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Toxigenic *Corynebacterium ulcerans* in a fatal human case and her feline contacts, France, March 2014

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In March 2014, a person in their eighties who was diagnosed with extensive cellulitis due to toxigenic *Corynebacterium ulcerans* died from multiple organ failure. Environmental investigation also isolated *C. ulcerans* in biological samples from two stray cats in contact with the case. This finding provides further evidence that pets can carry toxigenic *C. ulcerans* and may be a source of the infection in humans.

In March 2014, the French Institute for Public Health Surveillance (Institut de Veille Sanitaire, InVS) was informed that a toxigenic *Corynebacterium ulcerans* had been isolated from soft tissue samples of a patient in their eighties with extensive cellulitis in their hand and arm. The patient had received a diphtheria vaccination booster in October 2003. It is not known whether this patient received at least three doses of a combined diphtheria, tetanus and polio (DTPolio) vaccine in childhood. After the onset of symptoms, the patient attended a hospital emergency department because of sepsis (hyperthermia and inflammation) and cellulitis.

C. ulcerans was not isolated from the surgical subcutaneous swab of the patient's right hand taken at admission on Day 0. Three blood cultures, performed on Day 0 in Bact/Alert bottles (BioMérieux) were also negative after five days incubation at 35°C. Both aerobic and anaerobic cultures were performed. In addition, three soft tissue samples from the patient's right hand, taken during surgery on Day 2, were cultured on sheep blood agar and chocolate agar. All were positive for *C. ulcerans*, identified using MALDI-TOF [1]. No other bacteria except *C. ulcerans* (present in pure culture) were isolated from the three soft tissue samples.

Intravenous antibiotic treatment was initiated with amoxicillin and clavulanic acid on Day 0 and

complemented on Day 1 with gentamicin. The patient was admitted into the intensive care unit as they presented signs of systemic infection with multiple organ failure on Day 3 (thrombocytopenia, renal failure, and arrhythmia). Antibiotic treatment was changed to clindamycin, piperacillin and tazobactam. Ventricular arrhythmia and cardiac failure occurred. The patient died on Day 6.

Microbiological investigation

One culture from each of the three soft tissue samples was sent to the National Reference Centre (NRC) and the identification of *C. ulcerans* was confirmed by a multiplex PCR [2]. The NRC detected the presence of the *tox* gene by end-point PCR [3] and the production of diphtheria toxin by the isolate using the modified Elek test [4]. The isolate was sensitive to a large spectrum of antibiotics (among others: penicillin, amoxicillin, gentamicin, erythromycin, clindamycin, azithromycin, cotrimoxazole, ciprofloxacin) but not fosfomycine. Multilocus sequence typing (MLST) was performed using the MLST methodology used for *C. diphtheria* [5].

Veterinary investigation

A follow-up investigation was conducted by the local health authorities. Two delivery drivers were identified who had been in close vicinity to the patient, but they were not considered as close enough contacts to be sampled. The patient had two pet cats and was taking care of three stray cats. At the end of March, all five cats were taken away by the veterinary services. Throat and ocular samples were taken from each animal. In addition, conjunctival swabs were systematically taken, even if the cats were asymptomatic. One of the stray cats had a wound on its neck which was also sampled.

The samples were sent to the NRC for culture. *C. ulcerans* carrying the *tox* gene was isolated from the ocular sample of the stray cat with the wound and from the throat sample of another stray cat. The isolates were characterised using the same methods used for the human isolate. The modified Elek test was positive for both isolates. The samples of the third stray cat and the two pet cats tested negative for *C. ulcerans*.

After the patient's death, the cats were taken to an animal shelter. The Direction for the protection of populations of Yvelines decided to start antibiotics treatment of the infected cats. They were treated with amoxicillin for 10 days and a post-treatment sampling control was performed. These cultures showed the persistence of a *C. ulcerans* bearing the *tox* gene in the pharynx of one infected cat despite antibiotic treatment. The other post-treatment cultures were negative, including those for the cat that previously had *C. ulcerans* isolated from an ocular sample.

Discussion

From 2002 to 2013, 28 autochthonous cases of diphtheria due to toxigenic *C. ulcerans* were reported in mainland France [6]. The affected patients were mostly women (18/28) over 60 years of age with comorbidity [6]. The vaccination status was known for only six cases, and only two had received a diphtheria booster in the 20 years before the event. In veterinary investigations performed on pets owned by 14 cases only two dogs tested positive for toxigenic *C. ulcerans* (*tox+*), one of them carrying an identical ribotype as the *C. ulcerans* isolated from the owner of one of these dogs [7].

For the present case, seven housekeeping genes were compared by MLST, and all alleles from the human and animal isolates were found to be identical and belonged to sequence type ST325. This number is deduced from the *C. diphtheriae* database (<http://pubmlst.org/cdiphtheriae/>) and only provisional because there is presently no MLST scheme for *C. ulcerans*.

Nevertheless, this result strongly suggests that transmission of *C. ulcerans tox+* occurred from a stray cat. Few studies have described toxigenic *C. ulcerans* in domestic cats [8-10]. Transmission from animal to human or from a common unknown source of infection cannot be formally ruled out as several recent studies have mentioned *C. ulcerans* carriage in different mammalian species [11,12].

Conclusion

The clinical course of events (sepsis and multiple organ failure) and the possible zoonotic transmission suggest that the infection by *C. ulcerans* probably led to the death of the patient. The discovery of the bacteria in the stray cats reinforces the need to strengthen the links between animal and human health research, to better characterise the circulation of the bacteria in animals. Despite national recommendations on the use of

diphtheria antitoxin and vaccination boosters, severe and lethal infections due to *C. ulcerans tox+* have been observed in France among elderly people who were in contact with cats and dogs [13].

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Conflict of interest

None declared.

Authors' contributions

All the authors contributed to the acquisition of data, analysis or interpretation; drafting the paper (or revising it critically) and approve the final version.

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Emergence of enterovirus 71 C4a in Denmark, 2009 to 2013

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Enterovirus (EV) 71 has emerged as a primary cause of severe neurologic enterovirus infection in the aftermath of the global polio eradication effort. Eleven subgenotypes of EV71 exist, the C4 subgenotype being associated with large outbreaks in Asia with high mortality rates. This subgenotype has rarely been reported in Europe. In the period between 1 January 2009 and 31 December 2013 a total of 1,447 EV positive samples from 1,143 individuals were sent to the Statens Serum Institute (SSI), and 938 samples from 913 patients were genotyped at the Danish National World Health Organization Reference laboratory for Poliovirus at SSI. Echovirus 6 (Eo6) (n=141 patients), echovirus 30 (E30) (n=114), coxsackievirus A6 (CAo6) (n=96) and EV71 (n=63) were the most prevalent genotypes. We observed a shift in circulating EV71 subgenotypes during the study period, with subgenotype C4 dominating in 2012. A total of 34 EV71 patients were found to be infected with strains of the C4 subgenotype, and phylogenetic analysis revealed that they belonged to the C4a lineage. In our study, the proportions of cases with cerebral and/or sepsis-like symptoms were similar in those affected by C4a (19/34) and those with C1 and C2 (15/35). The majority (n=30) of the 34 EV71 C4 cases were children ≤5 years of age, and males (n=22) were over-represented. Continued EV surveillance is required to monitor the spread of EV71 C4 in Denmark and the rest of Europe.

Introduction

Human enteroviruses (EV) are small, single-stranded RNA-viruses from the Enterovirus genus of the Picornaviridae family. They can cause a range of clinical manifestations from mild mucocutaneous and/or gastrointestinal symptoms, to visceral and severe neurologic diseases with involvement of central nervous system (CNS). Polioviruses used to be the most important EV due to widespread outbreaks of paralytic disease. A rather successful global effort to eradicate polio has now made EV71 the primary cause of severe neurotropic EV-associated infectious diseases [1]. EV71 variants have been classified into three genogroups (GgA, GgB, and GgC), and the latter two are

further subdivided into subgenotypes B1 to B5, and C1 to C5. Currently genogroups B and C are co-circulating worldwide. Subgenotype C1 is predominating in Europe, but it can also be found in Australia, Malaysia and Singapore. The C4 subgenotype has predominantly been identified in large outbreaks of hand, foot and mouth disease (HFMD) in Asia, and in particular mainland China, where severe cases and a rather high mortality rate have been reported [2-4]. In 2004 the C4 subgenotype was detected for the first time in Europe, and has to date only been reported in a total of nine cases in Austria, Croatia, and Hungary, respectively [2-4]. In February 2012, the first EV71 C4 case was detected in Denmark in a Serbian infant admitted to the paediatric ward at Hospital A with fever and CNS symptoms. In the following months more EV71 C4 cases were detected in the same geographical area as the hospital. The Virology Surveillance and Research Section (VOF) at the Department of Microbiological Diagnostics and Virology, Statens Serum Institut (SSI) serves as the National World Health Organization (WHO) Reference Laboratory for Poliovirus in Denmark. The Danish EV surveillance is implemented to monitor poliomyelitis as part of the poliomyelitis elimination efforts in Denmark. We took advantage of the well-functioning EV-surveillance system to characterise the emergence of EV71 C4 strains in Denmark.

Methods

Enterovirus surveillance system

The national EV surveillance system in Denmark is conducted in a joint effort by the National WHO Poliovirus Reference Laboratory at VOF, SSI and the Infectious Disease Epidemiology Department (IDED) at SSI. The system is voluntary and all types of EV positive sample material may be submitted for characterisation. However, as part of the global poliovirus elimination programme, in case a patient is diagnosed with EV in the cerebrospinal fluid (CSF), it is highly requested that CSF and stool are forwarded to the Danish WHO Reference Laboratory for Poliovirus for virus characterisation including culture and viral protein (VP)1/

VP2 sequence-based typing. Samples for diagnostic testing may also be sent directly to SSI from general practitioners and hospitals. Once weekly, VOF, SSI reports the new EV positive cases to the WHO Regional Office for Europe (EURO) in Copenhagen, as well as to the IDED. To ensure that relevant clinical information is archived in the national EV surveillance database, the IDED sends a letter with a standardised questionnaire regarding information on the clinical symptoms (including information on acute flaccid paralysis) and a reminder to send stool for virus characterisation directly to the doctors/departments in charge of the EV-positive patients. Completed questionnaires are returned and data entered in the database by the IDED, SSI. To ensure completeness of clinical data information for this paper, we have contacted relevant hospital departments and asked for relevant information on all EV71 cases where clinical information was missing.

Enterovirus characterisation

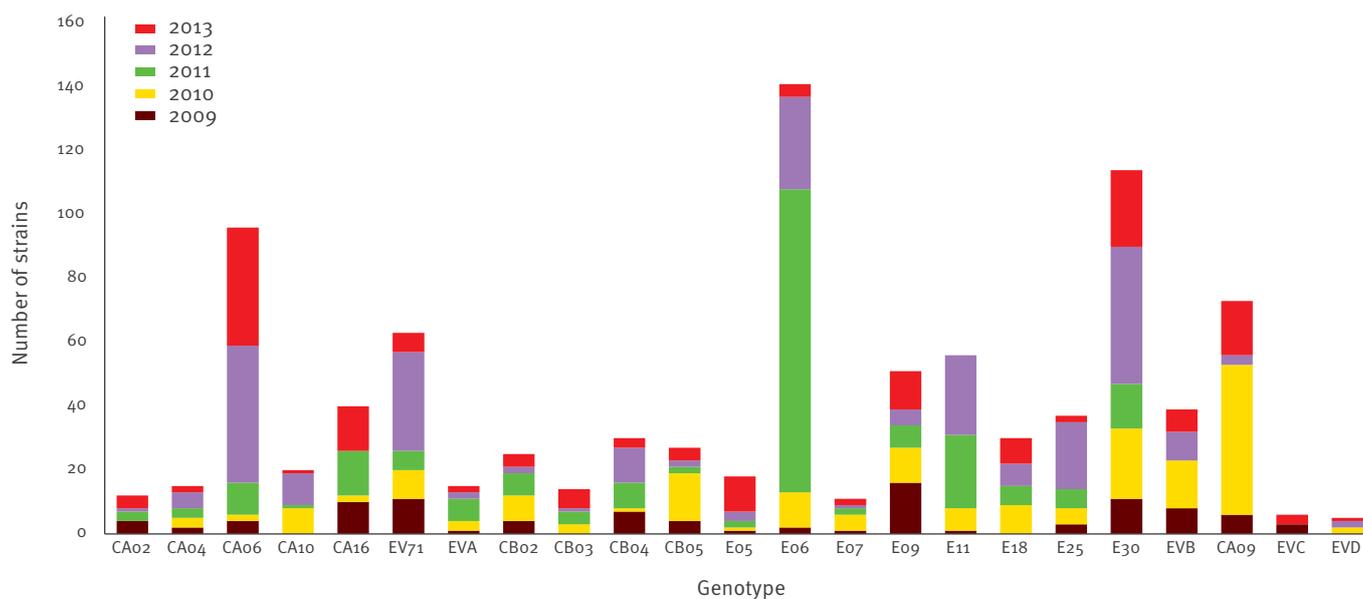
Isolates from all severe (i.e. with meningitis, encephalitis and sepsis-like illness) EV positive cases are routinely typed centrally at the VOF at SSI by sequencing part of the VP2 gene from the polymerase chain reaction (PCR) product obtained directly from the diagnostic sample, as VP2 sequencing has been demonstrated to be more sensitive than VP1 sequencing [5]. VP1 sequencing is performed in cases where VP2 typing is unsuccessful, or for specific typing analyses. Non-typable virus isolates are cultivated in two cell-lines

according to WHO guidelines [6] and then characterised by VP1 and VP2 sequencing. For this study, all samples that were positive for EV71 in diagnostic PCR were tested by VP1/VP2 sequencing. VP1 typing and sequencing was applied to comply with international EV characterisation standards. The sample materials for EV71 C4 positive patients are further described (results section).

RNA was extracted from 200 µl of CSF using the QiaCube with the Qia AMP DNA Blood Mini Kit (QIAGEN Nordic, Copenhagen, Denmark), or from 200 µl of other sample material (such as faeces, swabs, biopsies) using the MagNa pure LC robot with the total nucleic acids kit (Roche Diagnostics A/S, Hvidovre, Denmark). Diagnostic PCR for EV was conducted as described previously [7]. 5 µl of the extraction was used as template for PCR amplifying part of the VP1 and VP2 gene, respectively in semi-nested PCR [5]. cDNA synthesis and first round PCR was carried out using a OneStep reverse transcription (RT)-PCR kit (QIAGEN Nordic, Copenhagen, Denmark), and second round amplification was carried out, producing a VP1 amplicon of 350 to 400 basepairs and a VP2 PCR amplicon of 368 basepairs.

Prior to sequencing, PCR products were purified using exo-SAP IT (GE Healthcare, Buckinghamshire, UK). Purified PCR products were sequenced in both directions. Phylogenetic analysis based on the VP1

FIGURE 1
Distribution of enterovirus genotypes, Denmark, 2009–2013 (n=938)^a

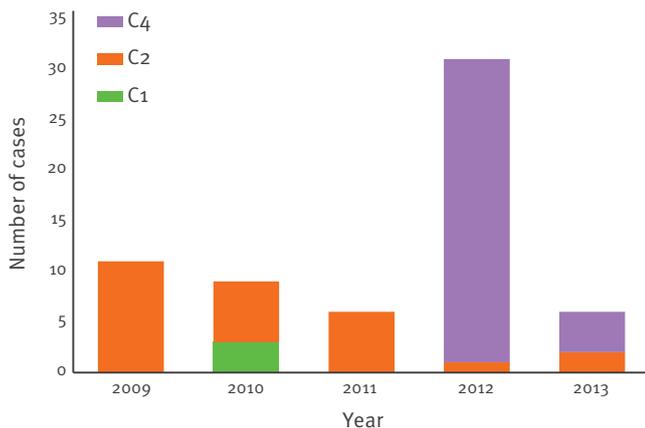


Genotypes for which 10 or more viruses were detected are shown. Genotypes for which fewer than 10 viruses were detected are grouped together according to which enterovirus (EV) species they belong to, i.e. EVA, EVB, EVC, and EVD. The viruses which are grouped according to species are shown immediately after the last genotype of that species in the chart.

^a A total of 938 strains were characterised from 913 individuals. Of the 938 strains, 25 represent either co-infections with two different genotypes, or a subsequent infection with a different genotype.

FIGURE 2

Distribution of enterovirus 71 subgenotypes C1, C2, and C4, Denmark, 2009–2013 (n=63)



sequences was carried out by maximum likelihood and the Kimura 2-parameter model with discrete gamma distribution and invariable sites, and 1,000 bootstrap replications using the Molecular Evolutionary Genetics Analysis (MEGA)5 software package [8]. VP1 sequence data from 23 of the 34 EV71 subgenotype C4 samples were of sufficient quality and length to be included in the phylogenetic analysis (sequence length 178–305 nucleotides). All sequences from this study have been submitted to GenBank. Two subgenotype C1 strains and 11 subgenotype C2 strains were also included, as were all publically available (GenBank) European EV71 C4 sequences. The analysis was supplemented with reference sequences obtained from GenBank representing EV71 subgenotypes A, B1 to B5, and C1 to C5 (including C4a and C4b).

Results

In the period from 1 January 2009 to 31 December, 2013 a total of 7,879 samples from 5,611 individuals were received at the Department of Microbiology Diagnostic and Virology at SSI for EV diagnostics. Of these, 984 (12%) samples from 779 (14%) different individuals were positive for EV. In the same period 544 EV positive samples from 427 individuals were received for our national surveillance, thus totalling 1,447 positive samples from 1,143 individuals. 938 EV samples from 913 individuals were successfully genotyped. In 25 individuals more than one EV genotype was detected. A total of 41 different genotypes were identified, the most prevalent being Eo6 (n=141/913, 15%), E30 (n=114/913, 12%), CAo6 (n=96/913, 11%), and EV71 (n=63/913, 7%) (Figure 1).

EV71 was detected throughout the entire period, however there was a marked shift in the subgenotype, from C1 (n=3) and C2 (n=23), being found mainly between 2009 and 2011 with additionally C2 in 2012 (n=1) and 2013 (n=2), to subgenotype C4 being found primarily in 2012 (n=30) but also in 2013 (n=4) (Figure 2). The 34 EV71 C4 infected individuals were unevenly distributed

with regard to sex, as 22 of the 34 cases were males. With regards to age, the majority of infected individuals were young children, with 30 of the 34 C4 cases \leq 5 years-old, and 16 $<$ 1 year-old.

With regard to the severity of symptoms, patients infected with the C4 subgenotype showed comparable symptoms to patients infected with subgenotypes C1 and/or C2 (Table 1 and 2). Nineteen of the 34 C4 patients had cerebral or sepsis-like symptoms. Additional symptoms among the EV71 C4 infected cases were gastroenteritis (n=7), and HFMD (n=4). EV71 C4 was detected primarily from stool samples (n=19/34, Table 1). Except for the single clustering of EV71 C4 cases in Funen during the months of July to December of 2012 (n=12), most single cases appear sporadically throughout the study period and from all five major geographical regions of Denmark (Table 1). Of the 12 clustered cases from Funen, 10 were admitted to the central hospital and one case was referred to this hospital from a nearby provincial hospital. The age range was 0 to 40 years (median: 2 years), with an uneven sex distribution of nine males, and three females.

The phylogenetic analysis revealed one major C4 lineage, containing all of the C4 strains reported in this study (Figure 3). These were determined to belong to the C4a lineage from Asia. Previously reported C4 strains from Europe belong to the C4b lineage [2-4].

Discussion

This study reports the finding of a new EV71 C4a subgenotype, detected in Denmark for the first time in the spring of 2012. As of December 2013 a further 33 EV71 C4 cases have been detected, the majority in infants with moderate to severe symptoms. EV71 C4 cases from Austria and Hungary were also found to be associated with severe symptoms such as meningitis and acute flaccid paralysis [2,3]. In Denmark, study material is based on cases referred for either diagnostic purposes, or submitted to the National WHO Polio Reference Laboratory at SSI, as part of the national EV surveillance. As a consequence, the detection of mild and/or asymptomatic cases of EV71 infection in the Danish population is not complete, and we can therefore not conclude that EV71 C4 is always associated with severe symptomatology. Only 6/63 EV71 cases were associated with HFMD. There is no specific surveillance for HFMD in Denmark, so the actual level of mild cases of EV71 in circulation may be underestimated.

The EV71 C4 strains identified in Denmark shared a surprisingly high sequence similarity with an EV71 C4a epidemic strain from China, 2008 (EU913466, Figure 3) [9-11]. So far, the relatively severe presentation, although with no fatalities, of 19 of the 34 EV71 C4 cases, with a temporal-spatial clustering of nine of the meningitis/encephalitis cases in Funen during the second half of 2012, suggests that the Danish emerging C4 strain has the same potential for a high transmission

TABLE 1

Characteristics of enterovirus 71 C4 infected patients, Denmark, 2009–2013 (n=34 patients)

Patient number	Age in Years	Sex	Date of sampling	Geographical region	Primary symptoms	Sample material
1	0	M	11-02-2012	Funen	Meningitis	Faecal swab
2	0	M	22-05-2012	Jutland	Meningitis	Stool
3	2	M	06-07-2012	Funen	Gastroenteritis	Swab
4	4	M	09-07-2012	Funen	Meningitis	Faecal swab
5	3	F	23-07-2012	Jutland	Encephalitis	Stool
6	0	F	01-08-2012	Jutland	Gastroenteritis	Stool
7	0	F	22-08-2012	Funen	Sepsis-like syndrome	Stool
8	40	M	30-08-2012	Funen	Meningitis	CSF
9	2	M	04-09-2012	Funen	Meningitis	Stool
10	0	F	11-09-2012	Zealand	Fever	Stool
11	1	M	13-09-2012	Zealand	Unknown	Fluid
12	6	M	27-09-2012	Jutland	HFMD	Swab
13	29	M	11-10-2012	Jutland	Respiratory symptoms	Swab
14	0	F	14-10-2012	Jutland	Gastroenteritis	Stool
15	0	M	17-10-2012	Funen	Encephalitis	Swab
16	3	M	26-10-2012	Funen	Meningitis	Swab
17	0	M	26-10-2012	Jutland	Meningitis	Stool
18	1	M	30-10-2012	Funen	Encephalitis	Swab
19	0	M	04-11-2012	Funen	Gastroenteritis	Stool
20	0	M	05-11-2012	Funen	Meningitis	Stool
21	0	M	07-11-2012	Zealand	Sepsis-like syndrome	Stool
22	0	F	08-11-2012	Zealand	Sepsis-like syndrome	Stool
23	0	M	09-11-2012	Funen	Meningitis	Stool
24	4	M	16-11-2012	Jutland	Meningitis	Stool
25	1	F	25-11-2012	Jutland	Fever	Stool
26	1	F	28-11-2012	Jutland	Gastroenteritis	Stool
27	33	F	06-12-2012	Zealand	HFMD	Vesicle fluid
28	2	M	07-12-2012	Zealand	HFMD	Swab
29	3	M	11-12-2012	Jutland	Gastroenteritis	Stool
30	4	F	18-12-2012	Funen	Meningitis	Swab
31	0	M	08-01-2013	Jutland	Gastroenteritis	Stool
32	2	F	16-07-2013	Jutland	Meningitis+HFMD	Faecal swab
33	0	M	22-08-2013	Zealand	Meningitis	CSF
34	0	F	03-10-2013	Jutland	Fever	Stool

CSF: cerebrospinal fluid; HFMD: hand foot and mouth disease; M: male; F: female.

rate and high pathogenicity as described previously for Asian EV71 C strains, including EV71 C4 [9-13].

Other EV71 C subgenotypes, namely C1 and C2, have previously been identified as occurring sporadically in Denmark throughout a four year study period (2005 to 2008), implying simultaneous circulation of these lineages without genetic selection of either strain based on VP2 sequences [14]. However, only one EV71 C2 case was identified during 2012 and two in 2013, suggesting that the introduction of C4 within the population of Denmark might have a suppressive impact on the circulation of other EV71 C subgenotypes. Furthermore,

the number of EV71 positive samples in 2012 (n=31) is in itself notable, as a total of only 29 EV71 cases were identified throughout the previous four-year study period [11]. It will be interesting to follow the emergence of C4, and see whether it will follow the typical trend of the other EV71 subgenotypes with only limited evolutionary change within its two lineages (C4a and C4b) over time, or whether this subgenotype will continue to dominate the future EV seasons and give rise to outbreaks of severe disease in Europe, as the C4s are known for in parts of Asia. The increasing number of EV71 C4 identified during the 2009 to 2013 surveillance period, and the initial clustering of 11 cases within one

TABLE 2

Main clinical presentation of enterovirus (EV)71 C4 and EV71 C1 and EV71 C2 patients, Denmark, 2009–2013 (n=63)

Symptom	C4 patients	C2+C1 patients
Meningitis	13	12
Gastroenteritis	7	7
HFMD	4	2
Encephalitis	3	2
Sepsis-like syndrome	3	1
Respiratory symptoms	1	1
Fever	3	6
Myoclonus	0	1
Unknown	1	1
Total number of patients	34 ^a	29 ^a

HFMD: hand foot and mouth disease.

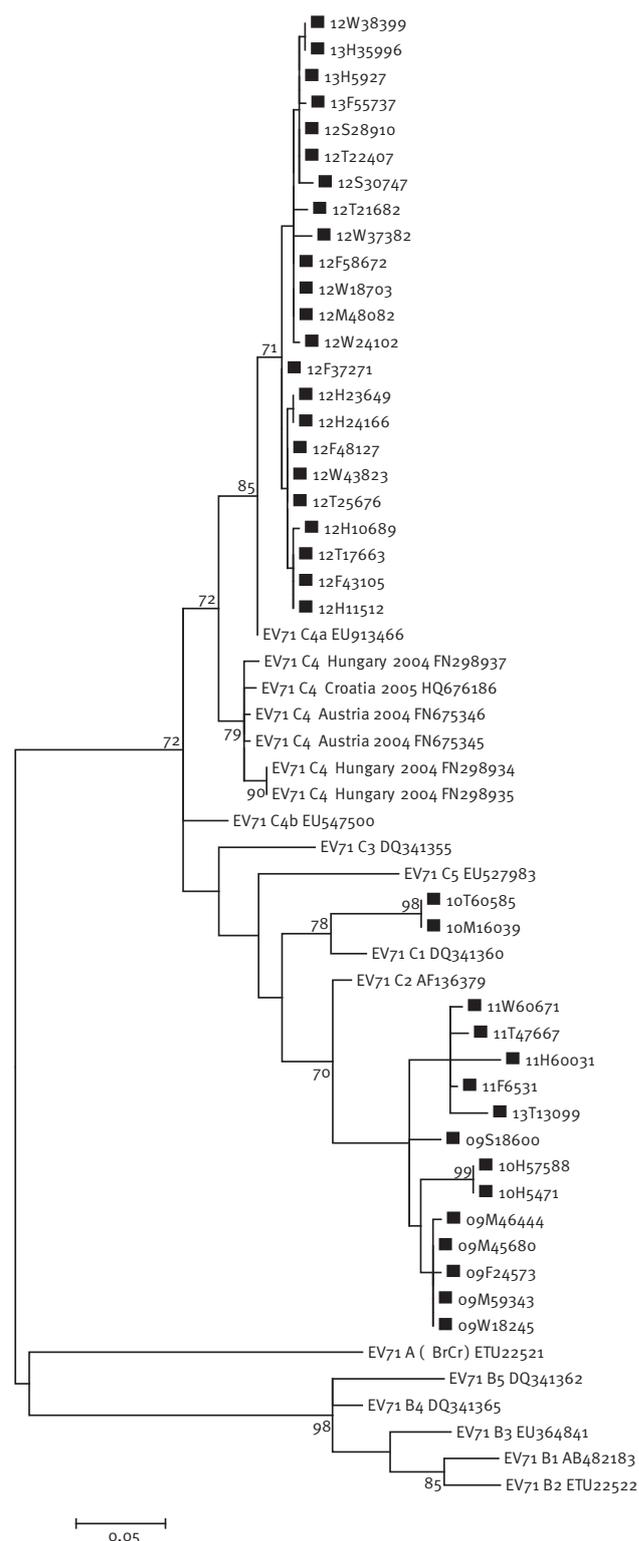
^a Some patients presented more than one symptom so the total numbers of patients are not equal to the sum of the numbers in the respective columns.

hospital during the second half of 2012, suggest that this subgenotype initially gave rise to a smaller localised outbreak and is potentially now establishing itself in this northern European population. In the recent ‘perspective’ from the Global Disease Detection Operations Center at the United States Centers for Disease Control and Prevention (CDC), EV71 was considered to be one of the top-five global infectious disease threats to watch, due to its propensity to cause large outbreaks and severe, life threatening, neurologic disease [15].

In conclusion, the circulation of EV71 subgenotype C4 in Denmark has been established. Based on observations using a wide range of different samples from patients with a broad range of EV symptoms, this subgenotype was found to coincide with severe disease, as were the other EV71 subgenotypes C1 and C2, detected in Denmark during the study period. EV surveillance of high quality and high sample volume is needed to closely monitor the continued emergence of EV71 C4 in the European population over the coming years to establish the pathogenicity and virulence of this subgenotype. A broader emergence of EV71 within Europe might potentially widen the focus of the current development of EV71 vaccines for targeted use in Asia, to a potential future benefit in Europe as well [16,17].

FIGURE 3

Phylogenetic analysis of viral protein 1 sequences from Danish enterovirus 71 strains, Denmark, 2009–2013



Danish enterovirus (EV)71 strains are represented with a black square. The sequence identifiers for the sequences obtained in this study are made up of a two figure prefix to denote the year of sample collection, followed by our internal sample number. Subgenotype C reference sequences were downloaded from GenBank (EV71 C1 accession number: DQ341360; EV71 C2: AF136379; EV71 C3: DQ341355; EV71 C4a: EU913466; EV71 C4b: EU547500; EV71 C5: EU527983). EV71 subgenotypes A (EV71A: ETU22521) and B (B1: AB482183; B2: ETU22522; B3: EU364841; B4: DQ341365; B5: DQ341362) were included to root the tree. Previously identified European C4 strains were also included in the analysis. The scale bar represents the number of nucleotide substitutions per site.

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Conflict of interest

None declared.

Authors' contributions

TKF: conceptualised the study, drafted the manuscript and headed the investigation; AYN: participated in the molecular and phylogenetic analyses, and has participated in the writing of the manuscript; TVS: collected clinical information on the most important samples; PHA: participated in the national enterovirus surveillance and in the registration of clinical symptoms; BA: conducted the laboratory characterisation of EV; SOI: headed the laboratory characterisation of EV and the phylogenetic analyses, and has participated in the writing of the manuscript.

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Pregnancy-related listeriosis in France, 1984 to 2011, with a focus on 606 cases from 1999 to 2011

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This study describes trends in the incidence of pregnancy-related listeriosis in France between 1984 and 2011, and presents the major characteristics of 606 cases reported between 1999 and 2011 to the French Institute for Public Health Surveillance through the mandatory notification system. The incidence of pregnancy-related listeriosis decreased by a factor of 12 from 1984 to 2011. This reduction was a result of progressive implementation of specific *Listeria monocytogenes* control measures in food production. A lower incidence of pregnancy-related listeriosis was observed in regions with a lower prevalence of toxoplasmosis. Given that dietary recommendations in pregnancy target both toxoplasmosis and listeriosis prevention, we suppose that recommendations may have been delivered and followed more frequently in these regions. Cases reported between 1999 and 2011 (n=606) were classified as maternal infections with ongoing pregnancy (n=89, 15%), fetal loss (n=166, 27%), or live-born neonatal listeriosis (n=351, 58%). The majority of live-born neonatal listeriosis cases (n=216, 64%) were preterm births (22–36 weeks of gestation), of whom 14% (n=30) were extremely preterm births (22–27 weeks of gestation). Eighty per cent of mothers reported having eaten high risk food during pregnancy. A better awareness of dietary recommendations in pregnant women is therefore necessary.

Introduction

Invasive listeriosis is a rare but severe infection caused by *Listeria monocytogenes*, a bacterium capable of growing at low temperatures but destroyed by heat. Human listeriosis is mainly transmitted by food [1,2] and generally affects immunocompromised individuals, pregnant women and newborns [3]. The symptoms of listeriosis in pregnant women are non-specific and often include an influenza-like syndrome. The main risk associated with listeriosis during pregnancy is haematogenous transmission to the fetus through the placenta. Listeriosis can develop at any time during pregnancy [4] and can result in fetal loss, preterm

birth and/or neonatal infection [5]. Transmission to the fetus can also occur through ingestion of amniotic fluid [6]. Nosocomial transmission is occasionally reported in maternity units [7,8]. The proportion of pregnancy-related listeriosis decreased strongly between the 1980s and 1997, from nearly 50% to less than 25% of all invasive listeriosis cases [9]. The objectives of this study were to describe trends in the incidence of pregnancy-related listeriosis in France between 1984 and 2011, and the major characteristics of the 606 cases recorded between 1999 and 2011.

Methods

Data sources

The main indicator used to describe the annual incidence of pregnancy-related listeriosis is the rate of number of cases per number of live births in the French population. The number of live births is recorded every year by the French Statistical Office (Institut National de la Statistique et des Etudes Economiques; INSEE) [10]. The number of cases was retrieved from different sources for the following periods:

- 1984–1991: studies by the French National Health Laboratory [9,11].
- 1992–1998: National Listeria Reference Centre (NLRC) database. NLRC started ascertaining cases nationwide in 1992 because of a listeriosis outbreak [12]. The number of cases related to that outbreak has not been considered for the calculation of the annual incidence rate. In 1997, the sensitivity estimation of case ascertainment was 76% [9].
- 1999–2011: mandatory notifications. Listeriosis has been listed for mandatory notification in France since 1999. Accordingly, each diagnosed case must be declared by clinicians and laboratories to the regional health agency. Sensitivity was estimated at 87% in 2001 [13] and at 92% in 2006 (data not shown), using the capture–recapture method. The mandatory notification includes demographic, clinical, and laboratory

information [14]. Moreover, upon diagnosis, mothers are asked to complete a standard food questionnaire on their eating habits in the past two months. After validation of the content, the mandatory notifications and the food questionnaires are sent by the regional health agencies to the French Institute for Public Health Surveillance (InVS).

The annual incidence rate was estimated from 1984 to 2011, considering the sensitivity of each data source.

Case definition

In France, the diagnosis of listeriosis is made when *L. monocytogenes* is isolated from a normally sterile site in a patient presenting symptoms clinically compatible with listeriosis. A case is considered pregnancy-related when it involves a pregnant woman, a miscarriage, a stillbirth, or a newborn less than 28 days-old. When *L. monocytogenes* is isolated from both the pregnant woman and her newborn child, this is counted as a single case. Gestational age is given by the number of weeks of amenorrhoea. According to the information on the mandatory notification form, we categorised each case as ongoing pregnancy (diagnosis of invasive listeriosis in a pregnant woman with no concomitant delivery), fetal loss (miscarriage if gestational age is less than 22 weeks of gestation (WG), stillbirth if

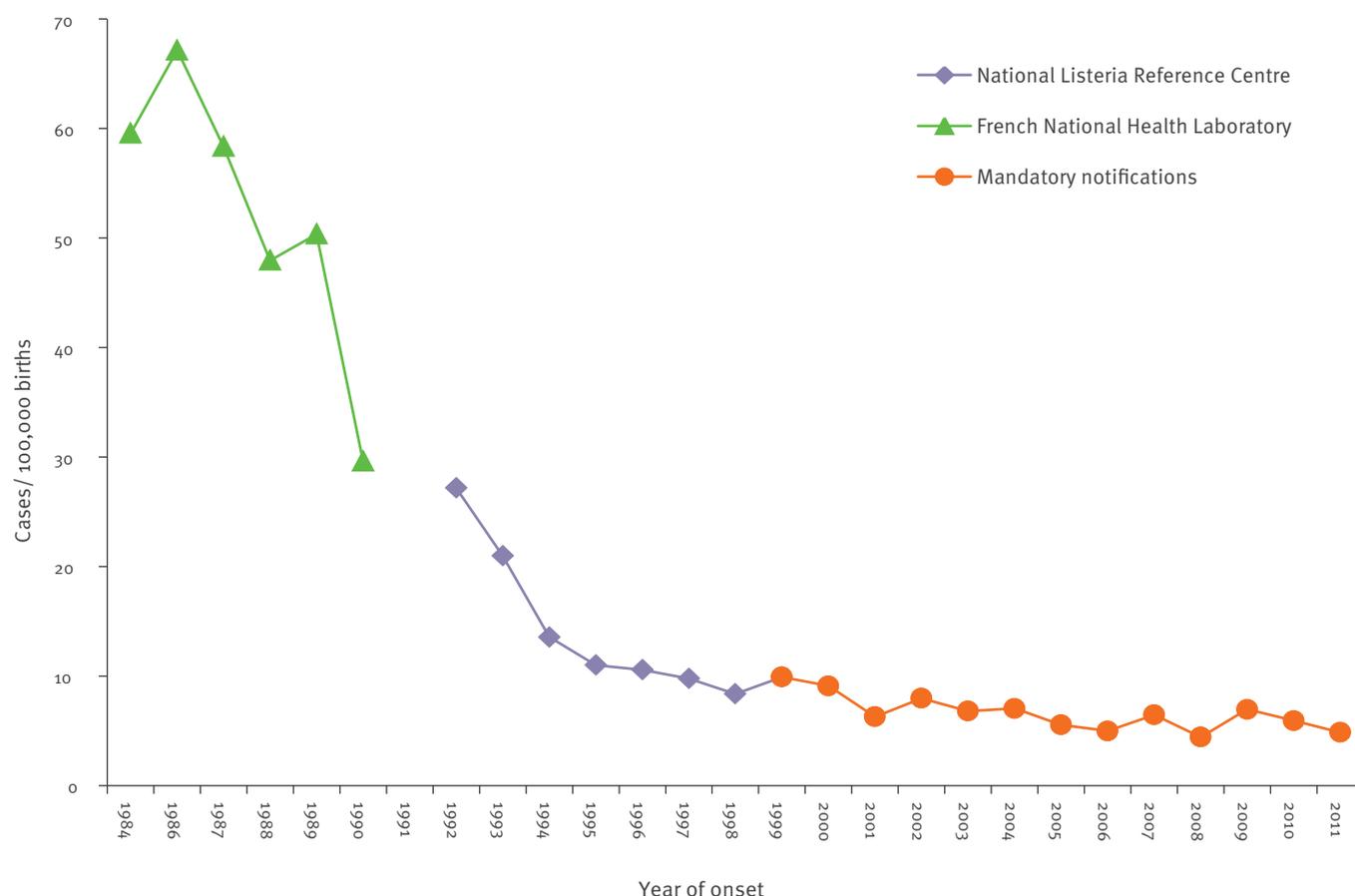
it is at least 22 WG), or live-born neonatal listeriosis (*L. monocytogenes* infection diagnosed in a newborn before 28 days of age). Live-born neonatal listeriosis was subclassified as ‘early neonatal cases’ (diagnosed between birth and day 6) or ‘late neonatal cases’ (diagnosed between day 7 and day 28). Early neonatal cases were classified as confirmed cases (*L. monocytogenes* isolated in the neonate’s cerebrospinal fluid (CSF) or the neonate’s blood), probable cases (*L. monocytogenes* isolated from placenta, the mother’s blood, or the neonate’s gastric aspirate), or possible cases (positive swab(s) from the neonate’s surface sites). Finally, neonatal listeriosis was defined as an infection of the newborn [≥ 22 WG independent of its vital status, i.e. stillbirths and live-born neonatal listeriosis].

Confirmation and characterisation of *L. monocytogenes* isolates by NLRC

Listeria isolates from pregnancy-related listeriosis referred to NLRC were confirmed with API Listeria (API, Appareil et Procédé d’Identification, bioMérieux, Marcy l’Etoile, France) [15] and serotyped by the slide agglutination method until January 2005 [16] and by multiplex PCR [17] starting February 2005. According to our experience, the PCR groups correspond fully to the four major serovars that cause human disease. Ongoing subtyping was conducted by

FIGURE 1

Incidence of pregnancy-related listeriosis, France, 1984–2011



DNA macrorestriction profiles analysis (pulsed-field gel electrophoresis; PFGE) according to standard protocols [18]. Isolates with indistinguishable *Apal* and *Ascl* DNA macrorestriction profiles, first based on visual comparison of banding patterns (since 2006 using BioNumerics 6.6 software; Applied Maths Saint-Martens-Latem, Belgium), were considered to be the same pulsovar. Susceptibility to a panel of 23 antibiotics was determined for each strain by disk diffusion according to guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) [19–21].

Statistical analyses

Results are expressed as numbers and percentages for categorical variables and as mean±standard deviation (SD) or median (range) for continuous variables as appropriate. Associations between categorical variables were assessed using the chi-squared test or Fischer's exact test as appropriate. Associations between continuous and categorical variables were assessed using Student's t-test or Kruskal–Wallis test as appropriate. Correlation between continuous variables was assessed using Pearson's or Spearman's correlation coefficients as appropriate. Statistical analysis was performed using Stata11.

Results

Incidence of pregnancy-related listeriosis

The annual incidence rate of pregnancy-related listeriosis per 100,000 live births fell from 60 (n=453) to 5 (n=35) cases per 100,000 live births between 1984 and 2011, a decline by a factor of 12. It decreased markedly between 1986 and 1996, gradually from 1996 to 2006 and was then stable until 2011 (Figure 1). From 1999 to 2011, the overall incidence rate of pregnancy-related listeriosis was 6.1 per 100,000 live births and varied according to the region from 2.2 to 13.6. It was highest in the Paris region and in the south-west of France (Figure 2). The incidence rate of pregnancy-related listeriosis was independent of the incidence rate of non-pregnancy-related listeriosis in all the regions of France ($r=0.16$, $p=0.07$). Pregnancy-related listeriosis was more frequent from July to September than during the rest of the year (mean: 5.0 ± 2.6 vs 3.5 ± 2.0 cases per month, $p<0.001$). Seasonal incidence of pregnancy-related listeriosis was not parallel to the incidence observed for non-pregnancy-related listeriosis which was higher from May to July than during the rest of the year (mean: 21.7 ± 1.2 vs 16.3 ± 2.7 cases, $p=0.008$) (Figure 3).

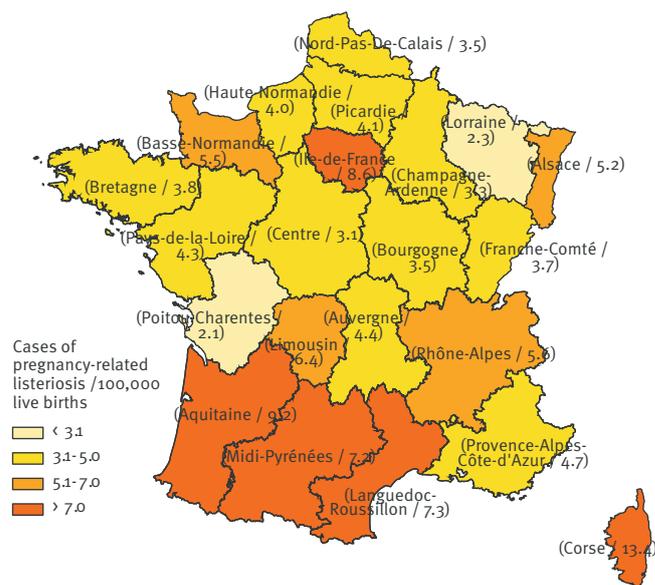
Cases from 1999 to 2011

We focused our study on the period 1999 to 2011, after the introduction of mandatory notification of listeriosis in France. A total of 3,413 cases of listeriosis were recorded from 1999 to 2011, of which 606 (18%) were considered pregnancy-related (Table 1). The mean age of the mothers was 29.5 ± 6.1 years. There was

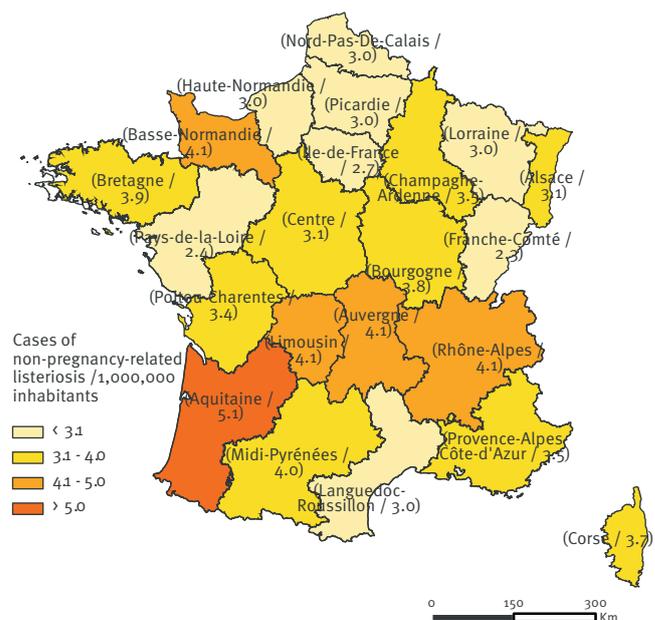
FIGURE 2

Average annual incidence rates of listeriosis, by Région, France, 1999–2011 (n=606)

A. Pregnancy-related listeriosis



B. Non-pregnancy-related listeriosis



Software used to draw the map: IGN-GéoFLA, using administrative boundaries from 1999.

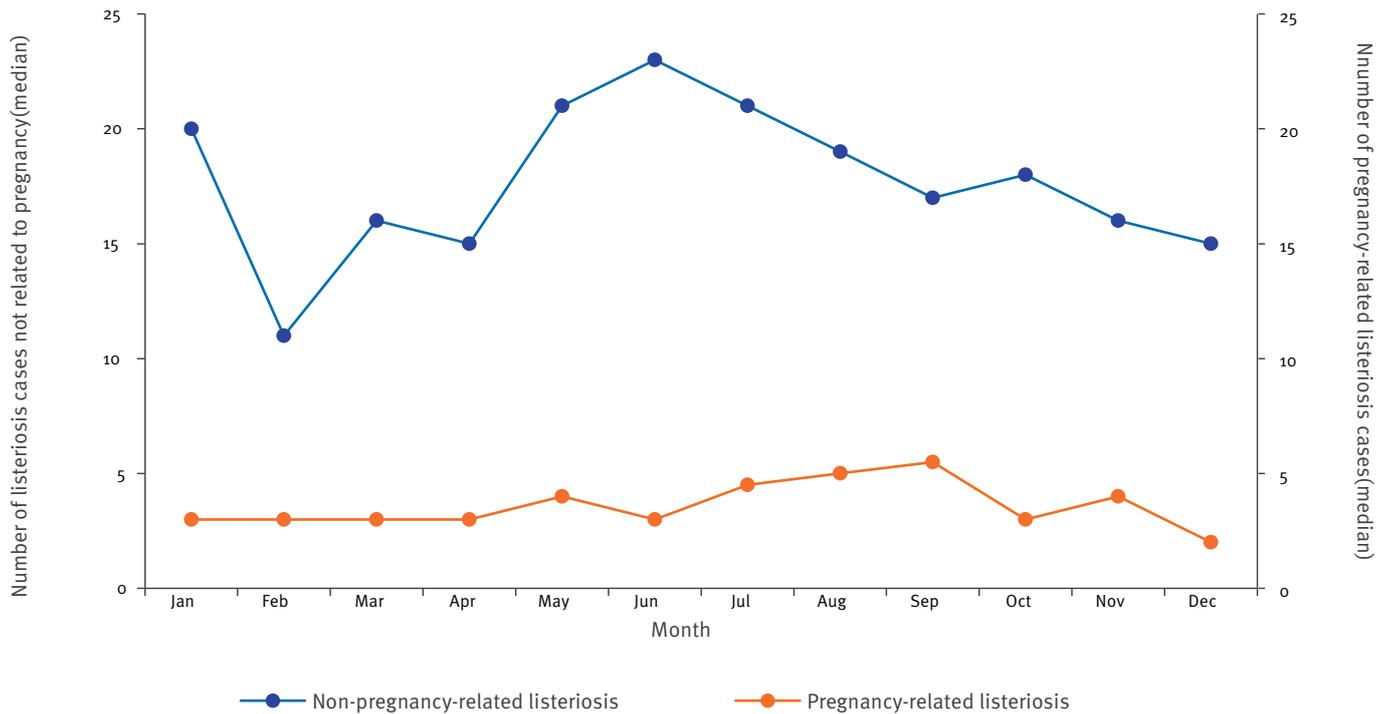
Data source: InVS, October 1013.

no significant difference to the mean age of mothers in the general population who gave birth in France in 2010 (mean: 29.7 ± 5.3) [22]. There were three twin pregnancies which resulted in six live-born neonatal listeriosis cases.

Among the 603 mothers with pregnancy-related listeriosis notified from 1999 to 2011, 15 (3%) were immunocompromised (eight human immunodeficiency virus (HIV)-positive including one with acquired

FIGURE 3

Median number of listeriosis cases per month, France, 1999–2011 (n=606)



immunodeficiency syndrome (AIDS), two with rheumatoid polyarthritis, two with haemorrhagic rectocolitis, two under immunosuppressive therapy but with unknown comorbidity, and one with chronic lymphocytic leukaemia). All mothers survived. Gestational age at diagnosis was recorded for 585 cases (Table 2). The median gestation period at diagnosis was 32 weeks (range: 5–41 weeks). Maternal infection was confirmed by *L. monocytogenes* isolates in blood (n=272, 45%) and/or placenta (n=215, 35%) and/or CSF (n=3, 0.01%). Of the women with meningitis, one lost the fetus at 12 WG, the two other women gave birth to a live neonate.

Among the 603 mothers, 509 (84%) completed the food questionnaire (Table 1). During the two months before diagnosis, 405 (80%) mothers had eaten at least one high risk product not recommended during pregnancy, mainly pâté (51%), and smoked salmon (33%). In south-western France, where listeriosis incidence is highest, mothers more often reported the consumption of high-risk products than in other regions, in particular of a type of pâté called rillettes (26% vs 16%, $p<0.05$), and Pyrénées' cheeses (20% vs 5%, $p<0.001$). They also reported eating uncooked meat more frequently (40% vs 17%, $p<0.001$).

Ongoing pregnancy

Ongoing pregnancies were infrequent (n=89/603, 15%) and were diagnosed at (median) 30 WG (range: 7–39 WG). All cases were confirmed by *L. monocytogenes*-positive blood culture. All mothers were treated with antibiotics (information on the type of antibiotic were not available to the authors) and only one of the

newborns was infected with *L. monocytogenes*. Review of this case revealed that the mother had had fever at 28 WG. She was treated with cephalosporins that are ineffective against *L. monocytogenes* [23]. When the result of the blood culture was available, she had no more fever and treatment was not changed. Three weeks later, she delivered a newborn with invasive listeriosis (positive blood culture).

Fetal loss

Pregnancies resulted in 166 fetal losses (27%). There was a median of 13 (range: 9–21) fetal losses per year. Fetal losses occurred at a median of 21 WG (range: 5–37 WG) and 90% occurred before 28 WG (Table 2). There were 95 (57%) miscarriages and 71 (43%) stillbirths which occurred at a median of 18 WG (range: 5–21 WG) and 25 WG (range: 22–37 WG) respectively. Fetal loss decreased significantly with gestational age at diagnosis ($p<0.001$) (Table 2).

Live-born neonatal listeriosis

Live-born neonatal listeriosis accounted for 58% (n=351) of pregnancy-related listeriosis and occurred at a median gestation period of 35 weeks (range to 22–41 weeks) in the 337 cases for whom gestational age was known. A majority of live-born neonates (n=216, 64%) were preterm births (i.e. 22–36 WG), of whom 14% (n=30) were extremely preterm births (i.e. 22–27 WG) (Table 2).

Among the neonatal cases, 329 (94%) were early neonatal cases. Among them, the median gestation period at birth was 35 WG (range: 22–41 WG) with

TABLE 1

Characteristics of pregnancy-related listeriosis cases, France, 1999–2011 (mothers n=603, births n=606)

Characteristics	Population	Missing data
Age of mothers, in years: mean (\pm SD)	29.5 (\pm 6.1)	10
Type of pregnancy-related listeriosis: n (%)		
Foetal loss (miscarriage or stillbirth)	166 (27%)	0
Live-born neonatal listeriosis	351 (58%)	0
Maternal infection with ongoing pregnancy	89 (15%)	0
Gestational age: median (range)	32 (5–41)	21
Completed food questionnaire: n (%)	509 (84%)	0
At least one not recommended product consumed: n (%)	405 (80%)	0
Number of different types of not recommended food products consumed: median (range)	3 (1–14)	0
Type of not recommended products consumed: n (%)		
Pâté	256 (51%)	0
Dried sausage	208 (41%)	0
Smoked salmon	165 (33%)	0
Unpasteurised cheeses	101 (20%)	0

95% (n=314) diagnosed less than 48 hours after birth. There were 109 (33%) confirmed invasive cases and among them 14 (13%) cases had *L. monocytogenes* culture-positive CSF. Among the 195 probable cases, there were 132 (68%) cases with maternal infection (placenta or maternal blood culture-positive) and 63 (32%) cases with no evidence of maternal infection but *L. monocytogenes* isolated from the neonate's gastric aspirate. There were only 25 (8%) possible cases with *L. monocytogenes* isolated exclusively from the neonate's surface swabs. Twenty-six (8%) early neonatal cases died. The median duration of life before death was 1 day (range: 0–24 days). Neonatal case fatality fell with gestational age, from 33% in highly preterm births (<28 WG) to 2% in infants born at term (p=0.05).

Among the 18 cases of late neonatal listeriosis, the median gestation period was 39 WG (range: 35–41 WG). It was significantly longer than the gestation period of the early neonatal listeriosis cases (p<0.001). All of them had *L. monocytogenes* culture-positive CSF. Three clusters of nosocomial infection by possible cross-infection between pairs of neonates born at the same time in the same hospital were identified. In all three clusters, the first baby had an early onset listeriosis and the second baby presented, several days later, a late neonatal listeriosis. In each pair the *L. monocytogenes* strains belonged to the same PCR serogroup and exhibited indistinguishable PFGE patterns. None of the late neonatal cases died. Information on treatment was not available to the authors.

Microbiological analyses

L. monocytogenes strains were sent to NLRC for 589 cases of pregnancy-related listeriosis: PCR serogroup IVB was predominant (n=362; 61%), followed by IIB

(n=111; 19%), IIA (n=109; 19%), and IIC (n=7; 1%). In the population with non-pregnancy-related listeriosis, the distribution of PCR serogroups was IVB (n=1,487; 46%), followed by IIA (n=810; 25%), IIC (n=521; 16%) and IIB (n=443; 14%) which differed significantly from the distribution in the population with pregnancy-related listeriosis (p<0.001). There was no association between PCR serogroup and fetal loss (p=0.17) or between PCR serogroup and neonatal death (p=0.08). For neonates, the PCR serogroup distribution was similar in cases with *L. monocytogenes* culture-positive CSF and for cases that were not neuro-invasive (p=0.43). No resistance was observed to any clinically relevant antibiotics recommended for the treatment of listeriosis.

Discussion

In France, the incidence of pregnancy-related listeriosis decreased markedly from 1986 to 1996. A similar reduction in the incidence of listeriosis was observed in the United States (US) between 1989 and 1993 and coincided with the implementation of industrial, regulatory, and educational measures [24]. Previous analyses have suggested that a substantial part of the decrease in illness due to *L. monocytogenes* from 1986 to 1996 in France was related to control measures implemented at the food production level [9]. The first *Listeria* control measures, implemented in France in 1986, targeted manufacturers producing cheese for exportation to the US, since American authorities had imposed a 'zero *Listeria*' rule on imported cheeses. These control measures were subsequently extended to all cheese producers in France in 1988. In 1992, a large outbreak involving 279 cases throughout France, including 92 pregnancy-related cases, prompted the French Ministry of Health to issue recommendations to pregnant women to avoid certain foods. After this

TABLE 2

Pregnancy-related listeriosis cases by gestational age at diagnosis, France, 1999–2011 (n=585)

Gestational age at diagnosis	All cases		Maternal infection with ongoing pregnancy		Foetal loss (miscarriage + stillbirth)		Live-born neonatal listeriosis		
	n=585	%	n=87	%	n=161	%	n=337	%	Deaths n=26
<14 WG	23	4%	8	9%	15	9%	0	0	0
14–21 WG	92	16%	17	20%	75	47%	0	0	0
22–27 WG	98	17%	13	15%	55	34%	30	9%	10
28–31 WG	79	14%	9	10%	10	6%	60	18%	10
32–36 WG	163	28%	32	37%	5	3%	126	37%	4
≥37 WG	130	22%	8	9%	1	1%	121	36%	2

WG: weeks of gestation.

outbreak, control measures were extended to include all foods potentially contaminated with *L. monocytogenes*, and hygiene measures were strengthened throughout the food distribution chain. Between 1992 and 1996, the proportion of highly contaminated food-stuffs (≥ 100 colony-forming units/g) fell substantially. Between 1994 and 2000, additional measures were implemented, such as systematic withdrawal of contaminated foods from the market and distribution of information leaflets to pregnant women by their physicians. The incidence of pregnancy-related listeriosis in France continued to fall gradually from 1996 to 2006 and has been stable since 2006. The incidence of pregnancy-related listeriosis in England, which was, in 1985, 10 times lower than in France, also decreased substantially over the same period, from 45 cases in 1985 to 15 to 20 cases per year in the early 2000s [25].

Over the last two decades, the sensitivity of the surveillance system has increased. Capture–recapture studies estimate that 76% of laboratory-confirmed cases were ascertained in 1997 [9], before introduction of the mandatory notification in 1998. This proportion was estimated at 87% in 2001 [13] and at 92% in 2006 (data not shown). Consequently, the decrease in incidence observed since 1997 is slightly underestimated.

The geographical distribution was not the same for pregnancy-related and other cases. The higher incidence seen in the south-west of France, both for pregnancy-related and other cases, is puzzling. As listeriosis is transmitted by food, this higher incidence could be a consequence of specific eating habits in this region. The food questionnaire highlighted a higher consumption only for a few high-risk products in this region. However, the questionnaire focused on food products that are mostly available throughout the country like pasteurised milk cheeses. Thus, it is possible that certain high-risk products available only in the south-west and not listed in the questionnaire, contributed to this higher incidence. Another hypothesis could be that mothers in the south-west were less aware of dietary preventive measures than in the

rest of the country. Indeed, the proportion of women in this region consuming rillettes, a high-risk product specifically targeted by the dietary recommendations, was higher than elsewhere. In France, dietary recommendations in pregnancy target both listeriosis and toxoplasmosis prevention. Interestingly, toxoplasmosis seroprevalence in pregnant women is higher in south-western France than in other regions of the country [26], supporting the hypothesis that mothers in the south-west may be less aware of dietary preventive measures. Furthermore, as women not immunised against toxoplasmosis are screened each month during their pregnancy in order to detect a seroconversion, they have a regular opportunity to receive these recommendations. We hypothesise that toxoplasmosis-positive pregnant women are less likely to be informed about dietary prevention measures than toxoplasmosis-negative pregnant women. This hypothesis is supported by the correlation between the regional incidence of pregnancy-related listeriosis and the regional seroprevalence of toxoplasmosis in pregnant women ($r=0.53$, $p=0.01$) [26].

Overall, most mothers had consumed several types of foods not recommended during pregnancy. This highlights the need to improve health education of mothers during pregnancy, in particular in certain regions. Regarding the seasonality, there is a time lag between the seasonal peak in pregnancy-related listeriosis cases and the other forms of listeriosis. As has been recently established, the incubation period for pregnancy-related listeriosis (median of 28 days, ranging from 17 days to 67 days), is much longer than the incubation period for other clinical forms of listeriosis [27]. This may explain that, even if the peak in exposure occurs in the same season, the peak in diagnosis of pregnancy-related cases is some weeks later than other cases.

Pregnancy-related listeriosis mostly affects healthy women without additional predisposing conditions [28,29]. Indeed, in our study, only 3% of the mothers had additional predisposing conditions.

From 1999 to 2010, 18% of reported listeriosis cases occurred in pregnant women or neonates. This proportion was similar in other countries such as the US (16%, from 2004 to 2007) [30], Germany (15%, from 2001 to 2005) [31], and England (12%, from 2001 to 2008) [25]. In our study, 27% (n=166) of pregnancy-related cases resulted in fetal loss, compared to 20% in the US (from 2004 to 2007) [30] and 33% in Denmark (from 1994 to 2005) [5]. The proportion of cases with ongoing pregnancy in our study (15%) was similar to the proportion reported in Denmark (13%) [5].

The proportion of preterm births among listeriosis cases is extremely high: 70% of 408 neonatal listeriosis cases were preterm births, compared with 7% of the total 14,832 births in a survey carried out in France in 2010 (relative risk (RR): 9.5; 95% confidence interval (CI): 8.5–10.8) [22]. The discrepancy is even higher for severely premature births (<32 WG): 38% of neonatal listeriosis cases vs 2% of all births in France (RR: 25.7; 95% CI: 20.9–31.6), and extremely premature births (22–27 WG): 21% versus 0.7% of all births in France (RR: 31.2; 95% CI: 23.8–42.6) [22]. The prognosis for fetal survival among pregnancy-related listeriosis improves with an increasing gestation period at diagnosis, in particular after 28 WG. Indeed, 87% of fetal losses were diagnosed before 28 WG. Compared with the study by Humbert et al on 601 pregnancy-related listeriosis cases in France between 1970 and 1975, our study shows that the proportion of preterm birth among live-born neonatal cases has not changed (64% vs 63%) [32]. However, the case fatality ratio has fallen dramatically: 33% in the period from 1970 to 1975 versus 4% in the period 1999 to 2011 ($p < 0.001$) [32], probably due to the progress in neonatal care. The proportion of stillbirths in preterm infants (22–31 WG) with neonatal listeriosis is higher than in preterm infants of a general population cohort (Epipage study) (39% vs 25%, $p < 0.001$) [33]. In contrast, the case fatality ratio of live-born preterm neonates with listeriosis is similar to the case fatality ratio of the live-born preterm neonates of Epipage (22% vs 20%, $p = 0.22$).

The PCR serogroup distribution differed significantly among pregnancy-related listeriosis and non-pregnancy-related listeriosis. Serogroup IVB, which is the most common PCR serogroup in human listeriosis [5,14,34,35], was more frequent than in non-pregnancy-related listeriosis and serogroup IIB was less frequent.

This study was based on mandatory notifications made by physicians and microbiologists in the context of the French national surveillance programme on listeriosis and therefore has some limitations. As no information on clinical symptoms was available, we considered neonates with *L. monocytogenes* isolated exclusively from surface swabs as possible listeriosis cases although they may not actually have been infected. Moreover, we have no information on the long-term sequelae in the newborn. The MONALISA

study (Multicentric Observational National Analysis of LIsteriosis and ListeriA; <http://clinicaltrials.gov/show/NCT01520597>), a prospective study on listeriosis, will present detailed clinical, biological and microbiological data of all incident cases of listeriosis in France from the end of November 2009 until 2013, including pregnancy-related cases, and provide extensive information on the prognosis of newborns.

Conclusion

Pregnancy-related *L. monocytogenes* infection is a rare but severe infection in pregnancy. The proportion of fetal loss (27%) and, for neonatal listeriosis, the proportion of preterm birth (64%) is extremely high. Fortunately, there has been a marked decrease in incidence from 1984 to 2006 related to the implementation of specific *L. monocytogenes* control measures at the food production level. It is important to maintain these measures, which have proven their efficacy. The incidence of pregnancy-related listeriosis was lower in regions where the prevalence of toxoplasmosis was lower, and this may be related to differences in the distributed information about preventing toxoplasmosis and listeriosis. This suggests that promotion of dietary recommendations could contribute to the prevention of listeriosis in pregnancy. As 80% of mothers reported having eaten high-risk food during pregnancy, fetal loss (13 cases/year) could be reduced by improving awareness of pregnant women, in particular about dietary recommendations.

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Conflict of interest

None declared.

Authors' contributions

Delphine Girard analysed and interpreted data, drafted and revised the manuscript. Alexandre Leclercq performed typing of the *Lm* strains, and participated in writing the manuscript. Edith Laurent collected and analysed data. Marc Lecuit and Henriette De Valk interpreted data and revised the article for intellectual content. Véronique Goulet conceptualised and designed the study, designed the data collection instruments, interpreted data, participated in writing the article. All authors approved the final manuscript as submitted.

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Letter to the editor: leptospirosis versus hantavirus infections in the Netherlands and in Belgium, 2000 to 2014

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To the editor: We fully agree with statements of Goeijenbier et al., who conducted a study on leptospirosis and hantavirus in the Netherlands [1], that hantavirus-induced haemorrhagic fever with renal syndrome (HFRS) often mimics leptospirosis [2]. Moreover, we confirm their findings with a parallel study in Belgium (Table), where, according to frequent practice, we screened for both pathogens from the start in sera of patients who presented with such similar symptoms. This resulted in only 55/1,580 (3%) patients serologically confirmed as having leptospirosis, whereas almost double, or 106 (7%), appeared compatible with acute HFRS (hantavirus IgM positivity). This percentage is more than triple that found in the current Dutch study (about 2%) [1]. In the authors view, this discrepancy is mainly due to a different screening practice, common in Belgium, and explaining the 1,525/1,580 (97%) leptospirosis-negativity. Moreover, in the Dutch study, leptospirosis-positive cases have not been taken into account. Concomitant acute HFRS–leptospirosis co-infections have however previously been described, a finding now confirmed again in five Belgian cases (Table).

These dual acute infections are probably even more frequent in highly endemic tropical regions, as demonstrated very recently in Sri-Lanka (Sunil-Chandra, data not shown) by proving concomitant enzyme-linked immunosorbent assay (ELISA) IgM-positivity for both pathogens in seven of 31 patients, hospitalised for leptospirosis. This illustrates that seroconfirmation (even by the gold standard microscopic agglutination test, MAT) of leptospirosis does not automatically exclude concomitant HFRS, a globally underestimated but evidence-based fact, having far-reaching epidemiological and therapeutical implications. Indeed, so-called ‘therapeutical failures’ with antibiotics in

TABLE

Serological results in a cohort of patients suspected for leptospirosis, or hantavirus infection, Belgium, 2000–2014 (n=1,580 patients)

Number of patients (%)	Leptospirosis ^a	Hantavirus IgG ^b	Hantavirus IgM ^b
1,390 (88)	Negative	Negative	Negative
78 (5)	Negative	Positive	Positive ^c
34 (2)	Negative	Positive	Negative
23 (1)	Negative	Negative	Positive ^c
50 (3)	Positive	Negative	Negative
3 (1)	Positive	Negative	Positive ^c
2 (1)	Positive	Positive	Positive ^c

Grand Total: 1,580.

^a Leptospirosis serology was performed by microscopic agglutination test (MAT) with a threshold dilution of 1/50 using eight to 10 strains belonging to between five and nine distinct serogroups. Leptospiral IgM presence was assessed by immunochromatographic assay (Core diagnostics, Birmingham, United Kingdom).

^b For hantavirus serology, the Institute of Tropical Medicine, Antwerp, Belgium used IgG and IgM immunofluorescence assay (IFA) from 2000 to 2007, followed by various commercial diagnostic enzyme-linked immunosorbent assays (ELISA), mostly and mainly based on both Korean prototype Hantaan virus (HTNV) and European Puumala virus (PUUV). The National Reference Centre for Hantaviruses, University of Leuven, Belgium, used, as a routine first screening step in hantavirus serology, HTNV and PUUV IgG and IgM ELISA (Progen, Heidelberg, Germany) (results in the current Table).

^c Results of eventually ensuing individual confirmation tests such as immunoblot, reverse transcription-polymerase chain reaction (RT-PCR), or focus reduction neutralisation tests (FRNT) were not considered for this Table. Consequently, IFA or ELISA results based only on positive IgM should be interpreted as very frequently, but not always, synonymous with acute hantavirus-induced haemorrhagic fever with renal syndrome.

leptospirosis, might partly be due to missed concomitant HFRS worldwide.

The persistently lower numbers of registered HFRS cases in the Netherlands, compared to neighbouring countries (Belgium and Germany) [1], are not so much due to lower medical awareness, but to an absent or dampened effect of so-called ‘mast years’, cyclic two to three yearly abundant autumnal production of beechnuts, leading to local HFRS peaks [3]. The Netherlands have a low beech tree coverage of only 10 to 14%, in contrast to Belgium with 24 to 33%, and particularly to south Germany with 43 to 56%, making south Germany the most HFRS-endemic area in west Europe [3]. It is probably not a coincidence that the very first (1988) cluster of Dutch HFRS cases was noted around Enschede and Oldenzaal in Twente [4]. This easternmost salient area of the Netherlands is the only part with a beech coverage of 24 to 33% [3]. We performed in 1988 a first local rodent capture action, confirming a high degree (40%) of hantavirus infection of bank voles in that region of Twente [4], still nowadays the most endemic part of the country.

Hantavirus screening in leptospirosis-suspected patients is an attractive idea, but it is not new. Van der Groen et al. tested 682 Belgian leptospirosis-suspected sera, documenting in 26 (4%) a IgG indirect fluorescent antibody (IFA) hantavirus-positivity, compared to only 21/950 (2%) in healthy blood donors [2]. Thus, already in 1983, a significantly higher hantavirus IgG prevalence in leptospirosis suspects, versus blood donors, was demonstrated (relative risk: 1.72; 95% confidence interval (CI): 1.08–2.76) [2]. Expansion of this basic strategy during 11 subsequent years resulted in the most important leptospirosis versus HFRS study so far, confirming IgG IFA hantavirus-positivity in 2% (44/2,055) of leptospirosis suspects, versus only 1% (124/9,413) in blood donors ($X^2=10.5$; $p<0.001$) in Belgium [5].

Evidence of a ‘new’ hantavirus, Seoul virus (SEOV), is not ‘mounting in Europe’, as the Dutch authors exemplify with two recent IFA-confirmed SEOV cases in England and Wales (References 22, 23 and 30 of the study under discussion). Use of exactly the same IFA technique, but expanded with a sensitive Chinese SEOV screening antigen R22, allowed, already two decades ago, the first discovery in Europe of 15 clinical SEOV-cases and one asymptomatic IgM-positive control in Northern Ireland [6]. Finally, this simple but almost never applied strategy for screening leptospirosis-suspected cohorts worldwide, enabled the first detection of clinically documented hantavirus cases in the New World (Brazil, 1993) [7], in India (2006) [8], and thus recently in Sri-Lanka.

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Conflict of interest

None declared.

Author contributions

J Clement conceived the idea and wrote the Letter, and did the bibliography. M Van Esbroeck performed the leptospirosis serology and part of the hantavirus serology, and helped writing the manuscript. K. Lagrou performed the hantavirus serology, initiated and supervised the hantavirus laboratory data bank screening at Leuven university, and constructed the Table. J Verschueren was responsible for the leptospirosis data bank screening. NP Sunil-Chandra was responsible for the mentioned leptospirosis and hantavirus data from Sri-Lanka. M Van Ranst supervised the hantavirus serology at Leuven university, and helped writing the manuscript.

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The AIRSAN Project - Efficient, coherent EU level response to public health threats in air transport

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The Ebola virus disease epidemic in West Africa since spring 2014 illustrates once again the need to be well prepared for cross-border public health threats. One challenge is to contain the spread of the disease by a coordinated international response which should entail sound cooperation between the public health and the aviation sector. The AIRSAN Project, funded by the European Commission, aims to ensure an efficient, coherent response at EU-level to public health threats in air transport. Project partners are public health authorities, airlines, airport managements and international organisations, e.g. the World Health Organization (WHO), the International Civil Aviation Organization (ICAO) and the International Air Transport Association (IATA).

The AIRSAN Project provides the AIRSAN website; an open-access website for dissemination of information for public health and civil aviation authorities, airlines and airports: <http://www.airsan.eu/>

The AIRSAN Project website also provides access to:

- **AIRSAN Guidance Documents:** The AIRSAN Project develops guidance documents that focus on managing public health threats in air transport which will be made available on the AIRSAN website. As an interim result, the AIRSAN bibliography has been created and is available online. The bibliography makes public health action-orientated information in the aviation sector quickly accessible: <http://www.airsan.eu/Resources/Bibliography/Search.aspx> In the current Ebola outbreak situation the following example illustrates the benefit of the AIRSAN bibliography: a competent public health authority wants to know how to manage a flight-passenger with suspected Ebola virus disease at an airport. The keyword-search “Management of suspect or affected travellers (at-airport)” reveals 14 documents with information about the specific topic. In case the flight-passenger is confirmed with Ebola virus disease the keyword “Contact tracing” can be searched and results include documents like the Risk Assessment Guidelines for Infectious Diseases Transmitted on Aircraft

(RAGIDA) [1] which gives specific advice on the definition of close contacts in cases of viral haemorrhagic fevers.

- **The AIRSAN Network:** The AIRSAN Project brings together competent public health authorities, civil aviation authorities, airport management and airlines across EU Member States in form of a network. Interested authorities are invited to register here for the AIRSAN Network: <http://www.airsan.eu/ContactUs/RegistertotheAirsanNetwork.aspx>. Registered members can use the password-protected AIRSAN Communication Platform to exchange information, e.g. on airport exercises or developed information material and to discuss topics concerning public health in the aviation sector.
- **The AIRSAN Training Tool:** The AIRSAN Project is developing a training tool that will support authorities and companies with the implementation of the AIRSAN Guidance Documents. The AIRSAN Training Tool will also be made available on the AIRSAN website.

In summary, the AIRSAN Project facilitates the implementation of the International Health Regulations (2005) [2] and the Decision 1082/2013 [3] in EU Member States.

References

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3. The European Parliament and of the Council of the European Union. Decision No 1082/2013/EU of the European Parliament and of the Council of 22 October 2013 on serious cross-border threats to health and repealing Decision No 2119/98/EC. Official Journal of the European Union. Luxembourg: Publications Office of the European Union. 5.11.2013:L 293. Available from: <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:293:0001:0015:EN:PDF>