Figure 1) [12].

However in 2011, by 4 October, the HPA national surveillance scheme had identified eight cases of LD in residents of England and Wales with onset of symptoms in the 14 days following travel to Corfu. As this exceeded the annual average, and all cases had an onset of symptoms within four weeks of each other, an incident meeting was convened involving representatives from public health authorities in the UK and Greece and from the European Centre for Disease Prevention and Control (ECDC).

Methods

Information from the national enhanced surveillance questionnaire for LD [13] was collected for each case and a further trawling questionnaire was administered in order to identify any common links between the cases in either Corfu or England and Wales. Regular

teleconferences were held with the Hellenic Center for Disease Control and Prevention (HCDCP), Greece, whereby data from questionnaires were shared in order to facilitate field investigations. Data exchange was also facilitated using the Epidemiological Intelligence Information System (EPIS), an international platform through which a network of experts can rapidly share data with other countries. Active case finding was undertaken by alerting the 29 member countries of the European Legionnaires' Disease Surveillance Network (ELDSNet) [14]. Both the UK and Greece enhanced case-finding activity by alerting clinicians and public health professionals through their national channels.

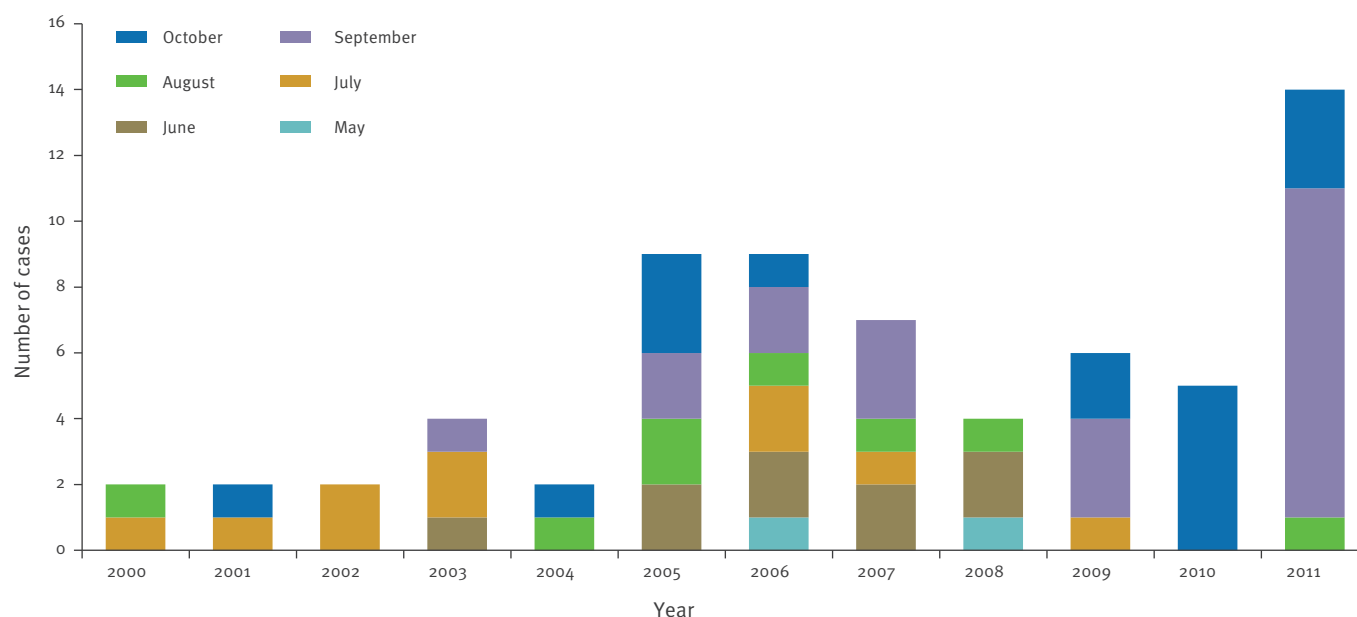
Environmental investigations in Corfu were undertaken by the Greek authorities. Where possible, hotel rooms and pool showers in all accommodation sites where cases reported having stayed overnight were sampled. In addition, samples were collected from public areas, including Corfu airport and fountains in Corfu city. A risk assessment was also carried out at each accommodation site.

In England and Wales, no public area was identified as a potential source of infection but Health Protection Units carried out, where possible, domestic sampling of cases' households through local environmental health departments.

Primary diagnosis of all patients was made in local microbiology laboratories using commercial *L. pneumophila* urinary antigen kits. Following standard practice, the local laboratories were asked to forward all clinical samples from cases to the national legionella reference laboratory in the HPA Respiratory and Systemic Infections Laboratory (RSIL) in London.

FIGURE 1

Cases of Legionnaires' disease in residents of England and Wales, with reported travel to Corfu, Greece, 2000–2011



Urine samples from all cases were examined using the RSIL in-house assay which is specific for *L. pneumophila* serogroup 1 strains of the mAb3/1 subgroup [15]. Sputum samples were requested for all urinary antigen positive patients for culture and any isolates obtained were characterised using monoclonal antibody (mAb) subgrouping [16] and SBT [9]. Samples which yielded a positive result in the *L. pneumophila* specific PCR but were culture-negative, were examined using nested-direct SBT [10].

Environmental samples were cultured by standard methods [17] in either the Central Laboratory of Public Health in Vari-Attica or the Laboratory of Public Health of Thessaly, Greece. At the time of this investigation these laboratories did not have the capacity to undertake full epidemiological typing of isolates, a representative selection of positive isolates obtained were then submitted to RSIL, as the ELDSNet co-ordinating laboratory, for further characterisation by mAb subgrouping and SBT.

Results

Fourteen confirmed cases of LD were detected in residents of England and Wales; a Greek national with LD was also identified. One case of non-pneumonic legionellosis was identified in a resident from Scotland. All cases had been in Corfu in the 14 days before onset of symptoms.

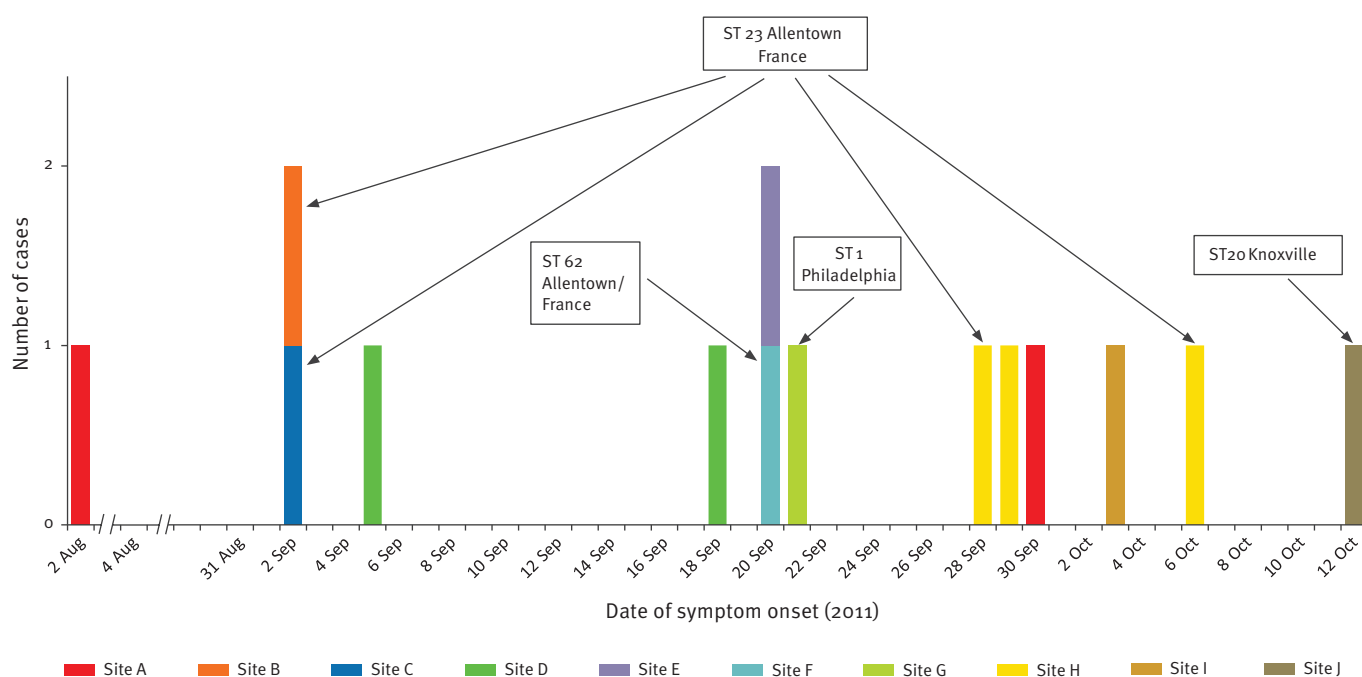
The earliest date of onset of symptoms of cases from England and Wales was 2 August 2011 and, the latest date was 12 October 2011 (Figure 2). The median age of cases was 61 years (range: 39–79), and eight of the cases were male. Seven patients had known co-morbidities. All cases were hospitalised and seven cases were admitted to intensive care units; one patient required extracorporeal membrane oxygenation. One case died 27 days after onset of symptoms.

A trawling questionnaire was completed for all 14 cases from England and Wales. Thirteen of the 14 cases had stayed in tourist accommodation. The remaining case had stayed in a private villa owned by a friend. In total, 10 different accommodation sites associated with cases were identified, and the locations of these were scattered across Corfu. There were three accommodation sites where more than one case had stayed: two cases stayed at Site A (early August and late September), two cases stayed at Site D (early September and mid-September) and three cases stayed at Site H (two in mid-September and one in late September); only the two cases in Site H stayed in accommodation at the same time. Cases did not appear to share a common airline tour company, or airport of departure or arrival in England or Wales, and no other common potential exposures were identified.

For three of the cases, domestic sampling of home residences in England and Wales was undertaken but

FIGURE 2

Cases of Legionnaires' disease in residents of England and Wales associated with travel to Corfu, Greece^a, August–October 2011 (n=14)



ST: sequence type.

^a All cases had been in Corfu up to 14 days before onset of symptoms.

legionellae were not recovered from any of these. The environmental investigations undertaken by the Greek authorities highlighted deficiencies in the disinfection and maintenance of water systems of the accommodation sites in Corfu. Issues with water temperature control were also identified. Environmental samples were obtained from nine of the 10 accommodation sites although for one site sampling was confined to samples taken from an external water tank, the pool filter and a pool shower since the business was insolvent and access to the hotel rooms was not possible. Excluding this latter site, sampling revealed that *L. pneumophila* serogroup 1 was present in all but one accommodation site, often in association with other *L. pneumophila* serogroups or *Legionella* species. The source of positive samples and the number of positive samples are indicated in the table. Samples from the airport and local town fountain were negative for legionellae.

All 14 cases were reported as *L. pneumophila* urinary antigen positive and samples from 13 of these were submitted to RSIL for confirmation. All 13 were confirmed as positive for *L. pneumophila* serogroup 1 mAb3/1+ve antigen. Sputum samples were requested from all the patients but were received from only nine: legionellae were grown from seven of these, the remaining two were both culture and *L. pneumophila* PCR negative. The typing results of both the clinical and environmental samples submitted by the Greek authorities to RSIL are summarised in the table. In four instances, clinical isolates and corresponding environmental isolates were available for comparison, and in two of these (Sites C and G) they were indistinguishable, having the same monoclonal antibody (mAb) subgroup and sequence type. Furthermore, for four of the five patients who stayed in Sites A, D and F,

TABLE

Results from the typing of environmental and clinical samples of England and Wales cases of Legionnaires' disease associated with travel to Corfu, Greece, August–October 2011

Accommodation	Number of positive samples	Sources of positive environmental samples submitted by Greek laboratories	Environmental isolates recovered	Patient	Clinical microbiology result
Site A ^a	7/9	Cold and hot water shower, swimming pool shower	L.p Sgp1, mAb 'Oxford/OLDA', ST1 L.p Sgp1, mAb 'Philadelphia', ST1 L.p Sgp3, ST388	1 ^a	L.p Sgp1, mAb3/1+ve
				11 ^a	L.p Sgp1, mAb3/1+ve
Site B	2/12	Cold water shower	L.p Sgp1, mAb 'Oxford/OLDA', ST1	2	L.p Sgp1, mAb 'Allentown/France', ST23
Site C ^b	1/4	Hot water shower	L.p Sgp1, mAb 'Allentown/France', ST23	3 ^b	L.p Sgp1, mAb 'Allentown/France', ST23
Site D	5/20	Cold and hot water shower	L.p Sgp1, mAb 'Allentown/France', ST23 L.p Sgp6, ST461 <i>Legionella anisa</i>	4 ^a	L.p Sgp1, mAb3/1+ve
				5 ^a	L.p Sgp1, mAb3/1+ve
Site E	4/8	Hot water shower	L.p Sgp1, mAb 'Oxford/OLDA', ST1 L.p Sgp6, ST461	6	L.p Sgp1, mAb 'Allentown/France', ST62
Site F ^a	11/19	Cold and hot water shower	L.p Sgp1, mAb 'Benidorm', ST42 L.p Sgp6, ST1260 L.p Sgp10, NT	7 ^a	L.p Sgp1, mAb3/1+ve
Site G ^b	6/8	Cold and hot water shower	L.p Sgp1, mAb 'Philadelphia', ST1 L.p Sgp6, NT	8 ^b	L.p Sgp1, mAb 'Philadelphia', ST1
Site H ^c	0/3	None identified	Legionellae not recovered from external samples	9	L.p Sgp1, mAb 'Allentown/France', ST23
				10	No samples submitted to Respiratory and Systemic Infections Laboratory
				13	L.p Sgp1, mAb 'Allentown/France', ST23
Site I	5/9	Cold and hot water shower, swimming pool	L.p Sgp1, mAb 'Oxford/OLDA', ST1 L.p Sgp3, NT	12	L.p Sgp1, mAb3/1+ve
Site J	0/12	None identified	Legionellae not recovered from any samples ^d	14	L.p Sgp1, mAb 'Knoxville', ST20

NT: not tested.

^a No clinical isolate available for typing but the urinary antigen result is consistent with infection being due to an isolate recovered from the site.

^b Clinical and environmental isolates match.

^c No samples could be obtained from hotel rooms but three swabs were collected from the pool and water tank outside the building.

^d Sampling was not performed according to protocol as the hotel was closed due to foreclosure procedures.

although no clinical isolates were obtained, the RSIL urinary antigen assay result confirmed that they were all infected with *L. pneumophila* serogroup 1 strains expressing the mAb3/1 epitope and among the environmental isolates recovered from each of these sites, there were strains that carried the same epitope (i.e. mAb Philadelphia, Allentown/France and Benidorm). Given that mAb3/1+ve strains are generally uncommon in the environment [16], these data are consistent with, and supportive of the view that these accommodation sites were the source of these patients' infection.

Control measures

In response to the outbreak, the HCDCP issued guidelines to all health professionals in the region underlining the importance of early recognition, prompt diagnosis, timely management and notification of LD. The Greek authorities started a communication campaign aimed at providing information on the use of effective measures to prevent legionellosis on all Greek islands at the beginning of the 2012 tourist season. Communication material has been forwarded to Hotel Associations and other tourist accommodation providers and information seminars for these groups were organised on several islands.

In addition to the communication campaign, all regional public health laboratories, in collaboration with local public health authorities, have been conducting risk assessments and environmental sampling in a representative number of hotels in touristic areas of Greece, for example, Crete, Halkidiki, Skiathos and Corfu.

Along with environmental sampling, a risk assessment for each accommodation site was carried out in accordance with the European Working Group for Legionella Infections (EWGLI) guidelines and recommendations made on control measures to be taken [18].

Conclusions

Historically, of all the clusters identified in Greece between 1980 and 2010, 12 occurred in different accommodation sites in Corfu Island and involved between two and four cases of LD in residents from England and Wales. There were no environmental samples for any of the 12 clusters, but two of the historic sites were involved in the 2011 cluster of cases. This paper highlights the improvement in reporting environmental samples to the HPA national surveillance system and the benefits of collaborative working between European countries.

An interesting observation made early on in this investigation was the absence of cases reported in residents outside England and Wales. We would have expected to have seen more cases in residents from other countries visiting the island or perhaps among the local population. ELDSNet alerted all collaborating countries of the increase in case numbers and requested immediate submission of any cases associated with travel to Greece; however, no cases were reported. There is

no evidence or reason to believe that travellers from England and Wales to Corfu Island are more susceptible to LD than travellers from any other country. Therefore, the reason behind this difference may be case ascertainment between health systems of other countries and England and Wales.

It is also important to note that only one death was associated with this incident. A plausible reason for this is that not all cases had clinically relevant comorbidities. In addition, increased awareness of the disease as a result of media interest may have altered clinical practice resulting in improved clinical outcome.

Given the high number of cases over a short period of time, initial concerns focused on the possibility of a point-source outbreak. The results of laboratory typing, however, indicated that simultaneous clusters of disease were occurring, as opposed to a common source outbreak. In most similar investigations few clinical isolates, few environmental isolates, or few of both, are available for comparison and this restricts the ability of investigators to detect multiple sources [19]. In this investigation we obtained clinical isolates from over 50% of cases and environmental samples from almost all potential sources. This high rate allowed us to obtain very strong evidence of the source for two cases and good, albeit indirect, evidence for a further five cases. This investigation illustrates both the value of subtyping in the corroboration of environmental results with clinical results, and the importance of obtaining sputum samples from patients in the context of an investigation. In six of the accommodation sites, multiple strains of *L. pneumophila* were recovered from the environment. While this is not an uncommon finding, it does illustrate that unless extensive environmental sampling, followed by characterisation of multiple isolates, is undertaken from potential sources, the infecting strain could easily be missed.

As the incident occurred during late summer and early autumn, the temperate weather conditions of that period may have encouraged the proliferation of the *Legionella* bacteria in water [20]. In combination with the lack of disinfection regimes in the accommodation sites, this may have been the underlying cause of the incident. All accommodation sites sampled were positive for *Legionella* indicating that, in general, standards for preventing *Legionella* colonisation were not adequate. It is therefore imperative that steps are taken to educate, support and inform all those working in the tourism industry about how to reduce the risk of *Legionella* infection, in particular during the summer season.

It is hoped that the communications campaign initiated by the Greek authorities will succeed in raising awareness. If it is demonstrated to have a positive impact on the number of cases of travel-associated Legionnaires' disease, there may be added value in further annual campaigns in reducing the burden of this infection

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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 Luxembourg [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 (EUptstrain) group in collaboration with the EUVAC.NET hub. The EUptstrain 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.

List of participants of the European Bordetella expert group

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Does the Japanese encephalitis virus (JEV) represent a threat for human health in Europe? Detection of JEV RNA sequences in birds collected in Italy.

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To the editor:

Autochthonous cases of Japanese encephalitis (JE) have never been reported in Europe, where there is virtually no circulation of the virus [1]. However, in the issue of *Eurosurveillance* published on 12 July 2012, Ravanini et al. [2] reported the detection of an RNA sequence of the Japanese encephalitis virus (JEV) NS5 gene in one pool of *Culex pipiens* mosquitoes collected in north-eastern Italy during the summer of 2010. Unfortunately, the sequence was relatively short (167 bp), and attempts to amplify longer sequences using primers targeted to E, NS5 and NS3 genes and to isolate the virus on cell cultures failed.

To support their findings, the authors cited a previous study reporting the identification of JEV RNA in bird samples collected in Tuscany, a neighbouring Italian region, a decade before [3], and noted the lack of information on viral sequences in that study. Here we provide the results of molecular testing on bird tissue samples, which appear to confirm recent findings on JEV presence in Italy.

Between 1997 and 2000 dead birds were collected to investigate episodes of bird mortality observed in Padule del Fucecchio, a wetland area in Tuscany where cases of West Nile virus (WNV) infection in horses were observed in 1998 [4]. The episodes involved mainly blackbirds (*Turdus merula*) and song thrushes (*Turdus philomelos*). Other species, less affected by mortality, were also sampled such as rock doves (*Columba livia*), redwings (*Turdus iliacus*), sparrows (*Passer italiae*), and starlings (*Sturnus vulgaris*).

In May 2001 formalin-fixed tissue samples from seven birds collected in 2000 that tested positive for JE group antigens by immunohistochemistry, were sent to the Central Research Institute of Epidemiology in Moscow, Russia to carry out molecular testing for flaviviruses. Later, in early 2003, samples from 14 birds collected in

1997 which had also tested positive, were sent to the same Institute.

The samples were subjected to RT-PCR assays, one targeting the nonstructural NS5 gene for the detection of most flaviviruses [5] and the other for detection of the WNV and JEV envelope (E) genes (the details of these in-house assays are available from the authors on request).

None of the samples tested positive for WNV, but PCR amplicons of the JEV NS5 gene (expected size, 215 bp) were obtained from tissues of six birds collected in 2000. Amplicons of the JEV E gene (expected size, 687 bp) were obtained from the same six birds and in one of the birds collected in 1997. Sequencing of 552 bp fragments of the JEV E gene from five of the samples (GenBank accession numbers AF501311-AF501315) and of NS5 amplicons from two samples showed maximum similarity (99%) with JEV genotype III, for example with the Nakayama strain [6].

Importantly, the JEV nucleotide sequence found in Italian *Culex pipiens* [2] differed from the JEV RNA sequence found in Italian birds only at two positions, which are shown bold and underlined (Italian birds, NS5 gene fragment:

```
C T T G G A G C A C G G T A T C T A G A G T T T G A
A G C T T T G G G G T T C C T G A A T G A A G A C C A T T
G G C T G A G C C G A G A G A A Y T C A G G A G G T
G G A G T G G A A G G C T C G G G C G T C C A A A A
G C T A G G A T A C A T C C T C C G T G A C A T A G
C A G G A A A G C A A G G A G G G A A A A T G T ) .
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However, it remains undefined whether the viruses were the same in the two episodes. To this date no human cases of JE have been reported in Italy. Thus, two different hypotheses may be considered:

- Unidentified flaviviruses highly similar to JEV but less or not virulent for humans, are circulating in Italy

(southern Europe). This hypothesis cannot be completely ruled out until further analyses are performed.

- Limited JEV circulation has occurred between birds and mosquitoes in Italy but no human cases have been observed, as in Australia since 1995 [7]. This may be due to the relatively low availability of amplifying hosts (pigs) in that area, low vector competence of European *Culex pipiens* [8], low capability of local birds to maintain a persistent JEV circulation or other factors suppressing the JEV epidemic cycle [1,6], and limited or absent human exposure.

In conclusion, our results seem to support the hypothesis of long distance spread of JEV from endemic areas to Europe (Italy) [1,7]. However, sporadic introduction of JEV to new areas by migratory birds or by other ways may not necessarily lead to local viral circulation. Moreover, the circulation of other flaviviruses closely related to JEV cannot be completely ruled out. Whether JEV represents a human health threat in Europe merits further investigation.

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Is Japanese encephalitis emerging in Europe?

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To the editor:

P. Ravanini et al. recently detected a small fragment of Japanese encephalitis virus (JEV)-like RNA in a *Culex pipiens* mosquito pool collected in autumn 2010 in northern Italy [1].

JEV is the prototype of a group of closely related flaviviruses which include West Nile virus (WNV) and Usutu virus (USUV). These viruses are circulating in birds, which are amplifying hosts, and in *Culex sp.* mosquitoes. Ardeid wading birds and pigs are amplifying hosts for JEV in Asia; humans and horses are sensitive hosts. Five genotypes of JEV have been described in Asia and some of them are widely distributed and most frequently associated with JE outbreaks and epidemics [2].

The spread of WNV in Europe over the last two decades with co-circulation of different viral lineages, as well as the emergence of USUV, initially in Austria in 2001, demonstrate that arboviral diseases of tropical origin may spread in temperate regions [3]. Environmental and climatic changes may also influence the distribution of these viruses in relation with migration patterns of birds.

As a consequence of the recent spread of WNV, entomological, and human/animal surveillance has increased in the recent years in several European and neighbouring countries. In addition, the use of generic PCR amplification techniques has widened the spectrum of viral investigations in collected specimens. Meanwhile the single detection of an RNA fragment of 157 bp with a sequence compatible with JEV has to be treated very cautiously in the absence of additional genomic amplifications of JEV RNA from the initial positive mosquito pool. In addition, contamination of the PCR cannot be completely excluded. This finding requires complementary studies to confirm the presence of JEV in Europe.

Previously Mani et al. [4] had reported JEV-like infection in passerine birds collected in Tuscany in 1996. The authors claimed that fragments on the viral E gene amplified from the organs of these birds were closely related to the Nakayama strain of JEV. This strain has

been commonly used for vaccine production in Asia. Additional studies in Tuscany were inconclusive.

Research into the possible introduction of JEV to Europe should be conducted. Entomological investigations should be strengthened in habitats potentially suitable for JEV transmission in Europe and the use of generic flavivirus RT-PCR assays should be extended. Serological surveys in birds (in particular Ardeid wading birds) should include the differential diagnosis between WNV, USUV and JEV antibodies. Suspected neuroinvasive infections in humans and/or horses not confirmed as WNV or USUV infections, should be tested for JEV. As a second priority, serosurveys in pig-breeding farms located in the proximity of potential mosquito-breeding habitats such as rice paddies may also be conducted.

If the presence of JEV is confirmed in northern Italy, a risk assessment at the human/animal interface would need to be conducted to evaluate the public health consequences. As a result, the strategy for the laboratory differential diagnosis of neuroinvasive cases occurring in humans and also horses during the mosquito season may have to be modified to include JEV in the panel of viruses under investigation.

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