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Since 1995, when a first pilot issue was published, *Eurosurveillance* has provided the European public health community with a platform to exchange relevant findings on communicable disease surveillance, prevention and control. From the outset, the journal has been open access and has not charged article processing costs.

In 2016, we celebrate 20 years of regular publication. A glimpse at the *Eurosurveillance* archives demonstrates how the journal has matured over the years in terms of format and content. It shows, for example, the merging of the formerly weekly and monthly issues, acceptance of the ‘weekly’ for indexing in PubMed/MEDLINE and the evolution from a print and online journal to a full online journal and a gradual geographical expansion of the origin of published articles.

However, already from the start, topics covered were remarkably similar to those that are high on the public health agenda today. One of the articles in the pilot issue in 1995 gave an overview of immunisation schedules in Europe [1], a topic still of interest nowadays. Our aim to provide insightful and balanced information on vaccination was shown after the later retracted publication by Wakefield et al. that included subsequently falsified claims of an association of measles mumps and rubella vaccines with autism [2]. Just one week afterwards, *Eurosurveillance* ran a commentary in its weekly edition, followed, two months later, by an entitled ‘Further evidence that MMR vaccine, inflammatory bowel disease, and autism are not linked’ [3,4]. The public health challenges that Europe faces in reaching the measles elimination goal in Europe were marked in a ‘Spotlight on measles’ series on ongoing outbreaks and their implications [5].

Rapid communications were an early feature for the journal at a time when rapid processing of articles was not a common element of scientific journals. The evolution, growth and opportunities offered by the Internet facilitated timely communication and fast turnaround times tremendously. The initially short news-like items are the element of the journal that has most evolved. Today, rapid communications are well-recognised short scientific dispatches. Several of them are among our most highly cited articles, but more importantly, their value has been in their impact on public health practice.

While we have been able to present ‘firsts’ on several occasions [22,23] and track epidemics and emerging diseases in a timely manner [24], we are publishing an increasing number of (systematic) reviews to provide sound evidence and support for decisionmaking [25].

Working with *Eurosurveillance* is rewarding. The journal has many supporters and collaborators in Europe and beyond whom we are not able to name individually. We would like to express our gratitude to them and also
thank our board members, colleagues and publisher wholeheartedly for their continued support. Our 20th anniversary is a reason to celebrate. We marked the occasion on Wednesday 30 November with a luncheon seminar ‘20 years of communicating facts and figures in a changing environment’, held on the margins of the European Scientific Conference on Applied Infectious Diseases Epidemiology (ESCAIDE). Two eminent speakers, David Heymann and Lawrence Madoff, highlighted changes in sharing information about communicable diseases from a public health perspective over the past 20 years. In addition, we present this selection of articles as a snapshot of the journal’s publications and evolution. The topics covered match those that have remained relevant over two decades and we hope our readers will enjoy browsing through this compilation.

References


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Since 2011, human immunodeficiency virus (HIV) incidence appears unchanged in the European Union/European Economic Area with between 29,000 and 33,000 new cases reported annually up to 2015. Despite evidence that HIV diagnosis is occurring earlier post-infection, the estimated number of people living with HIV (PLHIV) who were unaware of being infected in 2015 was 122,000, or 15% of all PLHIV (n=810,000). This is concerning as such individuals cannot benefit from highly effective treatment and may unknowingly sustain transmission.

Although preventable through effective public health measures, human immunodeficiency virus (HIV) persists in the 31 countries of the European Union and European Economic Area (EU/EEA) [1]. In this report an analysis of EU/EEA HIV and acquired immunodeficiency syndrome (AIDS) surveillance data from 2015 as well as from prior years is presented. We estimate that, in 2015, 15% (122,000/810,000) of people living with HIV (PLHIV) in the EU/EEA were unaware of their infection.

Analysis of annual surveillance data
HIV and AIDS surveillance data are reported annually by EU/EEA countries to a joint database for HIV/AIDS within the European Surveillance System (TESSy) coordinated by the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO) Regional Office for Europe [1].

Annual data on HIV diagnoses from 2003 to 2015 were stratified by the presence of a concurrent AIDS diagnosis, i.e. an AIDS-defining event within 3 months of HIV diagnosis, and, for individuals without AIDS, by CD4 cell count (<500, 500–499, 200–349, ≤200 cells/mm3) at the time of diagnosis [2].

The ECDC HIV Modelling Tool version 1.2.2 was used to derive both the estimates of annual HIV incidences, as well as those of the average times from infection to HIV diagnosis each year [3]. These two types of estimates are only presented for the period from 2011 to 2015 due to greater uncertainty of data from the previous years of the study.

The number of PLHIV in 2015 who were not yet diagnosed was obtained by fitting to data on HIV diagnoses from 2003 to 2011, adjusted for reporting delay, using the "Incidence Method", a CD4 cell count-based back-calculation method [4].

Data on the estimated number of diagnosed PLHIV were reported for 2015 by nominated contact points in EU/EEA countries to ECDC as part of the Dublin Declaration monitoring process in 2016 [5]. In the three countries (Iceland, Liechtenstein, and Norway) not reporting estimates of diagnosed PLHIV, data on cumulative HIV cases reported to TESSy through 2015 minus the number of persons reported to have died, were used as a proxy for diagnosed PLHIV.

The estimated number of diagnosed PLHIV from the Dublin Declaration monitoring reports and the undiagnosed PLHIV estimate from the model were summed to obtain the total number of PLHIV in the EU/EEA for 2015. This was used to derive the proportion undiagnosed PLHIV in that year.

Comparable estimates of the number of diagnosed PLHIV from the Dublin Declaration monitoring reports are not available for earlier years than 2015, thus the estimates of PLHIV overall and of the proportion of PLHIV unaware of their infection could only be calculated for 2015.
In 2015, 29,727 cases of HIV were diagnosed and reported in the EU/EEA, resulting in a rate of 6.3 per 100,000 population when adjusted for reporting delay. The notification rate and the number of new HIV diagnoses reported have remained unchanged since 2011, with between 29,000 and 33,000 new cases reported annually (notification rates of between 6.3 and 6.5 per 100,000 population) [1].

HIV incidence estimates present a stable trend similar to that of HIV cases notified via the surveillance system, with an estimated 30,000 new infections (95% confidence interval (CI): 25,000–37,000) for the year 2015 (Figure 1).

**Evolution of CD4 cell count at diagnosis and of the delay between infection and diagnosis in years up to 2015**

Late diagnosis is a persistent issue in EU/EEA countries. In the 24 EU/EEA countries reporting data on CD4 cell count at diagnosis among 18,103 persons ≥15 years-old diagnosed in 2015, nearly half (n=8,490; 47%) of all cases had a CD4 cell count of less than 350 cells/mm³, while 28% (n=5,094) had advanced HIV infection (CD4<200 cells/mm³). In the thirteen countries reporting the CD4 cell count consistently over time, the median CD4 cell count at diagnosis increased significantly from 314 cells/mm³ in 2005 to 377 cells/mm³ in 2015 (p<0.001).

Meanwhile, the estimated expected time from HIV infection to diagnosis decreased from 4.2 years (95% CI: 4.1–4.3) on average in 2011 to 3.8 years (95% CI: 3.6–4.0) in 2015 (Figure 2).

**Estimated number of persons living with undiagnosed infection**

The number of people living with undiagnosed HIV in the EU/EEA in 2015 was estimated at 122,000 (95% CI: 111,000–136,000). The total estimated number of PLHIV in the EU/EEA was 810,000 (0.2% of adult population ≥15 years-old). The resulting estimated proportion of those living with undiagnosed HIV was 15% (95% CI: 14–17%).

**Background and discussion**

The Joint United Nations Programme on HIV/AIDS (UNAIDS) has set forth ambitious global targets to end AIDS by 2030 and established ‘90–90–90’ targets for 2020 (90% of all people living with HIV will know their status; 90% of people aware of their status will receive sustained antiretroviral treatment; and 90% of those on antiretroviral treatment will have viral suppression) [6]. To better understand HIV trends and estimate the status of the first target (90% of people living with HIV aware of their status) in the EU/EEA, we analysed HIV and AIDS surveillance data through 2015. Despite high treatment coverage [7], earlier diagnosis, and concerted prevention efforts, there is no decline in the number of HIV diagnoses or the number of HIV infections in the EU/EEA in recent years.

This analysis shows that the estimated proportion of all PLHIV in the EU/EEA who are living with undiagnosed HIV is 15%. Using a similar CD4 back-calculation approach on surveillance data, it was estimated that 16% of PLHIV in the United States in 2013 were undiagnosed [8]. The estimate presented here for the EU/EEA is considerably lower than the previous estimate of 30%, which is based on data from 2005 [9]. This could be a result of several factors. First, this might be a reflection of increased or more targeted testing as supported by the observed increase in the CD4 cell counts at diagnosis and decreased time from HIV infection to diagnosis. With treatment guidelines moving towards earlier treatment, and growing awareness of the benefits of early antiretroviral treatment, more persons at higher risk of infection may get tested more frequently. Second, the annual number of new infections is approximately the same as the number of new diagnoses. Thus the number living with undiagnosed HIV remains relatively stable and as people on treatment live longer with HIV, the proportion of undiagnosed persons with HIV will naturally become smaller in relation to the ever-increasing population of diagnosed PLHIV [1]. Third, new methods to estimate the undiagnosed fraction are available and these are informed by improved surveillance data.

While approximately 85% of those living with HIV in the EU/EEA are estimated to be diagnosed, it remains to be seen whether it is possible for the EU/EEA to reach the UNAIDS first ‘90’ target by 2020. A more appropriate measure to gauge progress may be to monitor the reduction in the number of undiagnosed individuals.
The average time between HIV infection and diagnosis, while improving, is still nearly four years. As starting antiretroviral treatment earlier reduces morbidity and mortality among HIV-positive individuals [10] and reduces HIV transmission to HIV-negative partners [11] it is essential that individuals are diagnosed early. In order to further reduce the time from HIV infection to diagnosis, countries should consider implementing and scaling up innovative approaches to promote greater access to and uptake of HIV testing by those most at risk, including community-based testing, self-testing and home sampling, as well as indicator-condition-guided testing.

This pooled EU/EEA estimate conceals differences between key populations, where the trend over time and proportion undiagnosed is likely to vary. An EU estimate is also more heavily weighted towards the situation of countries with larger populations. The proportion of persons remaining undiagnosed is diverse across countries that have carried out national analyses [12-17] and is likely to be significantly higher than 15% in many countries and among some key population groups. In Europe, further work is needed to carry out key population-specific and country-level estimates of HIV incidence and the undiagnosed number in a standardised manner in order to more accurately monitor progress and inform testing programmes.

This analysis has several important limitations. It was not possible to adjust the data for countries that did not have full coverage of HIV surveillance prior to 2012 (such as Italy and Spain) and this may have resulted in an underestimation of PLHIV. Conversely, PLHIV may have been overestimated due to the inability of many countries to fully link their death, emigration and surveillance registries and, thus, accurately measure the number of those diagnosed still living with HIV. For these reasons, it was not possible to obtain a reliable estimate of PLHIV using only HIV notification data reported to TESSy. Instead, data reported by countries through the Dublin Declaration monitoring process on people diagnosed and living with HIV were used, and these were obtained using different methods, with some countries unable to completely remove all cases who had died or emigrated from the number diagnosed. Until approaches to estimate diagnosed PLHIV can be further standardised, country-reported data provide the best current estimate in the EU/EEA.

Conclusions

Overall, this analysis demonstrates that recent HIV incidence is constant in the EU/EEA, and that a substantial number of people are living with undiagnosed HIV. Efforts to obtain better national and key population-specific estimates and to further increase the offer and uptake of HIV testing among those most at risk remain key to informing HIV prevention efforts and achieving global targets to reduce HIV incidence and the number of persons remaining undiagnosed in the EU/EEA.

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Conflict of interest

None declared.

Authors’ contributions

The ECDC HIV/AIDS Surveillance and Dublin Declaration networks supplied the data and provided comments on the manuscript. All co-authors developed the concept of the manuscript. AvS carried out the modelling analysis and AP the remaining analysis. AP wrote the first draft and responded to reviewers comments. All authors have read and approved the final manuscript.

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Severe acute respiratory infection caused by swine influenza virus in a child necessitating extracorporeal membrane oxygenation (ECMO), the Netherlands, October 2016

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In October 2016, a severe infection with swine influenza A(H1N1) virus of the Eurasian avian lineage occurred in a child with a previous history of eczema in the Netherlands, following contact to pigs. The patient’s condition deteriorated rapidly and required life support through extracorporeal membrane oxygenation. After start of oseltamivir treatment and removal of mucus plugs, the patient fully recovered. Monitoring of more than 80 close unprotected contacts revealed no secondary cases.

We here report a patient with severe acute respiratory infection as a result of swine influenza virus infection in the Netherlands.

Case description
A school-aged patient with a previous history of mild eczema developed a respiratory tract infection in October 2016, a couple of days after visiting a pig farm. The child had entered the pigsty but had not been in direct contact with pigs. Despite early prescription of antibiotics by the general practitioner the child’s clinical situation rapidly deteriorated. Within three days after onset of disease the child was transferred to a paediatric intensive care unit (PICU) for non-invasive ventilation support and intensive monitoring. Despite these efforts, the patient deteriorated further and was intubated in order to start mechanical ventilation. Bronchoscopy following intubation revealed large amounts of highly viscous mucus in the airways. Efforts to remove this mucus failed to improve ventilation. Mechanical ventilation became increasingly complex and it was decided to initiate veno-venous extracorporeal membrane oxygenation (ECMO) and to transfer to a quaternary PICU. Due to ECMO, blood oxygenation was secured and extensive bronchoscopy could be performed, during which topical DNAse (Dornase alpha, Pulmozyme, Roche) was instilled to decrease viscosity and facilitate removal of obstructing mucus plugs. On the following day, bronchoscopy was repeated and additional mucus was removed.

In the days following these procedures, the patient improved rapidly. ECMO was discontinued five days after start and the patient could be extubated. For the entire duration of hospitalisation, the patient had received broad-spectrum antibiotics, although all bacterial cultures remained negative. Throat swabs had been collected at initial admission and tested positive for influenza A virus, of which the quaternary PICU
was informed on the day after the patient transfer. Oseltamivir treatment (60 mg twice daily) was started hours after initiation of ECMO and transport. It was continued for a total of 7 days when a nasal swab tested negative for influenza virus. At the time of submission of this report, the child was recovering well.

Virological results
The initial diagnostic routine was limited to testing for the influenza A virus matrix gene, without subtyping. In view of the severe course of illness, the child was resampled for repeated testing including typing of the haemagglutinin (HA) gene by quantitative real-time PCR for H1 (seasonal and pdm2009), H3, H5, H7 and H9. All typing PCRs were negative.

We determined the full virus genome sequences of a cell culture isolate derived from a respiratory tract sample using Illumina MiSeq. All gene segments (GenBank accession numbers KY250316-KY250323) were 97–98% and 98–100% identical at, respectively, nucleotide and amino acid level to publicly available SIV sequences from the Netherlands (GISAID accession numbers EPI639351, EPI639914, EPI639917, EPI639930, EPI640657, EPI640912, EPI641210, EPI641215). The gene segments were all of the Eurasian avian A(H1N1) SIV lineage that has been circulating in pigs since 1979 [1]. Pigs at the farm visited by the patient tested positive for the same SIV (curation of full genome sequence data is in progress). The virus isolate from the patient, A/Netherlands/3315/2016, was sensitive to oseltamivir and zanamivir by NA-star neuraminidase inhibitor resistance detection assay (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands).

Public health measures
Zoonotic influenza is a notifiable disease in the Netherlands. Following confirmation of the zoonotic SIV infection, the national and relevant municipal public health authorities were notified and a teleconference was organised to decide on measures. The risk for human-to-human transmission was considered very low, given the enzootic presence of swine influenza viruses and the fact that zoonotic infections are seldom diagnosed.

In order to detect human-to-human transmission at an early stage, it was decided to contact all individuals that had been in close direct contact with the patient without wearing personal protective equipment, and monitor them for symptoms of possible SIV infection (cough, fever or conjunctivitis) for 10 days after exposure. In total, more than 80 contacts were monitored. These included the patient’s family members living in the same household, persons living and working on the pig farm, and healthcare workers who cared for the patient without wearing personal protective equipment (i.e. before the influenza diagnosis). Six contacts developed mild respiratory symptoms including cough, coryza and conjunctivitis during the monitoring period but all tested negative for influenza A virus.

According to the international health regulations, this case has been notified to the European Union Member States and the European Centre for Disease Prevention and Control (ECDC) through the Early Warning and Response System (EWRS) and to the World Health Organization (WHO).

Discussion
Incidental cases of human infection with SIV have been reported worldwide since the late 1950s. Most of these were in individuals exposed to pigs. Apart from one isolated incident in military barracks in the United States (US), sustained and efficient human-to-human transmission had not been documented before 2009, when an influenza virus of swine origin triggered the first influenza pandemic of the 21st century. Indeed it is speculated that pigs may serve as a mixing vessel for the development of a pandemic influenza strain [2-5].

In addition, SIV infections account for roughly one third of all laboratory-confirmed zoonotic influenza events reported in the scientific literature [3]. This may be a gross underestimation of the actual number as there are no typical signs and symptoms that distinguish SIV infections in humans from those caused by seasonal influenza viruses [4]. Indeed several sero-epidemiological studies suggest that SIV infection in people with occupational swine exposure is common [6-8]. In the US, there is a routine surveillance for swine influenza in pigs, and 400 patients with a swine influenza infection have been reported through this system since 2005 [9]. Our case shows that careful assessment of airway disease in individuals exposed to pigs continues to be important, especially considering the importance of starting of antiviral treatment early.

Conclusion
We here describe that transmission of SIV to humans, though rare, can occur and cause severe disease requiring life support through ECMO. Monitoring of people in direct contact and not wearing personal protective equipment revealed no secondary cases.

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Conflict of interest
None declared.

Authors’ contributions

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All authors contributed to the text of the manuscript. PLF, EDW, RJH, IK took care of the patient. CJH, HCJ, PT were involved in case finding. PLF, SDP, TMB, RR, JJK, RAF, MVTP, BOM, MC, NB, MPK were involved in lab testing. HK, AT, CMS, were involved in outbreak management.

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More than 20 years after re-emerging in the 1990s, diphtheria remains a public health problem in Latvia

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In 1994, the World Health Organization (WHO) declared the goal of eliminating diphtheria within the WHO European Region by the year 2000. However, in 1990 an epidemic emerged within the Russian Federation and spread to other countries, including Latvia, by 1994. We describe national surveillance and immunisation coverage data in Latvia from 1994 to 2014 and present historical data from 1946. We defined a laboratory-confirmed case as a clinical case in which toxin-producing Corynebacterium diphtheriae, C. ulcerans or C. pseudotuberculosis was isolated. From 1994 to 2014, 1,515 cases were reported, giving an average annual incidence of 3.2 cases per 100,000 inhabitants (range 0.1–14.8), with the highest incidence in age groups 5–19 and 40–49 years (4.4 and 4.3/100,000, respectively); 111 deaths were reported, 83.8% cases were laboratory-confirmed. Most cases occurred in unvaccinated adults. To improve disease control a supplementary immunisation campaign for adults was initiated in 1995, and by the end of 1998 national coverage among adults reached 70%, and reached 77% in 2003, but declined to 59% by 2014. Diphtheria remains a problem in Latvia with continued circulation of toxin-producing strains of C. diphtheriae. We recommend to strengthen immunisation to cover adults, as well as the education of health professionals and a serological survey.

Introduction

Diphtheria is a highly contagious communicable disease caused by toxin-producing strains of Corynebacterium diphtheriae (or rarely by Corynebacterium ulcerans or Corynebacterium pseudotuberculosis) and transmitted through respiratory droplets during close contact, primarily infecting the pharynx, tonsils and nose. Diphtheria toxin is absorbed at the site of the lesions and may affect other organs far from the initial area of infection, such as the heart, nervous system, and kidneys. Diphtheria antitoxin is the specific treatment for diphtheria and must be given immediately when clinicians suspect a diphtheria case. Successful treatment of diphtheria depends on rapid administration of equine diphtheria antitoxin in combination with antibiotics [1]. Diphtheria can be prevented by vaccination.

Many countries have progressed towards the elimination of diphtheria. However, inadequate healthcare delivery systems, poverty and other social factors have led to diphtheria re-emerging and remaining endemic in many regions of the world [2]. Diphtheria still circulates in several countries in Africa, the eastern Mediterranean, eastern Europe, South America, southeast Asia and the South Pacific [3,4].

It was thought that indigenous diphtheria would be eliminated within the World Health Organization (WHO) European Region by the year 2000 following the success of the mass immunisation programme introduced more than 60 years ago [5,6]. In 1994, the WHO European Region proposed elimination of indigenous diphtheria by the year 2000 [6]. However, an epidemic had already emerged in 1990 in the Russian Federation and from 1991 to 1993 spread to neighbouring countries [7,8]. Although the affected countries succeeded in reducing diphtheria incidence, diphtheria remained endemic in Belarus, Georgia, Latvia, the Russian Federation and Ukraine. Other European countries reported sporadic imported cases between the years 2000 and 2013 (Belgium, Estonia, Finland, France, Germany, Lithuania, Netherlands, Sweden, UK) [3,9].

Despite the fact that diphtheria is a somewhat forgotten disease in many European countries, it remains a serious health problem in endemic countries and a potential threat for other countries considered to be disease-free. More recently, awareness has increased due to several sporadic cases being reported in Europe, and in particular a recent fatal case in Spain and cutaneous diphtheria cases in refugees and asylum seekers in Denmark, Germany and Sweden; the issue of
shortages of diphtheria antitoxin was also highlighted as an European Union priority [10,11].

In 1994, an epidemic started in the Baltic States, and Latvia was the most affected of these three countries [12]. The supplementary immunisation campaign initiated in 1995 led to improved disease control but vaccination coverage was not sufficient for eradication [13]. Between 1999 and 2014, Latvia reported the highest annual incidence of diphtheria in the WHO European Region [3].

Here, we describe trends over time based on national surveillance data and data on immunisation coverage from 1994 to 2014 in Latvia, complemented by historical data since 1946, to provide insight into the epidemiology of diphtheria more than 20 years after its re-emergence and to better target future prevention strategies.

Methods
Our study period is from 1994 to 2014, and we also describe historical data from 1946 onwards. We obtained and analysed national surveillance data. From 1946 to 2001 data was available in aggregated form and case-based data were available from 2002 to 2014.

Case definition
The case definition used for surveillance of diphtheria has changed between 1994 and 2014. Since 2002, we have used the European Union case definition for reporting to the Community network [14]. Cases included in annual reports before 2002 did not use a standardised case definition. In this paper, we analysed all reported clinically and/or laboratory-confirmed cases included in our annual statistical reports from 1994 to 2014. For our study we defined a clinically confirmed or suspected case as diagnosed by a physician with a typical clinical picture, e.g. upper respiratory tract illness with laryngitis or nasopharyngitis or tonsillitis with or without an adherent membrane/pseudomembrane, and for cutaneous diphtheria skin lesion diphtheria of other sites - conjunctiva or mucous membranes. We defined a laboratory-confirmed case as a case with clinical picture and the isolation of toxin-producing C. diphtheriae, C. ulcerans or C. pseudotuberculosis from a clinical specimen.

Case and contact management
According to the Latvian procedures for registration of infectious diseases, all cases, suspected and confirmed, should be notified within 1 working day to the local public health structure [15]. Physicians should take swabs to confirm the diagnosis before antibiotic treatment is started. Depending on the clinical condition of the patient, diphtheria antitoxin may be given. Patients should be immunised in the convalescent stage.

Swabs should be taken from all close contacts, who should be provided with prophylactic antibiotics and monitored daily for at least 7 days. Immunisation should be offered if contacts have not been vaccinated [16].

Description of surveillance
During the study period, physicians notified all suspected cases of diphtheria to local public health structure using standardised notification forms according
to the Regulations of the Cabinet of Ministers of Latvia valid at the time of reporting [17].

Following notification, the local epidemiologist began the investigation using a dedicated case investigation form and after completion, submitted this to the national level using electronic surveillance system. This case investigation form included information on clinical signs and symptoms, outcome, complications, laboratory data, vaccination status, history of travel, management of the case and contacts, etc.

Vaccination status
Regional epidemiologists ascertained vaccination status by checking patients’ medical cards. This ascertaintment took into account that in the first year of life children should receive the primary three-dose immunisation course of diphtheria vaccination. By the age of 12 months to 15 years, children should have received an additional three booster doses. It is recommended that adults over the age of 25 years have a booster dose every 10 years, free of charge. If more than 10 years had elapsed since the last booster dose, two doses of vaccine are recommended, with the second dose given 4–6 weeks after the first dose.

An unvaccinated adult was defined as an individual who had not previously been immunised against diphtheria, had not received a booster vaccination for more than 10 years or whose vaccine status was unknown [18]. A partially vaccinated individual was defined as a person who had started vaccination and received at least one dose of vaccine against diphtheria, but missed one or more doses of primary immunisation or booster dose for children or the second booster dose for adults (i.e. when an adult had received the most recent booster dose more than 10 years ago).

There were only slight changes in the Latvian immunisation programme between 1994 and 2014 (Table 1) [18,19].

Severity of disease
Symptoms of diphtheria can vary from mild to severe. Physicians defined severity of disease according to the distribution of the membrane and severity of symptoms of intoxication. Mild disease was defined as localised (affects only the nose, tonsils, or nose and throat) and moderate disease as a case with a more widely distributed membrane (affecting the nose, tonsils, throat and the entire tracheobronchial tree). Severe disease was defined as a case with widely distributed membrane and severe intoxication and/or systemic complications (myocarditis, neuritis and other systemic toxic effects) and/or death.

Laboratory investigation
Clinical specimens were taken from suspected diphtheria cases by clinicians for microbiological analysis (isolation and toxigenicity testing). All private and hospital laboratories in Latvia submitted cultures to the national reference laboratory for identification and toxigenicity testing.

Immunisation coverage
Immunisation coverage in children was routinely determined for each dose of vaccine by the National Public Health Institute. For the numerator, we used the number of vaccine doses administered by vaccination services annually, based on monthly reports. For the denominator, we used population estimates from the Central Statistical Bureau of Latvia [20].

The Institute also measured vaccination coverage among adults. To assess vaccination coverage among adults aged ≥ 25 years we divided the number of adults who received a third dose (of the primary three-dose immunisation course) or booster dose in the previous 10 years in the age group ≥ 25 years by the number of adults in that age group at the beginning of the reference year.

Statistical analysis
To describe trends over time and to provide the current epidemiology of diphtheria, existing surveillance data was summarised. We analysed cases’ vaccination status and age with clinical presentation of disease in terms of the frequency of severity of disease.

Categorical variables were summarised using frequencies and proportions. To calculate the incidence, the resident population estimates for each year obtained from the Central Statistical Bureau of Latvia were used [20]. There have been changes over time among the Latvian population due to emigration, low birth rate and other factors. The population shrank from 2.5 million inhabitants at the beginning of 1994 to 2.0 million at the beginning of 2014 [20].

### Table 1

Diphtheria immunisation programme in Latvia, 1994–2014

<table>
<thead>
<tr>
<th>Immunisation dose</th>
<th>Age of immunisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st dose</td>
<td>1994–1997</td>
</tr>
<tr>
<td></td>
<td>1998–2008</td>
</tr>
<tr>
<td></td>
<td>2009–2014</td>
</tr>
<tr>
<td>3rd dose</td>
<td>3 months</td>
</tr>
<tr>
<td></td>
<td>3 months</td>
</tr>
<tr>
<td></td>
<td>2 months</td>
</tr>
<tr>
<td>1st dose</td>
<td>4.5 months</td>
</tr>
<tr>
<td>2nd dose</td>
<td>6 months</td>
</tr>
<tr>
<td></td>
<td>6 months</td>
</tr>
<tr>
<td>3rd dose</td>
<td>12–15 months</td>
</tr>
<tr>
<td>1st booster dose</td>
<td>18 months</td>
</tr>
<tr>
<td>2nd booster dose</td>
<td>9 years</td>
</tr>
<tr>
<td></td>
<td>7 years</td>
</tr>
<tr>
<td>3rd booster dose</td>
<td>15–16 years</td>
</tr>
<tr>
<td></td>
<td>14 years</td>
</tr>
<tr>
<td>Adult booster dose</td>
<td>Every 10 years</td>
</tr>
</tbody>
</table>

*If more than 10 years have elapsed since the last booster dose, two doses of vaccine are recommended, with the second dose given 4–6 weeks after the first dose.*
Results

Historical trends in Latvia
At the end of the 1940s, diphtheria incidence was very high, reaching 108.9 per 100,000 inhabitants in 1946. From 1968 to 1985, no diphtheria cases were reported but there were 51 cases registered from 1986 to 1993 (Figure).

There was a sharp increase in incidence from 0.5 per 100,000 inhabitants in 1993 to 4.5 per 100,000 inhabitants. A second wave of increasing incidence was observed in 2000 (11.1/100,000). In 2000, an outbreak occurred among highly vaccinated trainees at a Latvian military academy; 45 cases were identified [21].

Cases from 1994 to 2014
From the beginning of the epidemic in 1994 to 2014, 1,515 cases were reported, giving an average annual incidence of 3.2 cases per 100,000 inhabitants (range: 0.1/100,000 (2010) to 14.8/100,000 (1995)).

Of these cases, 56.3% were female and 43.7% male. The highest incidence was in the age groups 5–19 and 40–49 years (4.4 and 4.3/100,000, respectively) (Table 2). Compared with 2009–2011, in 2012–2014 more cases were recorded among persons aged under 18 years.

Vaccination status
Of 1,515 cases, 68.0% were unvaccinated, 4.4% were partially vaccinated and 27.6% were fully vaccinated. Of all fatal cases (n=111) only one was fully vaccinated and the remainder were unvaccinated.

Outcome and severity of disease
A total of 111 deaths were reported, of which 33.3% were in the age group 40–49 years. The case fatality rate was 7.3%, varying from 0.5% to 14.6% in different age groups. The highest case fatality rate was among adults in the age group ≥ 60 years (14.6%), 50–59 years (13.3%) and among children under 5 years (13.1%) (Table 2).

Among reported cases, 21.0% were ascertained as severe, 47.5% as moderate and 31.5% as mild (Table 3). Severe forms of disease represented 23.4% of diphtheria cases among adults and 12.6% among children aged 0–17 years. Of all cases with severe form of disease 93.7% were partially vaccinated or unvaccinated and 6.3% vaccinated.

Laboratory investigations
From 1994 to 2014, 83.8% of all cases (1,270/1,515) were laboratory confirmed. Of these C. diphtheria cases, 92.4% had biovar gravis and 5.2% were biovar mitis. A toxigenic strain of C. ulcerans was identified only from one case in 2009. Biovar gravis was prevalent during the epidemic period. Although in the pre-epidemic period 1986–1993 biovar mitis dominated; 54.1% of strains identified were biovar mitis, and 45.9% of strains were biovar gravis.

Seasonality
More cases had their onset of symptoms during the autumn (September, October, November; n=583; 38.5%), but between other seasons there were no apparent differences.
Immunisation programme

Childhood vaccination coverage with three, five or six doses of diphtheria vaccine fell from 1989 to 1995.

Mass immunisation of adults was initiated in 1995. By the end of 1998 the national coverage among adults was 70%. The immunisation programme achieved high national vaccination coverage for adults of 77%, in 2003 but it deterio rated to 59% in 2014.

From 2000 to 2014, childhood vaccination coverage with a third dose ranged from 91% to 98% and with a fifth dose from 92% to 98%. From 2000 to 2014, vaccination coverage for adolescents (sixth dose at 15 years) ranged from 86% to 96%, decrease in coverage occurred from 96% in 2007 to 86% in 2014.

Discussion

Starting from 1994 Latvia experienced an increase in diphtheria cases, and during 1999–2014, Latvia reported the highest annual incidence of diphtheria within the EU and in the WHO European Region [3]. Although in European countries diphtheria is an uncommon disease, it is still endemic in Latvia [22]. Despite high vaccination coverage, incidence increased from 0.1 per 100,000 inhabitants in 2010 to 0.7 in 2013. The highest incidence was among the age groups 5–19 and 40–49 years. No cases in children were observed from 2009 to 2011, but new cases have emerged since 2012. Most cases occurred in adults who were either unvaccinated or incompletely vaccinated, and these subgroups had the most severe outcomes. The proportion of severe forms was six times higher among those who were unvaccinated of partly vaccinated in comparison to those who were fully vaccinated. Only 4.3% of vaccinated cases had the severe form of diphtheria and one case was fatal. This indicated that the disease in vaccinated individuals was milder and less fatal. The case fatality rate in the unvaccinated was more than 50 times higher compared with those vaccinated (10.0% vs 0.2%). The highest case fatality rate was among adults in the age groups ≥ 50 years; and among children under 5 years old. These population groups, children and older adults who did not have up-to-date immunisations, were defined as the high-risk groups [23]. From 1996 to 2003 annual seroepidemiological studies were carried out in Latvia. Studies in European countries have indicated that immunity levels below the protective level (> 0.1 IU) increased with age of adults [24]. On average in Latvia, for 23% of adults the immunity level was lower than protective and for 30% of adults it was protective. The highest number of seronegative adults was detected in adults aged ≥ 50 years. This may explain the large number of severe cases and high morbidity and mortality rate among adults over 50 years old.

Our investigation had some limitations. A lack of case-based data before 2002 required us to limit the scope of our analysis. Misclassification of vaccination status may have occurred due to poor documentation of vaccinations and this may have led to an overestimating of the rate of unvaccinated individuals.

Diphtheria remains a public health problem in Latvia with continued circulation of toxin-producing stains of C. diphtheriae. Maintaining high vaccination coverage is essential to prevent the re-emergence of C. diphteriae. This was exemplified by the re-emergence of diphtheria parallel with a decline of childhood vaccination

<table>
<thead>
<tr>
<th>Severity of disease</th>
<th>Number of severe cases</th>
<th>%</th>
<th>Number of moderate cases</th>
<th>%</th>
<th>Number of mild cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–4</td>
<td>22</td>
<td>28.9%</td>
<td>25</td>
<td>32.9%</td>
<td>29</td>
<td>38.2%</td>
</tr>
<tr>
<td>5–9</td>
<td>14</td>
<td>12.1%</td>
<td>33</td>
<td>28.4%</td>
<td>69</td>
<td>59.5%</td>
</tr>
<tr>
<td>10–14</td>
<td>3</td>
<td>3.2%</td>
<td>30</td>
<td>32.3%</td>
<td>60</td>
<td>64.5%</td>
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<tr>
<td>15–17</td>
<td>4</td>
<td>7.1%</td>
<td>20</td>
<td>35.7%</td>
<td>32</td>
<td>57.1%</td>
</tr>
<tr>
<td>18–19</td>
<td>5</td>
<td>3.6%</td>
<td>104</td>
<td>74.3%</td>
<td>31</td>
<td>22.1%</td>
</tr>
<tr>
<td>20–29</td>
<td>5</td>
<td>2.7%</td>
<td>99</td>
<td>52.7%</td>
<td>84</td>
<td>44.7%</td>
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<td>30–39</td>
<td>26</td>
<td>13.2%</td>
<td>91</td>
<td>46.2%</td>
<td>80</td>
<td>40.6%</td>
</tr>
<tr>
<td>40–49</td>
<td>95</td>
<td>33.9%</td>
<td>138</td>
<td>49.3%</td>
<td>47</td>
<td>16.8%</td>
</tr>
<tr>
<td>50–59</td>
<td>81</td>
<td>37.3%</td>
<td>105</td>
<td>48.4%</td>
<td>31</td>
<td>14.3%</td>
</tr>
<tr>
<td>≥ 60</td>
<td>63</td>
<td>41.4%</td>
<td>74</td>
<td>48.7%</td>
<td>15</td>
<td>9.9%</td>
</tr>
</tbody>
</table>

Children 0–17 years

Adults ≥18 years

Vaccination status

<table>
<thead>
<tr>
<th>Vaccination status</th>
<th>Number of severe cases</th>
<th>%</th>
<th>Number of moderate cases</th>
<th>%</th>
<th>Number of mild cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated</td>
<td>20</td>
<td>4.8%</td>
<td>203</td>
<td>48.6%</td>
<td>195</td>
<td>46.6%</td>
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<tr>
<td>Partially vaccinated or unvaccinated</td>
<td>298</td>
<td>27.2%</td>
<td>516</td>
<td>47.0%</td>
<td>283</td>
<td>25.8%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
coverage with three doses of vaccine during the first year of life from 90% in 1989 to 77% in 1995, and for the fifth dose at the age of 9 years from 97% to 90%, and for the sixth dose at the age 15 years from 98% to 80% [13]. This supports the WHO recommendation of achieving vaccination coverage above 90% for children and at least 75% for the adult population to eliminate the disease [6]. According to the goals of the national public health strategy for 2014 to 2020 we should achieve vaccination coverage for at least 95% of children and at least 62–65% of the adult population in Latvia [25].

The National Public Health Institute recommends to strengthen immunisation to cover adults with adequate booster dose(s) or three doses and continuous education of health professionals on how to talk with patients about their concerns of vaccines. We also suggest conducting a serological survey to document the current immunity to diphtheria.

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Conflict of interest
None declared.

Authors’ contributions
IK did data analysis and wrote the paper. The paper was reviewed by IL and JP.

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The aim of this study was to identify and characterise *Bacillus cereus* from a unique national collection of 564 strains associated with 140 strong-evidence food-borne outbreaks (FBOs) occurring in France during 2007 to 2014. Starchy food and vegetables were the most frequent food vehicles identified; 747 of 911 human cases occurred in institutional catering contexts. Incubation period was significantly shorter for emetic strains compared with diarrhoeal strains. A sub-panel of 149 strains strictly associated to 74 FBOs and selected on Coliphage M13-PCR pattern, was studied for detection of the genes encoding cereulide, diarrhoeic toxins (Nhe, Hbl, CytK1 and CytK2) and haemolysin (HlyII), as well as *panC* phylogenetic classification. This clustered the strains into 12 genetic signatures (GSs) highlighting the virulence potential of each strain. GS1 (*nhe* genes only) and GS2 (*nhe*, *hbl* and *cytK2*), were the most prevalent GS and may have a large impact on human health as they were present in 28% and 31% of FBOs, respectively. Our study provides a convenient molecular scheme for characterisation of *B. cereus* strains responsible for FBOs in order to improve the monitoring and investigation of *B. cereus*-induced FBOs, assess emerging clusters and diversity of strains.

**Introduction**

The *Bacillus cereus* sensu lato group includes the following closely related spore-forming species: *B. cereus* sensu stricto, *B. thuringiensis*, *B. cytotoxicus*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycooides* and *B. anthracis* [1]. The first four species are known to be involved in food poisoning [1]. *B. thuringiensis* is also mainly known as a biopesticide due to production of insecticidal toxins [2]. *B. anthracis* is highly virulent in mammals and is the causative agent of anthrax [3]. *B. cytotoxicus* is a newly identified group of strains that induce severe food poisoning. They are characterised by the production of cytotoxin K-1 (CytK-1) and a relatively high genomic diversity compared with other *B. cereus* strains [1].

*B. cereus* is currently the second most frequently found causative agent of confirmed and suspected food-borne outbreaks (FBOs) in France after *Staphylococcus aureus* [4]. Depending on the evidence implicating a food vehicle source during epidemiological and microbiological FBO investigations, the outbreaks are referred as a strong-evidence or weak-evidence FBO. Briefly, an FBO is defined as ‘strong-evidence’ when the following information is known and reported: food vehicle, food source, the link between outbreak cases and the food vehicle, place of exposure, and contributory factors. When several parts of the information are missing, the FBO is considered as ‘weak-evidence’ FBO [5].

Between 2006 and 2014 in France, *B. cereus* was recorded as the second or third major cause in weak-evidence FBOs. In 2014, *B. cereus* represented the second cause in weak-evidence FBOs, with 1,902 human cases for 224 FBOs, and the second cause of strong-evidence FBOs, with 23 FBOs accounting for 447 human cases and 18 hospitalisations [4]. The increase in *B. cereus*-induced FBOs is partly due to the input of national health and food safety authorities in the epidemiological and microbiological investigations of suspected FBOs. Indeed, *B. cereus* strains isolated from foodstuffs suspected of being involved in an FBO are now usually collected by the laboratory for food safety in ANSES. To illustrate this, during 1996 to 2005, only 94 strong-evidence and 196 weak-evidence FBOs were reported, whereas for 2014 alone, 23 and 241 strong- and weak-evidence FBOs were notified, respectively showing the high input of the authorities. Nevertheless, the number of total human *B. cereus* cases is likely to
B. cereus can induce two types of gastrointestinal disease, leading to emetic or diarrhoeal syndromes. The symptoms associated with B. cereus infection are generally mild and self-limiting, but more serious and even fatal cases have been described in France and around the world [6]. The emetic syndrome is characterised by vomiting and nausea, usually 30 minutes to 6 hours after ingestion, and can be confused with FBOs caused by Staphylococcus aureus. This syndrome is due to the ingestion of a thermostable toxin known as ceruleid, pre-formed in food before ingestion of contaminated foods. The emetic B. cereus strains represent a cluster of strains characterised by the presence of the plasmid-located ces gene encoding an enzyme involved in ceruleide synthesis [7].

Diarrhoeic symptoms are characterised by abdominal cramps and watery diarrhoea within 8 to 16 hours after ingestion of contaminated foods. These diarrhoeal symptoms and incubation periods can be easily confused with those caused by Clostridium perfringens food poisoning. More precise information about diarrhoeic strains is thus necessary to discriminate between possible causative agents and allow better diagnosis during FBOs. The diarrhoeal syndrome occurs after ingestion of vegetative cells or spores of diarrhoeic strains. This syndrome is generally attributed to at least three enterotoxins: haemolysin BL (Hbl), which has three components B, L1 and L2; non-haemolytic enterotoxin (Nhe) with its three components Nhe-A, Nhe-B and Nhe-C, and cytotoxin K (CytK). Two forms of cytotoxin K have been described, CytK-1 and CytK-2, the former being more cytotoxic than the latter [8]. In addition, B. cereus produces other toxins such as haemolysin II (HlyII), metalloproteases such as InhA1 and InhA2, and the cell wall peptidase FM (CwpFM), which may also be involved in pathogenicity [9-11]. The pathogenic spectrum of B. cereus ranges from strains used as probiotics to strains that are lethal to humans and it remains difficult to predict the pathogenic potential of a strain. Apart from strains encoding ces or cytK-1 genes, which are virulent and well described in the literature [8,12], the pathogenicity of B. cereus diarrhoeal strains is not fully understood and there are currently no specific markers to unambiguously differentiate between pathogenic and harmless strains. Indeed, the genetic studies carried out to date have been inconclusive and, regardless of the diseases they cause, all strains seem to carry genes encoding at least one of the known diarrhoeal toxins [13]. However, highly toxic strains do not necessarily overproduce these toxins [14]. The aim of this study was therefore to identify and characterise B. cereus strains from a unique national collection of 564 strains strongly related to 140 FBOs that occurred in France during 2007 to 2014 in order to improve the monitoring and investigation of B. cereus-induced FBOs, assess the risk of emerging clusters of strains and identify strain variability.

Methods

Epidemiological data

The epidemiological data related to each FBO were mainly collected through interviews or questionnaires by local health authorities. The suspected food in each FBO was traced by the local services of the French Ministry of Agriculture and Food (DDPP, Department for protection of populations). Collected data included a record of the type of suspected food, preparation location and date, type of packaging, number of human cases, symptoms and incubation periods. Then, a database of ANSES (French Agency for Food, Environmental and Occupational Health and Safety) was built, gathering epidemiological data as well as analytical results of B. cereus enumeration in food, strain characterisation and toxin production.

Strain collection

For each FBO, all bacterial strains from suspected food were isolated by plating leftovers on selective media plates allowing the discrimination of B. cereus from other bacterial pathogens (S. aureus, C. perfringens, etc). Identification and enumeration of one to five B. cereus strains per FBO were conducted by plating the strains on selective B. cereus agar media (MYP agar media: mannitol-phenol red-egg yolk medium (Biokar) according to the International Organization for Standardization (ISO) 7932 standard method or BACARA (BioMérieux), previously certified commercial
alternative method (AES 10/10–07/10). All isolates were tested for haemolytic activity on sheep blood agar [15], lecithinase production on MYP agar media and starch hydrolysis on plate count agar (BioMérieux).

DNA extraction
DNA was extracted after overnight incubation of the strains at 30 °C on trypticase soy agar with 0.6% yeast extract (Sigma-Aldrich) using the DNeasy Blood and Tissue Kit (Qiagen). DNA was quantified by absorbance at 260 nm on a Nanodrop1000 spectrophotometer (Thermo scientific).

Coliphage M13 sequence-based PCR typing
To study strain diversity and discriminate between strains isolated in samples within the same FBO, B. cereus strains were typed using coliphage M13 sequence-based PCR (M13-PCR) derived from an RAPD technique and adapted from [16]. The PCR mix contained 40 ng of DNA template, 0.9 mM dNTP mix (Roche Diagnostics), 4 mM MgCl2, 2 µM primer (GAGGGTGGCGGCTCT), 2.5 U Goldstar DNA polymerase, and Goldstar buffer (Eurogentec). Thermal cycling using the Veriti Thermal Cycler (Applied Biosystems) included a denaturation step at 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, 8 min at 68 °C and an elongation step at 68 °C for 8 min. The amplified DNA was analysed by SDS-PAGE electrophoresis. The M13-PCR patterns were visualised using ChemiDoc XRS imaging system. Then, DNA profiles were analysed with BioNumerics 7.1 software (Applied Maths).

panC gene sequencing
B. cereus strains were assigned to the seven known phylogenetic groups according to partial sequencing of the panC gene [17]. The sequencing was carried out by a commercial facility (Eurofins MWG Operon). The classification into the phylogenetic groups was performed using the algorithm described in [17]. The two typing methods panC gene sequencing and M13-PCR typing were used for separate objectives. This study did not explore the correlation between the two methods.

Virulence gene detection
The presence of potential virulence genes cytK-1, cytK-2, hblA, hblC, hblD, nheA, nheB, nheC, hlyII and ces [10,13] was evaluated by PCR. As the genetic diversity of B. cytotoxicus strains possessing cytK-1 is substantial, the primers used to detect the other virulence genes were not suitable for those particular strains. The PCR was performed with the Veriti Thermal Cycler. The final reaction mixture (25 µL) contained 200 µM dNTPs, 1X PCR buffer, 1 U FastStart Taq DNA Polymerase (Roche), 200–1,000 nM primers, and 2 µL (ca 10 ng) template DNA. The amplification protocol comprised initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 90 s and final extension at 72 °C for 7 min. PCR products were analysed by SDS-PAGE electrophoresis.

Enterotoxin quantification
The production of the enterotoxins Nhe and Hbl was tested using two immunological tests, the BCET-RPLA Toxin detection kit (Oxoïd) and Tecra kit (BDE VIA, 3M-Tecra), respectively, after culture in brain heart infusion broth (BioMérieux) for 6 hours at 30 °C with stirring [18].

Database and statistical analysis
Strain characterisation results and epidemiological data were entered into a central database using BioNumerics software. The distribution of mean incubation periods, i.e. the time between ingesting contaminated food and symptom onset, was characterised using R 3.1 software and the ‘fitdistrplus’ package [19]. The log-normal was fitted to data according to maximum-likelihood estimation. To study seasonal variation in the occurrence of FBOs, the distribution of FBO dates was analysed throughout the year according to a previously described method [20].

Results

Epidemiological and clinical data
We studied a collection of 564 B. cereus strains associated with 140 FBO that occurred in France during 2007 to 2014. In 66 of the FBOs, B. cereus was isolated concomitantly with other bacterial species (including S. aureus and C. perfringens) during microbiological investigations, making it impossible to affirm that B. cereus was the cause of these FBOs. Our study therefore focused on 339 B. cereus strains isolated from food samples analysed during 74 FBOs where no other pathogenic bacteria were detected in the food during microbiological investigations (Table 1). These 74 FBOs resulted in 911 human cases. Data on sex and age of
the cases were not always available and could therefore not be included in the study.

Over the eight years of the survey, the occurrence of FBOs was not subject to any seasonal effect (Figure 1). Emetic and diarrhoeal symptoms of human cases were often present at the same time and were reported for 57% of FBOs (42/74), whereas abdominal pains, diarrhoeic or emetic syndromes alone occurred in 4% (36/911), 12% (109/911) and 13% (118/911) total human cases, respectively.

Between 400 and $10^9$ B. cereus CFU/g were found in the incriminated foods. Levels lower than $10^5$ CFU/g were observed in 48/57 FBOs due to diarrhoeal strains and in 11/17 FBOs due to emetic strains (Table 1). The incubation period (time between ingestion of contaminated food and symptom onset) varied from less than 3 hours to 21 hours (Figure 2). The mean incubation period was 5.7 hours (standard deviation (SD) 1.3) and could vary within the same FBO (Table 1). However, the incubation period was significantly shorter for emetic strains (carrying the ces gene) – mean: 2.6 hours (SD: 2.1) – compared with diarrhoeal strains (mean: 6.6 hours (SD: 1.4)).

A single food source was incriminated for 57% of FBOs (42/74), of which 14/42 were associated with starchy food, 8/42 and 7/42 FBOs with vegetables and with mixed dishes composed of starchy food or vegetables, respectively (Table 1). Only 14% (10/74) of FBOs were associated with foodstuffs of animal origin.

Furthermore, 60% of FBOs (44/74) occurred in institutional catering, involving 82% (747/911) of the human cases. FBOs were poorly reported in a family context, which represented 13% of the FBOs (10/74) and 7% (64/911) of the human cases (Table 1). The remaining 27% (20/74) of FBOs occurred in a commercial catering context, involving 11% (100/911) of cases.

**Strain characterisation**

Phenotypic analysis of the strains showed that 92% (312/339) of the strains produced lecithinase. Haemolytic activity on sheep blood agar was detected for 87% (295/339). Some 48% (163/339) of strains were able to hydrolyse starch (data not shown). The panC gene sequences were used to assign B. cereus strains to one of the seven previously described phylogenetic groups I to VII (Table 2). Group I was not represented in the strains analysed. Group III was the most represented (46%; 156/339). Groups IV and II represented 24% (81/339) and 19% (64/339), respectively. The distribution of strains in groups VII, VI and V were 5% (17/339), 4% (14/339) and 2% (7/339), respectively.

M13-PCR typing and genetic characterisation were conducted on all 339 B. cereus isolates from the 74 FBOs in order to discriminate different patterns and genetic profiles. Up to five isolates from each FBO were subjected to M13-PCR typing. For 42 FBOs, a unique M13 pattern was identified among all isolates recovered from samples within the same FBO (such as FBO number 5, Figure 3A). In the remaining 32 FBOs, several M13 patterns were observed in samples within the same FBO (such as FBO number 6 with four different M13 patterns, Figure 3B). Thus, a total of 159 representative strains gathering 42 strains from the 42 FBOs of unique M13 pattern and 117 strains representative of the M13 pattern diversity from the remaining 32 FBOs, were selected for further characterisation (Figure 4).
The presence of major virulence genes was investigated (Table 2). The \textit{ces} gene was detected in 16% (25/159) of the \textit{B. cereus} strains, meaning they were emetic strains. All the emetic strains belonged to phylogenetic group III. The \textit{cytK-1} gene was detected in 5% (8/159) of strains, strictly associated with group VII and classified as \textit{B. cytotoxicus} strains.

The most frequently distributed genes were those encoding enterotoxin Nhe, namely \textit{nheC}, \textit{nheB} and \textit{nheA} genes detected in respectively 100% (159/159), 99% (157/159) and 96% (153/159) of the tested strains.

The \textit{hblA}, \textit{hblD} and \textit{hblC} genes encoding enterotoxin \textit{Hbl} were detected in 44% (70/159), 44% (70/159) and 40% (64/159) of the strains, respectively. The \textit{cytK-2} gene was detected in 42% (67/159) of strains and 23% (37/159) of strains carried \textit{hlyII}.

These genetic features allowed to cluster the strains into 12 pathogenicity or ‘genetic signatures’ (GSs), GS1 to GS12 (Table 2). Some 84% (133/159) of the strains belonged to GS1 to GS6. The most frequent GS encountered in the collection was GS1, which accounted for 21% (34/159) of strains. In GS1, only Nhe-encoding genes were detected. The \textit{ces}-positive strains were all placed in GS3 (except a single one in GS11) and possessed \textit{nhe} genes in addition to the \textit{ces} gene. GS11 also displayed the \textit{cytK-2} gene. GS7 contained all the \textit{B. cytotoxicus} strains carrying the \textit{cytK-1} gene. GS8 was characterised by strains carrying \textit{nheB} and \textit{nheC} genes, and \textit{hblA} and \textit{hblD} genes. All the strains in this group belonged to phylogenetic group VI (Table 2). Several GSs defined in this study were associated with a single \textit{panC} phylogenetic group, i.e. GS2 (IV), GS203 (III) GS7 (VII), GS8 (VI), GS11 (III) and GS12 (II).

**Discussion**

Food-borne infections are a common yet distressing and sometimes life-threatening problem for millions of people throughout the world [21]. \textit{B. cereus} is reported to be the fourth major cause of notified FBOs in the European Union and the second in France [4,5]. However, \textit{B. cereus}-associated outbreaks are likely to be underestimated, as they usually remain undiagnosed and therefore under-reported. If \textit{B. cereus} is suspected, several identification tests can be performed: morphology tests on selective media, resistance to polymyxin B, lecithinase synthesis, haemolytic capacity, mannitol fermentation and starch hydrolysis [22]. These tests do not, however, reveal whether the isolated strains are pathogenic nor their genetic features.

The main strengths of our study are the unique national \textit{B. cereus} strain collection linked to strong-evidence FBOs, the long period covered and an accurate epidemiological and strain characterisation. The study of symptoms does not readily allow the identification of the pathogen causing the FBO because gastroenteritis symptoms are also characteristic of other food-borne pathogens, especially \textit{S. aureus} or \textit{C. perfringens} [22]. However, phenotypic analysis and species discrimination allowed us to collect isolates and epidemiological data from 140 FBOs, of which 74 were strictly associated with \textit{B. cereus} and affected 911 human cases. Considering food safety issues, this provides confirmation that \textit{B. cereus} must be considered an important food-borne pathogen, and underlines the need to improve monitoring.

For 32 of these 74 FBOs, several strain patterns were distinguished from samples of a single FBO and it was not possible to discriminate which strain or which combination of strains was responsible for the outbreak.
### Table 1a

Epidemiological and microbiological data of food-borne outbreaks associated solely with *Bacillus cereus*, France, 2007–2014 (74 outbreaks, 339 strains)

<table>
<thead>
<tr>
<th>FBO</th>
<th>Year</th>
<th>Incriminated food</th>
<th>Human cases</th>
<th>Incubation period in hours</th>
<th>Symptoms</th>
<th>Strain patterns identified</th>
<th>Outbreak setting</th>
<th>CFU/g</th>
<th>Genetic signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2007</td>
<td>Semolina</td>
<td>5</td>
<td>0–3</td>
<td>Vomiting</td>
<td>1</td>
<td>Commercial catering</td>
<td>1.20E+07</td>
<td>GS3</td>
</tr>
<tr>
<td>2</td>
<td>2007</td>
<td>Shrimp</td>
<td>12</td>
<td>21–24</td>
<td>Vomiting, diarrhoea</td>
<td>1</td>
<td>Commercial catering</td>
<td>6.80E+04</td>
<td>GS1</td>
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<tr>
<td>3</td>
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<td>Tomatoes</td>
<td>4</td>
<td>0–3</td>
<td>Vomiting, diarrhoea</td>
<td>1</td>
<td>Commercial catering</td>
<td>7.00E+02</td>
<td>GS4</td>
</tr>
<tr>
<td>4</td>
<td>2008</td>
<td>Semolina</td>
<td>40</td>
<td>12–15</td>
<td>Diarrhoea</td>
<td>1</td>
<td>Staff canteen</td>
<td>1.20E+03</td>
<td>GS1</td>
</tr>
<tr>
<td>5</td>
<td>2008</td>
<td>Tabbouleh and minced beef</td>
<td>NK</td>
<td>NK</td>
<td>Abdominal pains, vomiting</td>
<td>1</td>
<td>Commercial catering</td>
<td>5.00E+03</td>
<td>GS2</td>
</tr>
<tr>
<td>6</td>
<td>2008</td>
<td>Mixed salad, goulash, mixed beef and mashed potatoes</td>
<td>19</td>
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<td>Vomiting, diarrhoea</td>
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<td>6.00E+02</td>
<td>GS1; GS2; GS7; GS12</td>
</tr>
<tr>
<td>7</td>
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<td>Mashed potatoes and boiled potatoes</td>
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<td>Medico-social institute</td>
<td>9.20E+05</td>
<td>GS7; GS8</td>
</tr>
<tr>
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<td>2008</td>
<td>Mixed salad (rice and corn)</td>
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<td>NK</td>
<td>Abdominal pains, vomiting</td>
<td>1</td>
<td>Staff canteen</td>
<td>1.90E+03</td>
<td>GS2</td>
</tr>
<tr>
<td>9</td>
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<td>13</td>
<td>12–15</td>
<td>Abdominal pains, vomiting, other</td>
<td>1</td>
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<td>2.00E+03</td>
<td>GS2</td>
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<tr>
<td>10</td>
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<td>Semolina</td>
<td>61</td>
<td>3–6</td>
<td>Abdominal pains, vomiting</td>
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<td>1.00E+04</td>
<td>GS7</td>
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<tr>
<td>11</td>
<td>2008</td>
<td>Semolina and lamb</td>
<td>4</td>
<td>0–3</td>
<td>Vomiting</td>
<td>1</td>
<td>Commercial catering</td>
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<td>12</td>
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<td>6–9</td>
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<td>Medico-social institute</td>
<td>1.50E+05</td>
<td>GS4; GS7</td>
</tr>
<tr>
<td>13</td>
<td>2008</td>
<td>Cream caramel and smoked salmon</td>
<td>11</td>
<td>9–12</td>
<td>Diarrhoea, other</td>
<td>3</td>
<td>Commercial catering</td>
<td>3.00E+03</td>
<td>GS2; GS8</td>
</tr>
<tr>
<td>14</td>
<td>2008</td>
<td>Fruit salad</td>
<td>70</td>
<td>NK</td>
<td>Vomiting, diarrhoea, other</td>
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<tr>
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<td>6–9</td>
<td>Vomiting, diarrhoea</td>
<td>1</td>
<td>Commercial catering</td>
<td>4.60E+03</td>
<td>GS6</td>
</tr>
<tr>
<td>16</td>
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<td>Wheat</td>
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<td>9–12</td>
<td>Diarrhoea</td>
<td>3</td>
<td>Commercial catering</td>
<td>1.60E+06</td>
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</tr>
<tr>
<td>17</td>
<td>2009</td>
<td>Tiramisu</td>
<td>15</td>
<td>0–3</td>
<td>Vomiting, diarrhoea</td>
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<td>Company canteen</td>
<td>8.00E+02</td>
<td>GS9</td>
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<tr>
<td>18</td>
<td>2009</td>
<td>Fish in coconut milk</td>
<td>2</td>
<td>0–3</td>
<td>Nausea, other</td>
<td>1</td>
<td>Commercial catering</td>
<td>1.10E+04</td>
<td>GS1</td>
</tr>
<tr>
<td>19</td>
<td>2009</td>
<td>Mashed potatoes</td>
<td>24</td>
<td>NK</td>
<td>Vomiting, diarrhoea</td>
<td>1</td>
<td>School canteen</td>
<td>4.00E+02</td>
<td>GS7</td>
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<tr>
<td>20</td>
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<td>Cantonese rice</td>
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<td>0–3</td>
<td>Vomiting, other</td>
<td>1</td>
<td>Family</td>
<td>1.60E+05</td>
<td>GS3</td>
</tr>
<tr>
<td>21</td>
<td>2009</td>
<td>Mashed potatoes, roast beef and French beans</td>
<td>7</td>
<td>6–9</td>
<td>Vomiting, diarrhoea</td>
<td>3</td>
<td>Medico-social institute</td>
<td>1.90E+03</td>
<td>GS3; GS5</td>
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<tr>
<td>22</td>
<td>2009</td>
<td>Quenelle of pike</td>
<td>15</td>
<td>0–3</td>
<td>Vomiting, diarrhoea, other</td>
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<td>GS6</td>
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<td>23</td>
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<td>Sandwich (tomato, carrots, chicken)</td>
<td>7</td>
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<td>Abdominal pains, nausea</td>
<td>4</td>
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<td>5.00E+03</td>
<td>GS1; GS2; GS6; GS10</td>
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<td>24</td>
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<td>1</td>
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<td>25</td>
<td>2009</td>
<td>Squid sauce</td>
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<td>9–12</td>
<td>Diarrhoea</td>
<td>1</td>
<td>Staff canteen</td>
<td>2.10E+05</td>
<td>GS12</td>
</tr>
<tr>
<td>26</td>
<td>2009</td>
<td>Sauteed shrimp</td>
<td>4</td>
<td>0–3</td>
<td>Vomiting, diarrhoea</td>
<td>7</td>
<td>Commercial catering</td>
<td>1.90E+04</td>
<td>GS1; GS4; GS6</td>
</tr>
<tr>
<td>27</td>
<td>2009</td>
<td>Semolina and peas</td>
<td>7</td>
<td>3–6</td>
<td>Nausea, diarrhoea, other</td>
<td>5</td>
<td>Staff canteen</td>
<td>2.00E+07</td>
<td>GS2; GS5</td>
</tr>
<tr>
<td>28</td>
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<td>Salad</td>
<td>44</td>
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<td>3</td>
<td>School canteen</td>
<td>1.00E+03</td>
<td>GS2</td>
</tr>
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</table>

FBO: food-borne outbreak; NK: not known.

* Medico-social institutes included centres for disabled people, leisure centres, retirement homes and other community facilities.
<table>
<thead>
<tr>
<th>FBO</th>
<th>Year</th>
<th>Incriminated food</th>
<th>Human cases n</th>
<th>Incubation period in hours</th>
<th>Symptoms</th>
<th>Strain patterns identified n</th>
<th>Outbreak setting</th>
<th>CFU/g</th>
<th>Genetic signature</th>
</tr>
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<td>29</td>
<td>2010</td>
<td>Pasta gratin</td>
<td>2</td>
<td>0–3</td>
<td>Vomiting, diarrhoea</td>
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<td>Family</td>
<td>1.50E + 07</td>
<td>GS5</td>
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<td>30</td>
<td>2010</td>
<td>Sausage and rice salad</td>
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<td>0–3</td>
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<td>Family</td>
<td>3.00E + 03</td>
<td>GS5</td>
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<td>31</td>
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<td>Paella</td>
<td>27</td>
<td>6–9</td>
<td>Diarrhoea</td>
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<td>2.80E + 04</td>
<td>GS2</td>
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<td>0–3</td>
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<td>13</td>
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<td>2.90E + 05</td>
<td>GS1; GS5; GS6; GS10</td>
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<td>8</td>
<td>3–6</td>
<td>Vomiting, diarrhoea</td>
<td>1</td>
<td>Family</td>
<td>6.50E + 04</td>
<td>GS3</td>
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<td>NK</td>
<td>GS2</td>
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<td>35</td>
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<td>1.20E + 04</td>
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<td>36</td>
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<td>Family</td>
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<td>GS3</td>
</tr>
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<td>Shrimp</td>
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<td>0–3</td>
<td>Abdominal pains, vomiting, other</td>
<td>2</td>
<td>Commercial catering</td>
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<td>GS1</td>
</tr>
<tr>
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<td>3–6</td>
<td>Abdominal pains</td>
<td>3</td>
<td>Commercial catering</td>
<td>8.20E + 04</td>
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<td>GS7</td>
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<tr>
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<td>12–15</td>
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<td>9–12</td>
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<td>3</td>
<td>Commercial catering</td>
<td>2.10E + 04</td>
<td>GS1; GS3; GS10</td>
</tr>
<tr>
<td>53</td>
<td>2012</td>
<td>Pasta</td>
<td>60</td>
<td>0–3</td>
<td>Vomiting, diarrhoea</td>
<td>3</td>
<td>School canteen</td>
<td>5.80E + 04</td>
<td>GS5</td>
</tr>
<tr>
<td>54</td>
<td>2012</td>
<td>Mixed salad</td>
<td>8</td>
<td>18–21</td>
<td>Abdominal pains, vomiting, other</td>
<td>1</td>
<td>Family</td>
<td>4.00E + 02</td>
<td>GS2</td>
</tr>
</tbody>
</table>

FBO: food-borne outbreak; NK: not known.

* Medico-social institutes included centres for disabled people, leisure centres, retirement homes and other community facilities.
highlighting the need for accurate data on the diversity of the isolated strains during FBO investigation. In contrast, for 42 of the 74 FBOs, a unique strain pattern was identified for each FBO, providing a valuable strain collection for further analysis of the correlation between *B. cereus* genotypic features and associated diseases. Thus, the design of this study strengthens the interpretation of results and avoids bias regarding the bacterial agent causing the FBO.

Our study described 74 FBOs in which only *B. cereus* was recovered. Nevertheless, a limitation of our study is the exhaustivity of the studied FBOs during the period, as the French institute for public health surveillance (InVS, since 2016 Santé publique France) notified 148 FBOs between 2007 and 2014, in which *B. cereus* was the confirmed causative agent (Figure 4). The number of FBOs notified to InVS was slightly higher than that of FBOs for which strains were received in ANSES and could be explained by the absence of microbiological investigation of such FBOs or the absence of isolation or sending *B. cereus* strains for further analysis.

Starchy food and vegetables were the most common food vehicles identified in our study. A previous study in commercial cooked chilled foods containing

<table>
<thead>
<tr>
<th>FBO</th>
<th>Year</th>
<th>Incriminated food</th>
<th>Human cases</th>
<th>Incubation period in hours</th>
<th>Symptoms</th>
<th>Strain patterns identified</th>
<th>Outbreak setting*</th>
<th>CFU/g</th>
<th>Genetic signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>2012</td>
<td>Chicken</td>
<td>NK</td>
<td>NK</td>
<td>Other</td>
<td>3</td>
<td>Commercial catering</td>
<td>4.00E+03</td>
<td>GS2; GS5</td>
</tr>
<tr>
<td>56</td>
<td>2012</td>
<td>Lamb meat</td>
<td>5</td>
<td>6–9</td>
<td>Vomiting, diarrhoea</td>
<td>1</td>
<td>Staff canteen</td>
<td>2.30E+03</td>
<td>GS2</td>
</tr>
<tr>
<td>57</td>
<td>2012</td>
<td>Mashed fish</td>
<td>18</td>
<td>9–12</td>
<td>Vomiting, diarrhoea</td>
<td>1</td>
<td>Medico-social institute</td>
<td>4.00E+02</td>
<td>GS7</td>
</tr>
<tr>
<td>58</td>
<td>2012</td>
<td>Diced mixed vegetables</td>
<td>14</td>
<td>9–12</td>
<td>Vomiting, diarrhoea</td>
<td>1</td>
<td>Medico-social institute</td>
<td>4.00E+02</td>
<td>GS2</td>
</tr>
<tr>
<td>59</td>
<td>2012</td>
<td>Millefeuille pastry</td>
<td>2</td>
<td>3–6</td>
<td>Nausea</td>
<td>1</td>
<td>Commercial catering</td>
<td>2.00E+03</td>
<td>GS2</td>
</tr>
<tr>
<td>60</td>
<td>2012</td>
<td>Onion soup</td>
<td>5</td>
<td>9–12</td>
<td>Vomiting</td>
<td>1</td>
<td>School canteen</td>
<td>4.00E+02</td>
<td>GS2</td>
</tr>
<tr>
<td>61</td>
<td>2013</td>
<td>Semolina</td>
<td>3</td>
<td>3–6</td>
<td>Vomiting, diarrhoea</td>
<td>2</td>
<td>Family</td>
<td>1.00E+04</td>
<td>GS5; GS10</td>
</tr>
<tr>
<td>62</td>
<td>2013</td>
<td>Grilled pork</td>
<td>2</td>
<td>6–9</td>
<td>Vomiting, diarrhoea</td>
<td>2</td>
<td>Family</td>
<td>1.80E+04</td>
<td>GS5; GS9</td>
</tr>
<tr>
<td>63</td>
<td>2013</td>
<td>Cheese-topped dish of</td>
<td>15</td>
<td>6–9</td>
<td>Diarrhoea, other</td>
<td>4</td>
<td>Staff canteen</td>
<td>6.50E+03</td>
<td>GS5; GS3; GS4</td>
</tr>
<tr>
<td>64</td>
<td>2013</td>
<td>Mashed potatoes</td>
<td>12</td>
<td>3–6</td>
<td>Vomiting, diarrhoea, other</td>
<td>2</td>
<td>Medico-social</td>
<td>2.90E+03</td>
<td>GS1; GS3</td>
</tr>
<tr>
<td>65</td>
<td>2013</td>
<td>Pineapple</td>
<td>5</td>
<td>NK</td>
<td>Other</td>
<td>2</td>
<td>School canteen</td>
<td>4.50E+02</td>
<td>GS1; GS9</td>
</tr>
<tr>
<td>66</td>
<td>2013</td>
<td>Mashed spinach</td>
<td>13</td>
<td>6–9</td>
<td>Vomiting, diarrhoea</td>
<td>3</td>
<td>Medico-social</td>
<td>1.00E+04</td>
<td>GS1; GS9</td>
</tr>
<tr>
<td>67</td>
<td>2013</td>
<td>Vegetable soup</td>
<td>10</td>
<td>15–18</td>
<td>Vomiting, diarrhoea</td>
<td>1</td>
<td>Medico-social</td>
<td>9.10E+02</td>
<td>GS2</td>
</tr>
<tr>
<td>68</td>
<td>2013</td>
<td>Mixed salad</td>
<td>NK</td>
<td>6–9</td>
<td>Abdominal pains</td>
<td>1</td>
<td>School canteen</td>
<td>5.50E+02</td>
<td>GS2</td>
</tr>
<tr>
<td>69</td>
<td>2013</td>
<td>Spinach</td>
<td>8</td>
<td>0–3</td>
<td>Vomiting, diarrhoea, other</td>
<td>2</td>
<td>Staff canteen</td>
<td>3.60E+02</td>
<td>GS5; GS10</td>
</tr>
<tr>
<td>70</td>
<td>2013</td>
<td>Mixed pie</td>
<td>19</td>
<td>12–15</td>
<td>Vomiting, diarrhoea</td>
<td>1</td>
<td>Medico-social</td>
<td>4.00E+02</td>
<td>GS1</td>
</tr>
<tr>
<td>71</td>
<td>2014</td>
<td>Mashed parsnips</td>
<td>11</td>
<td>0–3</td>
<td>Vomiting</td>
<td>2</td>
<td>School canteen</td>
<td>4.00E+02</td>
<td>GS3</td>
</tr>
<tr>
<td>72</td>
<td>2014</td>
<td>Shrimp</td>
<td>6</td>
<td>0–3</td>
<td>Abdominal pains, vomiting</td>
<td>2</td>
<td>School canteen</td>
<td>7.70E+03</td>
<td>GS1</td>
</tr>
<tr>
<td>73</td>
<td>2014</td>
<td>Polenta</td>
<td>25</td>
<td>18–21</td>
<td>Abdominal pains, diarrhoea</td>
<td>1</td>
<td>Medico-social</td>
<td>9.00E+03</td>
<td>GS5</td>
</tr>
<tr>
<td>74</td>
<td>2014</td>
<td>Semolina and ginger</td>
<td>11</td>
<td>0–3</td>
<td>Vomiting, diarrhoea</td>
<td>2</td>
<td>Family</td>
<td>1.50E+06</td>
<td>GS3; GS6</td>
</tr>
</tbody>
</table>

FBO: food-borne outbreak; NK: not known.

Medico-social institutes included centres for disabled people, leisure centres, retirement homes and other community facilities.
vegetables had shown high \textit{B. cereus} contamination levels in raw vegetables [23]. Thus, particular attention should be taken during sampling and epidemiological investigation into potential \textit{B. cereus} contamination of vegetables and starchy food. In our study, 60\% (44/74) of FBOs occurred in an institutional catering context. In the family context, 40\% (26/64) of the cases were caused by emetic strains. Incorrect cooling of food during preparation or the conservation of cooked dishes at room temperature is thought to be the cause of cereulide production [24]. Moreover, the severity of symptoms associated with emetic strains might explain an increased reporting of these strains in the family context, compared with diarrhoeic strains which may remain undiagnosed and therefore under-reported.

Epidemiological and clinical data show that the type of symptom could not be specifically associated with the presence of emetic or diarrhoeic strains. Indeed, 57\% (n=42) of the 74 FBOs shared both diarrhoeic and emetic syndromes although they were caused by only one type of strain. This may be partially explained by the fact that the emetic GS3 strains strongly produce Nhe enterotoxin (data not shown). We suspect that emetic strains may be ingested concomitantly with cereulide preformed in food, increasing pathogenicity and causing a mix of symptoms.

A significant difference was observed for the incubation period according to the type of strain. This is in accordance with previous findings showing that rapid onset of an emetic syndrome indicates intoxication by cereulide [25]. In contrast, ingestion of diarrhoeic bacteria can induce pathology via the production of enterotoxins in the small intestine, leading to a longer incubation period [26]. In some FBOs, the strains had short incubation periods (0–3 hours) without involvement of emetic strains. We hypothesise that those strains might be responsible for rapid vomiting despite absence of the ces gene as previously described [27], or alternatively that the emetic toxin was concomitantly ingested with the contaminated food in addition to a ces-negative strain, or that unknown factors were responsible for vomiting symptoms.

Diarrhoeal diseases are often associated with \textit{B. cereus} counts of $10^3$ to $10^8$ cells or spores [28]. In our study, concentrations below $10^3$ CFU/g were found in 12 of 57 foods related to diarrhoeal FBOs. This challenges the concept of a minimum infectious dose for \textit{B. cereus} in diarrhoeal FBOs. A mathematical model based on systematic data collection of \textit{B. cereus} concentrations in food implicated in outbreaks could be developed for dose–response assessment, in order to quantify infectivity associated with single cells [29]. Levels of at least $10^5$ CFU/g have generally been reported in the implicated food linked to an emetic syndrome [30]. In our study, levels of as few as 400 CFU/g were implicated. This could be explained by cereulide’s strong resistance to various treatments, underlining the importance of quantifying cereulide in foods. We cannot exclude the possibility that the CFUs recovered from leftover food accurately corresponded to the initial ingested CFUs. Indeed, food processing and storage before tests may have injured vegetative bacteria. However, we suspect that the spores, which are resistant to storage, are likely to be responsible for food-borne infections.

The genetic diversity of \textit{B. cereus} strains involved in FBOs was revealed in our study by characterisation of strains based on the detection of the genes encoding cereulide, diarrhoeic toxins (Nhe, Hbl, CytK-1 and CytK-2) and Haemolysin (HlyII) and by phylogenetic classification. A total of 12 pathogenicity signatures based on

### Table 2

<table>
<thead>
<tr>
<th>Genetic signature</th>
<th>Number of strains</th>
<th>$cytk\text{-}1$</th>
<th>$cytk\text{-}2$</th>
<th>$ces$</th>
<th>$hylI$</th>
<th>nheABC</th>
<th>hblCDA</th>
<th>panC phylogenetic groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS1</td>
<td>34</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>II - III - IV</td>
</tr>
<tr>
<td>GS2</td>
<td>28</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>IV</td>
</tr>
<tr>
<td>GS3</td>
<td>25</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>III</td>
</tr>
<tr>
<td>GS4</td>
<td>18</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>II - III</td>
</tr>
<tr>
<td>GS5</td>
<td>18</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>II - III</td>
</tr>
<tr>
<td>GS6</td>
<td>10</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>II - IV</td>
</tr>
<tr>
<td>GS7</td>
<td>8</td>
<td>Pos</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>VII</td>
</tr>
<tr>
<td>GS8</td>
<td>6</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>BC</td>
<td>AD</td>
</tr>
<tr>
<td>GS9</td>
<td>4</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>II - III</td>
</tr>
<tr>
<td>GS10</td>
<td>5</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>IV - V</td>
</tr>
<tr>
<td>GS11</td>
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<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>III</td>
</tr>
<tr>
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<td>2</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
<td>II</td>
</tr>
</tbody>
</table>

AD: only $hblA$ and $hblD$ detected; BC: only $nheB$ and $nheC$ detected; ND: primers used are unable to detect these genes in GS7 group strains; Neg: negative; Pos: positive.
The diarrhoeic strains were more polymorphic than the emetic strains, displaying nine different genetic signatures, although six accounted for 84% (105/125) of the strains. Genes encoding Nhe were present in all GSs, but had variable Nhe production (data not shown), suggesting that other factors may be involved in pathogenicity. GS1 (nhe genes only) and GS2 (nhe, hbl and cytK2) were the most prevalent GSs and may have a large impact on human health: they were present in 28% (20/74) and 31% (23/74) of FBOs, respectively. This is consistent with previous findings showing 28% and 24% of B. cereus strains belonging to GS1 and GS2, respectively [13]. Unlike GS1 strains, which were divided into three different phylogenetic groups, all GS2 strains belonged to phylogenetic group IV. These strains produce high concentrations of Hbl, are strongly cytotoxic to Caco2 cells and are more prevalent among strains responsible for food poisoning [12]. These characteristics might partially explain the pathogenic potential of strains of GS2, although a synergistic effect of Hbl and Nhe on pathogenicity was not observed [32].

GS7 contained all the B. cytotoxicus strains carrying the cytK-1 gene, which were related to phylogenetic group VII. Strains carrying cytK-1 were mainly found in vegetable purées, corroborating results of a study showing that 35% of B. cereus strains found in commercial potato products taken on retail level or from catering establishments, possess cytK-1 [33].

Several studies suggest that the pathogenic potential of group VI strains is very low [12]. In our study, these GS8 strains were involved in two FBOs in association with other strains belonging to GS2 and GS7, (FBO 7 and 13, respectively). Thus, it was not proven that GS8 strains were responsible for the symptoms. However, FBO 40, with 18 human cases, was caused by a unique GS8 strain, suggesting a virulence potential of this group [12].

Taken together, assignation of the strains according to genetic signature showed a high genetic diversity of B. cereus strains involved in FBOs and their pathogenic potential. Our results underline that B. cereus is a foodborne pathogen with a substantial impact on human health that should be investigated when a FBO is suspected. We propose an approach based on reported symptoms and incubation period. Particular attention should be given to vegetables and starchy food during the sampling as part of the investigation. We recommend collecting at least five colonies from each food sample potentially contaminated with B. cereus detection and transmitting isolates together with epidemiological data to the Laboratory for Food Safety. We also address special thanks to Dr Marie Laure De Buyser, who initiated the strain collection and the Central Veterinary Services Laboratory Unit of the Laboratory for Food safety.

Acknowledgements

The authors would like to warmly thank all the district veterinary and food analysis laboratories for carrying out B. cereus detection and transmitting isolates together with epidemiological data to the Laboratory for Food Safety. We also address special thanks to Dr Marie Laure De Buyser, who initiated the strain collection and the Central Veterinary Services Laboratory Unit of the Laboratory for Food safety.

Conflict of interest

None declared.

Authors’ contributions

BG participated in the design of the study and the draft of all the manuscript, he conducted the microbial analysis of the strain collection and molecular characterisation. MLV participated in the design and coordination of the study, and the draft of all the manuscript. AB participated in the design and coordination of the study, data interpretation and the draft of all the manuscript. LG carried out the statistical analysis on the clinical and epidemiological data and took part in the draft of the manuscript. SCS took part in building the strain collection and molecular characterisation. VM and JAH participated in the design of the study and took part in the draft of the manuscript. NR participated in the design of the study, data interpretation and the draft of all the manuscript.

References


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Meeting report

Monitoring of HIV testing services in the EU/EEA

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Background

HIV continues to be a serious public health issue in the European Union/European Economic Area (EU/EEA) and, despite concerted prevention efforts, the number of new HIV diagnoses reported each year has remained largely unchanged over the last decade [1]. The European region is increasing its efforts to reach the 90–90–90 targets advocated by the Joint United Nations Programme on HIV/AIDS (UNAIDS) [2]. One of the major challenges many European countries face is the high proportion of undiagnosed people living with HIV [3] and the high rates of late diagnosis [4,5]. In the past years, testing programmes have improved in terms of their accessibility and coverage, yet it remains difficult to monitor and evaluate the performance of testing programmes at all levels as a consequence of significant gaps in the data available on testing services [4,6].

In October 2016, the European Centre for Disease Prevention and Control (ECDC) convened an expert consultation, attended by representatives from a range of constituencies (national institutions, community organisations, healthcare workers) from 14 Member States and international organisations, to explore how to strengthen monitoring of HIV testing in the EU/EEA. The consultation’s aims were to (i) share experiences on how HIV testing is currently monitored, (ii) reflect on the need, scope and feasibility of a common approach to monitor HIV testing and (iii) formulate recommendations on how to improve the monitoring of HIV testing in the EU/EEA.

Strategic information and targeted HIV testing: what is needed?

Representatives of different constituencies from four countries made paired presentations on the need for and the use of strategic information at country level. For each country there were unique positions on the challenges of collecting and using strategic information but a clear consensus emerged that more and better national data were needed to monitor and implement an effective HIV testing strategy.

Susan Cowan (Statens Serum Institut, Denmark) and Per Slaaen Kaye (AIDS-Fondet) emphasised the importance of pushing beyond existing approaches to HIV testing in order to reduce the number of undiagnosed people. They noted that alternative approaches to testing, including, for example, home testing, are likely to be even more difficult to monitor than existing approaches. Cost-per-test and cost-per-case-detected is considered an important element in the assessment of testing approaches. Nevertheless, it was noted that the cost-per-case of finding new cases is likely to increase as the number of undiagnosed people declines.

Florence Lot (Agence nationale de santé publique, France) and Richard Stranz (AIDES) made complementary presentations about the current situation in France. They shared concerns about the large number of undiagnosed HIV cases and the high rates of undiagnosed prevalence among three populations: men who have sex with men (MSM), heterosexual women born abroad and heterosexual men born abroad. Intense community outreach and localised testing are being implemented in France to improve knowledge and testing uptake among these key populations.

Olivia Castillo Soria (Ministry of Health, Social Services and Equality, Spain) and Jordi Casabona (Centre d’Estudis Epidemiològics sobre les ITS i la Sida de Catalunya) described the importance of community HIV testing in Spain and presented an ongoing ministerial initiative to map and geo-reference community-based testing sites in the country and collect standardised information on HIV community testing programmes like number of test and result, testing and counselling and linkage to care. The long-standing experience of the HIV-DEVO Project [7] in Catalonia was presented as an example of a successful approach to monitor community-based testing. According to the latest data ca
20% of the new HIV cases in the region were diagnosed within the network.

Alison Brown (Public Health England), Cary James (Terence Higgins Trust) and Ann Sullivan (Chelsea and Westminster Hospital) presented on the challenges and opportunities for expanded testing in the UK in the context of high rates of undiagnosed and late diagnosis of HIV. New testing guidelines developed by the National Institute for Health and Care Excellence (NICE), to be released in December 2016, recognise the importance of expanding HIV testing outside of traditional settings. Innovative approaches such as home sampling and self-testing have great potential, with one initiative managing to distribute ca 4,000 self-tests in only 10 days in the country. A majority of those using the tests shared their results afterwards, providing a positive indication in terms of monitoring opportunities of this testing approach.

Key points identified through the presentations and ensuing discussion included:

- Taking a pragmatic approach and making use of readily available data, including surveillance and programmatic data are highly important.

- Better estimates of key population size, their geographic distribution within countries, and the relative proportion of undiagnosed cases are crucial to target testing services.

- The substantial contribution of community-based testing in detecting new HIV cases where it has been introduced at scale, e.g. Spain, France, Greece and Portugal, was recognised. It was noted that, while community testing sites often generate good monitoring data, the lack of consistency in the metrics used across single sites undermines the ability to estimate the relative contribution to overall testing efforts in a country, with some notable exceptions at national (e.g. Rede de Rastreio, http://www.gatportugal.org/noticias/rede-de-rastreio-comunitaria-resultados_83, Portugal), sub-national (e.g. the HIV-DEVO Project, Spain) and European level (HIV community-based testing practices in Europe [HIV-COBATEST] network) (8).

Existing efforts to monitor HIV testing

Representatives from three countries and two EU projects presented their experiences in monitoring HIV testing: Magdalena Pylli (Hellenic Centre for Diseases Control and Prevention, Greece), Kristi Rüütel (National Institute for Health Development, Estonia) and Daniel Simões (Grupo de Ativistas em Tratamentos (GAT), Portugal); Dorthe Raben (Optimising testing and linkage to care for HIV (OptTEST: http://opttest.eu/)) and Laura Fernández-López (Operational knowledge to improve HIV early diagnosis and treatment among vulnerable groups in Europe (Euro HIV-EDAT: https://eurohivedat.eu/)). There were substantial similarities in the metrics collected for monitoring HIV testing across the presentations, the most common being the number of screening tests, confirmatory assays, positive tests, reason(s) for testing, sex, age and site/setting (e.g. laboratory, community site, hospital, ante-natal care services). Other metrics were additional socio-demo-graphic data, previous testing history, risk behaviour and risk exposure, knowledge and use of biomedical HIV prevention (e.g. Post-Exposure Prophylaxis), linkage to care, cost per test and cost per diagnosis. Furthermore, measuring the rate at which service providers offer HIV test to eligible patients was deemed a key monitoring element for indicator-condition guided testing(9).

A number of data sources were mentioned, including reference laboratories and primary laboratories, health facilities, hospitals, national health insurance databases, cross-sectional and ad hoc studies, national and international networks of community-based testing sites that collect monitoring data using online platforms [8]. Among the challenges, poor data quality, uncertainty around representativeness of the data, limited availability and implementation of quality control measures were mentioned by all presenters. Lack of integration with national monitoring data was identified as a major challenge for community-based and indicator-condition guided testing initiatives. Additional challenges identified included: (i) tracking retesting by the same individual, (ii) lack of demographic data from anonymous tests, and (iii) the (lack of) reporting culture among service providers and limited financial resources.

Suitable metrics and data sources for monitoring HIV testing in the EU/EEA

In working groups, participants focused on suitable metrics and data sources for monitoring HIV testing in the EU/EEA.

Key recommendations concerning metrics and data sources included:

- Promote the use of a limited number of metrics that can be easily and widely tracked. There was general consensus around four metrics: (i) number of tests, (ii) basic demographic data of the tester (e.g. age, sex and population group), (iii) location/setting of the test, and (iv) number of reactive/positive tests.

- Use existing data sources to limit additional burden. While the exact data sources will vary by country, existing ones should be able to generate the data for the core metrics.

- Integrate all applicable data in a country to produce meaningful national datasets that capture the activities of the various organisations conducting HIV tests. The critical example is the integration of national monitoring data with those generated from community-based testing sites. It was suggested to promote collation of community-based organisations’ data at country
level as a first step towards effective integration with national data.

- Determine how to integrate data on home sampling and self-testing into the monitoring approach. One suggestion was to work with industry/private sector to collect indicative (e.g. sales) data.

The participants agreed that the core metrics should be scalable and flexible. In terms of scalability, the metrics would need to be feasible and meaningful to collect at the site level (e.g. by contributing to quality improvement cycle) but could also be scaled up for use at national and international levels with a comparable level of usefulness. They should also be flexible to allow use in specific settings and with specific populations, such as a network of community-based testing sites serving MSM or of health facilities implementing indicator-condition guided testing which can collect, aggregate and compare data points from these metrics to assess the implementation and effectiveness of the initiatives.

Several other metrics were identified as potentially useful for monitoring HIV testing, including linkage to care, site/setting of first reactive test/diagnosis, and reason for test. Linkage to care was recognised to be a vital data point for community organisations to monitor the ability of effectively referring to care of newly diagnosed individuals. There was general agreement that site/setting of first reactive test and/or diagnosis could be collected as an additional variable within the European HIV surveillance dataset to gather information on testing sites (as a proxy for testing modalities) and their relative yield of positive diagnoses.

While there was consensus that key populations’ size estimates and relative undiagnosed fractions would be extremely valuable instruments to monitor impact of testing programmes, there were concerns about their accuracy and robustness. The ECDC HIV modelling tool [9] [10] [8] is a valuable asset in supporting Members States with a standardised method and an easy-to-use online tool to produce national estimates. Data on late HIV diagnoses and the relative proportion among key diagnosed individuals. There was general agreement that site/setting of first reactive test and/or diagnosis could be collected as an additional variable within the European HIV surveillance dataset to gather information on testing sites (as a proxy for testing modalities) and their relative yield of positive diagnoses.

Conclusions
Expanding the availability and improving the targeting of HIV testing will reduce the percentage of late HIV diagnoses as well as the overall number of undiagnosed cases in EU/EEA countries. Among the innovative modalities of HIV testing, self-sampling and self-testing programmes as well as community-based voluntary counselling and testing have been shown to expand availability and improve targeting of HIV testing, particularly among key populations who are most affected by HIV.

Improving testing policies, planning, resource allocation and programme performance needs timely, accurate and high-quality data on HIV testing locally, nationally and regionally. Continuous efforts in developing accurate and robust estimates of people living with HIV, the size of key populations and relative undiagnosed fractions should be pursued to enable better assessment of the impact of testing activities. Increasing the utility of already collected metrics, such as the proportion of late diagnoses, could effectively help targeting testing efforts to key sub-groups.

A small, core set of metrics that are straightforward to collect and are broadly useful have been identified and should strengthen the capacity to monitor and evaluate testing programmes at local, national and regional levels. Data from the full range of national HIV testing initiatives, particularly healthcare and community activities, should be routinely aggregated where available to ensure that countries have a complete picture of their situation. The ongoing separation of datasets undermines the ability of all stakeholders to understand and assess the opportunities and challenges facing HIV testing programmes.

**Erratum**
The author name of David Hales was added on 6 December 2016.

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**Conflict of interest**
None declared

**Authors’ contributions**
LT and DH wrote the manuscript. Both authors read and approved the final manuscript.
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