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Emergence of enterovirus D68 in Denmark, June 2014 to February 2015

S E Midgley (soi@ssi.dk)[1], C B Christiansen[2], M W Poulsen[1], C H Hansen[1], T K Fischer[1,3]
1. Section for Virus Surveillance and Research, Department of Microbiological Diagnostics and Virology, Statens Serum Institut, Copenhagen, Denmark
2. Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark
3. Center for Global Health, Clinical Institute, Syddansk University, Odense, Denmark

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From June 2014 through February 2015, respiratory samples from 130 Danish patients were screened for enterovirus D68 (EV-D68). Fourteen EV-D68 cases were detected, of which 12 presented with respiratory symptoms, and eight had known underlying disease. The median age of EV-D68 cases was three years (interquartile range: 0–30 years). Acute flaccid paralysis (AFP) was not detected although Danish EV-D68 strains showed › 98% nt identity with EV-D68-strains from AFP cases from the United States and France.

This study reports the burden and characteristics of enterovirus D68 (EV-D68) disease in Denmark from June 2014 through February 2015. A retro- and prospective EV-D68 surveillance study was implemented at the National Danish World Health Organization (WHO) Reference Laboratory for Poliovirus at Statens Serum Institut (SSI) Copenhagen, in September 2014 as a result of the extensive outbreak of severe respiratory disease caused by EV-D68 in the United States (US) [1] and Canada [2] that started in July 2014. Surveillance for EV-D68 in respiratory samples has continued in Denmark since then. A number of neurological cases associated with EV-D68 have also been reported [3-5], as well as a small number of likely EV-D68 associated fatalities [6].

Laboratory investigation

In the study, we included a total of 1,322 samples, predominantly of respiratory origin, but also cerebrospinal fluid and unspecified swabs from patients from general practitioners (GPs) (26%) and hospital inpatients (74%). Samples were submitted to SSI for diagnostic testing for respiratory viruses, and respiratory samples of both GP (2%) and hospital origin (98%) were submitted for EV genotyping as part of the national EV surveillance [7], between 1 June 2014 and 28 February 2015. Informed consent from patients was not required according to Danish legislation regarding use of samples collected for surveillance purposes. Human rhinovirus (HRV) ribonucleic acid (RNA) was detected using an in-house real-time RT (reverse transcriptase)-PCR assay, and EV RNA was detected using primers described previously [8], both assays targeting the 5'non-translated region (NTR) and expected to detect all HRV and EV genotypes. A total of 130 samples representing 119 individuals tested positive for either HRV, EV, or HRV and EV. Of the 130 samples, 61 (47%) were EV-positive only, 41 (31.5%) were HRV positive only, and 28 (21.5%) were both EV and HRV positive (Table 1). Nasopharyngeal secretion was the most commonly submitted sample material, followed by tracheal secretion.

Ninety-two samples were from children (range 0–15 years of age) and 38 samples were from adults (range 21–88 years of age). The sex distribution was slightly

Table 1

Clinical samples screened for enterovirus D68, Denmark, 1 June 2014 to 28 February 2015 (n=130)

Sample material	Diagnostic finding			Total
	EV	EV and HRV	HRV	
BAL	1	0	5	6
Biopsy[a]	4	0	0	4
CSF	2	0	0	2
Expectorate	2	2	0	4
Nasopharyngeal secretion	28	13	11	52
Swab[b]	1	4	3	8
Tracheal secretion	17	5	10	32
Unspecified	6	4	12	22
Total	61	28	41	130

BAL: bronchoalveolar lavage; CSF: cerebrospinal fluid; EV: enterovirus; HRV: human rhinovirus.

[a] Biopsy materials included lung and lymph node tissue.

[b] Swabs were taken from unspecified locations and tongue.

Table 2

Enterovirus D68-positive cases Denmark, detected between September and November 2014 (n=14)

Case	Clinical information	Underlying disease	Age	Sampling date	Sample material	Diagnostic findings
1	Pneumonia and respiratory failure lasting four weeks	Pulmonary defect and asthma	2	24 Sep 2014	Tracheal secretion	EV and HRV-positive
2	Acute bronchitis, repeated admissions during a three-month period	Pulmonary defect and/or chronic lung disease	0	30 Sep 2014	Tracheal secretion	EV- and HRV-positive
3	Cough and fever	Malignancy	1	9 Oct 2014	Nasopharyngeal secretion	EV-positive
4	Pneumonia	Pulmonary defect and/or chronic lung disease	7	13 Oct 2014	Nasopharyngeal secretion	EV-positive
5	Acute respiratory failure	Cardiac disease and/or chronic lung disease	4	14 Oct 2014	Nasopharyngeal secretion	EV- and HRV positive
6	Coughing and fever lasting three weeks	Cardiac disease and/or chronic lung disease	0	20 Oct 2014	Nasopharyngeal secretion	EV-positive
7	Asthmatic wheezing for seven days, not responding to standard inhalation treatment	None	2	24 Oct 2014	Nasopharyngeal secretion	EV-positive
8	Low grade[a] fever for four days	None	3	27 Oct 2014	Nasopharyngeal secretion	EV- and HRV-positive
9	Acute onset, throat pain, coughing, muscle pain, fatigue, fever 38–39 °C, severe congestion and runny nose, lasting eight days	None	61	3 Nov 2014	Expectorate	EV- and HRV-positive
10	Mild pharyngitis for five days	None	30	4 Nov 2014	Unspecified	EV- and HRV-positive
11	Cough and fever for one week	None	0	26 Nov 2014	Tracheal secretion	EV- and HRV-positive
12	Cough and fever for two weeks; acute breathing difficulties resulting in CPAP treatment upon hospital admission (nine days).	None	68	26 Nov 2014	Unspecified	EV- and HRV-positive
13	Cough, congestion and fever lasting 21 days	Malignancy	3	26 Nov 2014	Nasopharyngeal secretion	EV-positive
14	Asthmatic cough and fever for four weeks	Malignancy	5	27 Nov 2014	Nasopharyngeal secretion	EV-positive

CPAP: continuous positive airway pressure; EV: enterovirus; HRV: human rhinovirus.

[a] Fever <38°C.

skewed as 51/92 of children were male and 22/38 adults were female.

All samples that tested positive for EV and/or HRV RNA in the diagnostic test were screened with an EV-D68 specific real-time RT-PCR [9]. As part of the Danish enterovirus surveillance system all EV positive samples were further characterised at the National WHO Reference Laboratory for Poliovirus at SSI, using the routine genotyping assay which amplifies part of the VP2 or VP1 region [10,11] followed by Sanger sequencing [7]. Fourteen patients were identified as EV-D68-positive (Table 2).

Twelve of these cases were detected using the EV-D68 specific real-time RT-PCR, two were detected by sequencing the amplicon from the VP2 PCR (data not shown). Eight were EV- and HRV-positive in the initial diagnostic test, the remaining six were EV-positive. All EV-D68 cases were detected between September and November 2014, which corresponds to the main peak of the EV season in Denmark 2014 (Figure 1, Table 2). Information on duration of illness was available for 10 patients, and patients with underlying conditions had a longer duration of illness (three weeks or longer,

compared with four to 14 days for patients with no underlying condition).

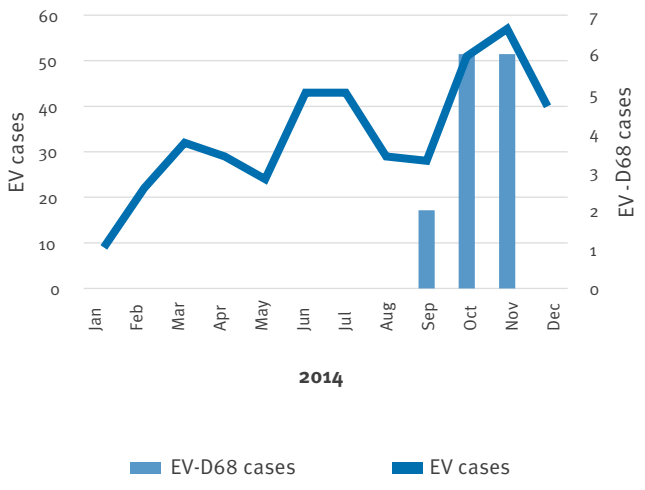
All EV-D68-positive samples from 2014, as well as historical samples, were characterised using an assay specific for the VP1 region of EV-D68 [12] and included in the phylogenetic analysis.
All PCR amplicons were sequenced using the forward and reverse primers from the VP1 and VP2 assays on an ABI 3500 automated sequencer (Applied Biosystems). Sequences were assembled in BioNumerics v6.6 (Applied Maths BV) and genotyped using BLAST analysis in the genotyping database and on GenBank. Sequences were aligned with published EV-D68 sequences downloaded from GenBank using the SSE v1.1 software [13]. The most appropriate phylogenetic model for the data was found using the model test function in MEGA6 [14], and phylogenetic analysis was carried out using maximum likelihood with the Kimura 2 parameter algorithm, gamma distribution, invariable sites and 1,000 bootstrap replications, also in MEGA6. Sequences obtained in this study were submitted to GenBank, accession numbers KP729103-KP729109, and KR108018 - KR108026. All primers used in this study can be seen in Table 3.

Phylogenetic analyses

All but two samples amplified successfully in the EV-D68 specific VP1 assay, producing sequences of around 800 nt. The two samples which could not be characterised using VP1 were the same two that failed to amplify in the real-time assay, and were confirmed to be EV-D68 by VP2 sequence analysis. Four historical EV-D68-positive samples were successfully amplified in the EV-D68 specific VP1 assay. BLAST analysis of the Danish VP1 sequences revealed that 6/12 of the Danish EV-D68 strains showed›98% homology in 100% of the sequence (between 716 and 839 nt in length) with the US 2014 outbreak strains, and 2/12 strains showed›98% homology with a EV-D68 strain from an acute flaccid paralysis (AFP) case in France case (LN626610). The remaining four Danish 2014 strains shared›98% homology with other French 2014 EV-D68 strains. Phylogenetic analysis of the 12 EV-D68 VP1 sequences identified eight clusters containing Danish strains of EV-D68 (Figure 2). Eleven of the Danish strains from 2014 cluster within clade B as described by Tokarz et al., 2012 [12], one clusters within clade A. Strains from 2008, 2010, and 2013 cluster within clade A.

Figure 1

Seasonal distribution of cases of enterovirus and enterovirus D68 infection, Denmark, 2014



EV: enterovirus.

Samples taken between January and May 2014 were not screened for the presence of enterovirus D68.

Discussion

EV-D68 which is a member of the large *picornaviridae* family of viruses, has primarily been associated with mild to severe respiratory infections [15,16]. Historically, EV-D68 was only sporadically detected worldwide during the usual EV seasonal epidemics, but since 2008 the EV-D68 has occasionally given rise to larger outbreaks globally [15,17], although no previous outbreak has seen neither the same scale nor severity as the North American EV-D68 outbreak in 2014 [1]. Shortly after the North American outbreak was announced in July, EV-D68 cases were detected in Europe, and the European Centre for Disease Prevention and Control (ECDC) issued a rapid risk assessment on 26 September 2014 [18]. Denmark joined an initiative started by the European Society for Clinical Virology (ESCV) to investigate the prevalence of EV-D68 in the European region in a retro- and prospective study covering June through November 2014.

In Denmark, ca 10% of respiratory samples tested between June 2014 and February 2015 were positive for EV and/or HRV, and of these 11% were determined to be EV-D68 by a combination of 5´NTR, VP1 and VP2 PCR and sequencing. This is a comparable detection rate to that described in other countries during the same time period [19]. No EV-D68 was detected in samples that were HRV-positive only, suggesting that the EV and HRV diagnostic result for EV-D68 cases is due to cross-reaction in the HRV assay. However, only 29.6% of double positive EV and HRV cases were associated with EV-D68. Other respiratory EV may also cross-react in an HRV assay in the 5'NTR, and, as a consequence, diagnostic laboratory reports from SSI for samples which are EV and HRV positive now contain a comment stating that this result may be due to infection with a virus detectable using both assays, rather than a double infection. The number of samples with this result has historically been small at SSI; should it continue to increase beyond the 10% rate identified in this study, the HRV assay may need to be revised.

Not all EV-D68 samples were detected using real-time and VP1 PCR, illustrating the difficulties in detecting and genotyping EVs in general due to the high level of diversity within this family of viruses. Danish strains

Table 3

Primers used for the detection and amplification of enterovirus D68

Assay	Target region	Forward primer[a]	Reverse primer[a]	Probe
EV diagnostic [8]	5' NTR	GGTGCGAAGAGTCTATTGAGC	CACCCAAAGTAGTCGGTTCC	FAM-CCGGCCCCTGAATG-MGB
EV-D68 specific diagnostic	5'NTR	TGTTCCCACGGTTGAAAACAA	TGTCTAGCGTCTCATGGTTTTCAC	FAM-TCCGCTATAGTACTTCG-MGB
				FAM-ACCGCTATAGTACTTCG-MGB
Nasri [10]	VP2	GARGCITGYGGITAYAGYGA	TTDATDATYTGRTGIGG	NA
		GARGARTGYGGITAYAGYGA	TTDATCCAYTGRTGIGG	
		GGITGGTGGTGGAARYTICC		
		GGITGGTAYTGGAARTTICC		
Nix [11]	VP1	GCIATGYTIGGIACICAYRT	GTYTGCCA	NA
		CCAGCACTGACAGCAGYNGARAYNGG	GAYTGCCA	
			CCRTCRTA	
			RCTYTGCCA	
			CICCIGGIGGIAYRWACAT	
			TACTGGACCACCTGGNGGNAYRWACAT	
Tokarz [12]	VP1	CCTTAATAGGGTTCATAGCAGC	CTGGGCCGGTGGTYACTA	NA
		ATGAGAGAYAGYCCTGACATTG	CATTGAGBGCATTTGGTGCT	

EV: enterovirus; NA: not applicable.

[a] Degenerate bases: I- inosine, B- C/G/T, D- A/G/T, N- A/C/G/T, R- A/G, Y- C/T, W-A/T.

were genetically very closely related to strains from AFP cases in the US and France, and AFP cases have also been described in Norway [19,20]. Strains from 2014 from countries other than Denmark also show a close genetic relationship to strains from AFP cases, suggesting host factors play an important role in the development of AFP as a result of EV-D68 infection. Underlying disease appears to increase the risk for severe infection, and therefore potentially also AFP. There is, however, no definite link between underlying disease and development of AFP, suggesting the involvement of other host factors. The level of viraemia may also contribute.

Following the detection of the first Danish EV-D68 cases during the fall of 2014, the National WHO Reference Laboratory for Poliovirus at SSI communicated these findings, alongside a description of the EV-D68 epidemic in the US, to all Danish clinicians including general practitioners and microbiologists with an appeal for vigilance regarding severe respiratory symptoms and AFP. Newsletters were posted on the National Clinical and Microbiology Societies websites and sent out using mailing lists, and an article was published in the weekly epidemiological Bulletin (EPI-NEWS) issued by the SSI [21]. Although Danish cases with EV-D68 and underlying conditions did experience severe respiratory symptoms, no cases of AFP were reported.

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Conflict of interest

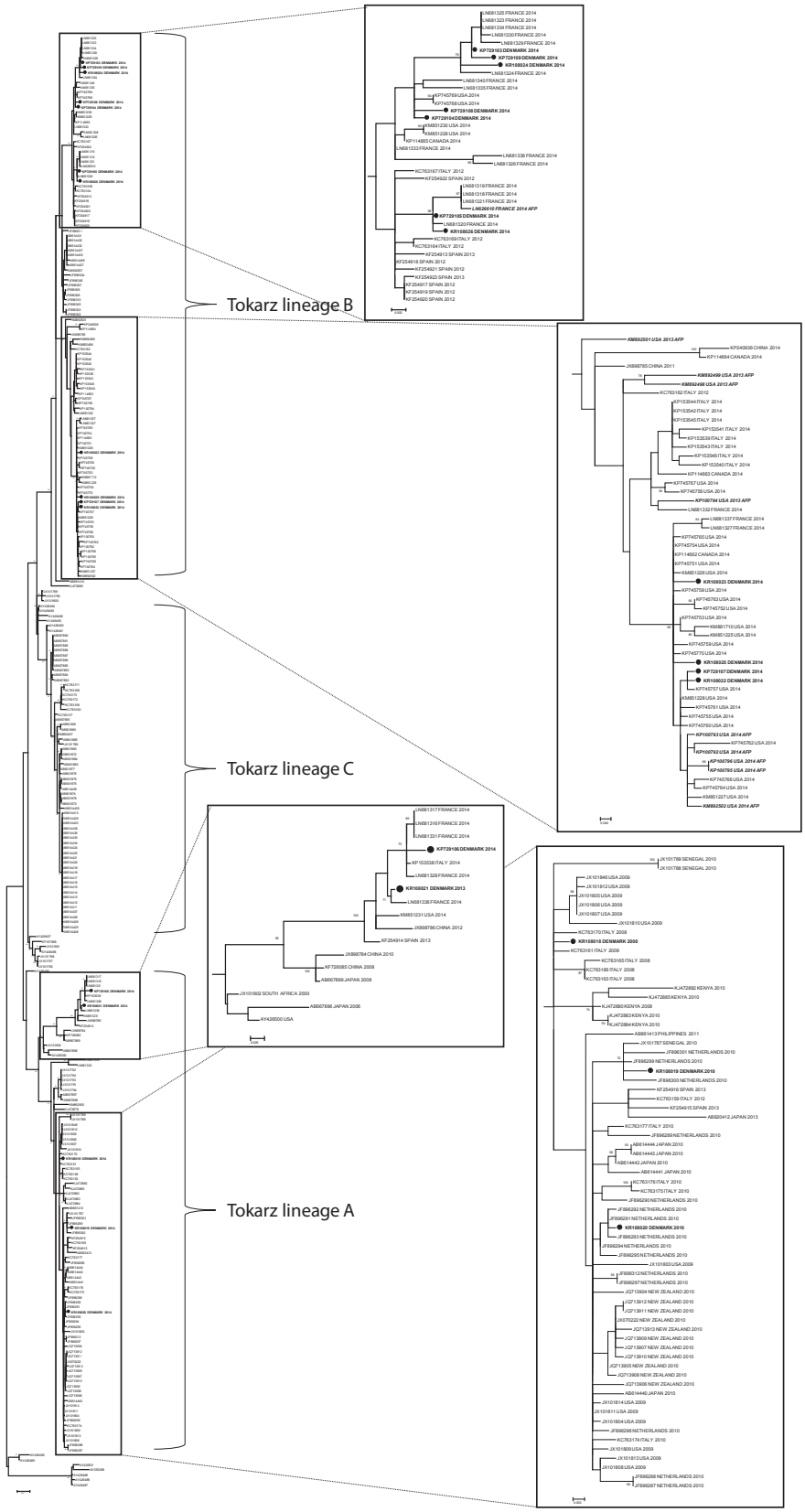
None declared.

Authors' contributions

SEM: drafted the manuscript and conducted the phylogenetic analyses.
CBC: provided respiratory samples for enterovirus characterisation and collected clinical information about EV-D68 cases.
MWP: conducted the laboratory characterisation of enteroviruses presented in this paper.
CHH: critical review of the literature regarding the 2014 outbreak.
TKF: conceptualised the study and has participated in writing of the manuscript together with SEM.

Figure 2

Phylogenetic analysis of enterovirus D68 VP1 sequences



The phylogenetic tree was constructed by maximum likelihood, with the Kimura 2-parameter algorithm, with gamma distribution, invariable sites and 1,000 bootstrap replicates, using MEGA6. Only bootstrap values›70% are indicated. Branch lengths are drawn to the indicated scale, proportion of nt substitutions per site. Sequences were 731 nt in length. Reference sequences were downloaded from GenBank; all complete or near complete genomes (n=50, downloaded 14 Apr 2015), as well as partial VP1 sequences›700 nt in length with complete overlap with the sequences from this study (n=232, downloaded 14 Apr 2015) were included, resulting in a dataset of 294 sequences. GenBank reference sequences and sequences from this study are identified by their accession numbers. Country of origin and detection year is specified after the sequence ID (where available) for references and study samples within defined clusters. Four EV-D68 sequences from the Danish genotyping database, identified between 2008 and 2013, were also included in the analysis. Sequences from this study are identified with filled circles and bold text. Reference sequences from known acute flaccid paralysis cases are identified with bold italics.

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Chikungunya outbreak in Montpellier, France, September to October 2014

E Delisle (delisle.elsa@gmail.com)[1], C Rousseau[1], B Broche[2], I Leparc-Goffart[3], G L'Ambert[4], A Cochet[1], C Prat[3], V Foulongne[5], J B Ferré[4], O Catelinois[1], O Flusin[3], E Tchernonog[5], I E Moussion[2], A Wiegandt[2], A Septfons[6], A Mendy[2], M B Moyano[2], L Laporte[2], J Maurel[2], F Jourdain[7], J Reynes[5], M C Paty[7], F Golliot[1]

1. Regional office of the French Institute for Public Health Surveillance (Cire Languedoc-Roussillon), Montpellier, France
2. Regional Health Agency of Languedoc-Roussillon, Montpellier, France
3. Institut de Recherche Biomédicale des Armées, National Reference Laboratory for arboviruses, Marseille, France
4. Entente Interdépartementale pour la Démoustication du littoral Méditerranéen (EID Méditerranée), Public mosquito control operator, Montpellier, France
5. Montpellier University Hospital, Montpellier, France
6. French Institute for Public Health Surveillance (Institut de Veille Sanitaire, InVS), Saint-Maurice, France
7. National Centre of Expertise on Vectors, Montpelier, France

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In October 2014, an outbreak of 12 autochthonous chikungunya cases, 11 confirmed and 1 probable, was detected in a district of Montpellier, a town in the south of France colonised by the vector *Aedes albopictus* since 2010. A case returning from Cameroon living in the affected district was identified as the primary case. The epidemiological investigations and the repeated vector control treatments performed in the area and around places frequented by cases helped to contain the outbreak. In 2014, the chikungunya and dengue surveillance system in mainland France was challenged by numerous imported cases due to the chikungunya epidemic ongoing in the Caribbean Islands. This first significant outbreak of chikungunya in Europe since the 2007 Italian epidemic, however, was due to an East Central South African (ECSA) strain, imported by a traveller returning from West Africa. Important lessons were learned from this episode, which reminds us that the threat of a chikungunya epidemic in southern Europe is real.

Background

Chikungunya (CHIK), as well as dengue virus which shares the same vector, has been identified as a threat for mainland France for several years, as all the prerequisites for autochthonous transmission of the virus are present in southern regions [1,2]. The mosquito vector, *Aedes albopictus*, first introduced in southern France in 2004, was established in 18 districts in 2014 [3]; and the virus is regularly introduced by viraemic travellers returning from endemic or epidemic countries to mainland France where the population is naive for chikungunya virus (CHIKV). This situation led to a first episode of autochthonous transmission of chikungunya in south-east France in 2010 [4]. Other countries in the south of Europe are also threatened by CHIK and dengue virus as the vector *Ae. albopictus* is established in large part around Mediterranean Sea and continues to disseminate further each year. This was demonstrated when an epidemic occurred in Italy in 2007, with more than 200 cases infected in three months [5].

In mainland France, since 2006, a chikungunya and dengue preparedness and contingency plan is implemented every year in vector-colonised districts. This plan aims to prevent the transmission and dissemination of these viruses [3,6]. It describes five risk levels defined according to entomological and epidemiological surveillance results. All clinically-suspected imported cases must be reported to the regional health authority in addition to mandatory notification of confirmed cases. Epidemiological investigations are then implemented as well as appropriate vector control measures in each location visited by patients during their period of viraemia. In addition, a network of laboratories practicing chikungunya and dengue diagnosis provides daily reports of their results to the French Institute of Public Health Surveillance regional boards. This enables identifying imported and autochthonous cases not already notified. A national database collects all suspected and confirmed cases identified during the season.

Here we report the epidemiological and entomological investigations of an outbreak of chikungunya which took place in Montpellier from September to October 2014 and try to explain the reasons why it occurred. Montpellier is a town of 400,000 inhabitants, located on the Mediterranean coast. *Ae. Albopictus*, identified in 2010, has progressively colonised the whole conurbation.

The alert

On 1 October 2014, a probable autochthonous chikungunya case was identified in Montpellier (case 1, index case). The patient, a woman in her 40s living near Montpellier, had developed 13 days earlier, on 18 September, a sudden fever, incapacitating arthralgia, myalgia and a rash. She had not travelled abroad during the two weeks before symptoms onset but had experienced multiple mosquito bites on 14 September in a residential area of Montpellier. She consulted her general practitioner (GP) who prescribed several serological tests, including for CHIKV. Analyses performed on a serum sample taken five days after symptoms onset identified IgM antibodies to CHIKV, that were confirmed by the National Reference Centre (NRC) in Marseille, on 13 October. Real-time reverse transcription PCR (RT-PCR) was negative for this sample.

While waiting for a second blood-sample to confirm the CHIKV infection by seroconversion, four additional suspected autochthonous chikungunya cases were notified on 16 October by the Infectious and Tropical Diseases Department of Montpellier University Hospital (UH) to the regional health authority. These cases (Cases 2 to 5) were adults from a same family and had not travelled abroad recently. They were not linked to the index case. They successively developed acute fever, painful joints and a rash between 20 September and 12 October (Figure).

They lived in a house located ca 150 m from the place where Case 1 (index case) experienced mosquito bites. On 17 October, a chikungunya real-time RT-PCR was positive for one of the family members at the Montpellier UH virology laboratory. The diagnosis of chikungunya was confirmed for all the family cases and Case 1 by the NRC, respectively on the 20 October and the 22 October (real-time RT-PCR and seroconversion, Table 1).

These geographically and temporally linked cases formed a cluster in a distinct area of Montpellier. According to the national contingency plan against the spread of CHIK and dengue viruses, these findings prompted immediate epidemiological and entomological investigations in order to contain the transmission.

Methods

Case definitions

For the investigation, the following case definition was applied. From 1 September to 30 November 2014, in Montpellier and conurbation:

- An autochthonous suspected case of chikungunya was defined by sudden onset of fever (≥ 38.5 °C) and arthralgia, not explained by another medical condition in a person without a history of foreign travel within 15 days prior to symptoms onset.
- An autochthonous probable case was defined as a suspected case with an epidemiological link to a confirmed case or a suspected case with positive chikungunya tests (real-time RT-PCR or serology) performed by a private laboratory, or hospital laboratory.
- An autochthonous confirmed case was defined as a suspected case with positive laboratory tests (real-time RT-PCR or serology positive for IgM and IgG antibodies to CHIKV) performed by the NRC or a suspected case with an epidemiological link to a confirmed case and a serology positive for IgM antibodies to CHIKV performed by the NRC.

Epidemiological investigations

Immediately after the identification of Case 1 (index case), the enhanced surveillance database was analysed to identify a potential primary case. Among all the imported chikungunya cases identified during the 2014 season, cases that had symptoms onset after 28 July and lived near or visited the same places as Case 1 were listed.

The time after 28 July was considered as the maximal period for viral transmission from symptoms onset of the primary case to symptoms onset of the second case, considering a mosquito lifespan of maximum one month, human incubation of maximum 12 days and human viraemia of seven days.

Active case finding of suspected autochthonous cases was implemented in Montpellier and conurbation: (i) a door-to-door investigation was conducted in the neighbourhood of the autochthonous cases' residences (200m radius). Nearly 1,000 households were targeted, representing ca 2,250 inhabitants; (ii) information on the event and a request to immediately notify all suspected autochthonous cases to the regional health authority was sent to all GPs and laboratories established in the Montpellier conurbation as well as all six emergency medical services (including the Montpellier UH). In addition, health professionals established within a 2km radius of the cases' residences were contacted by telephone (30 GPs and 11 laboratories).

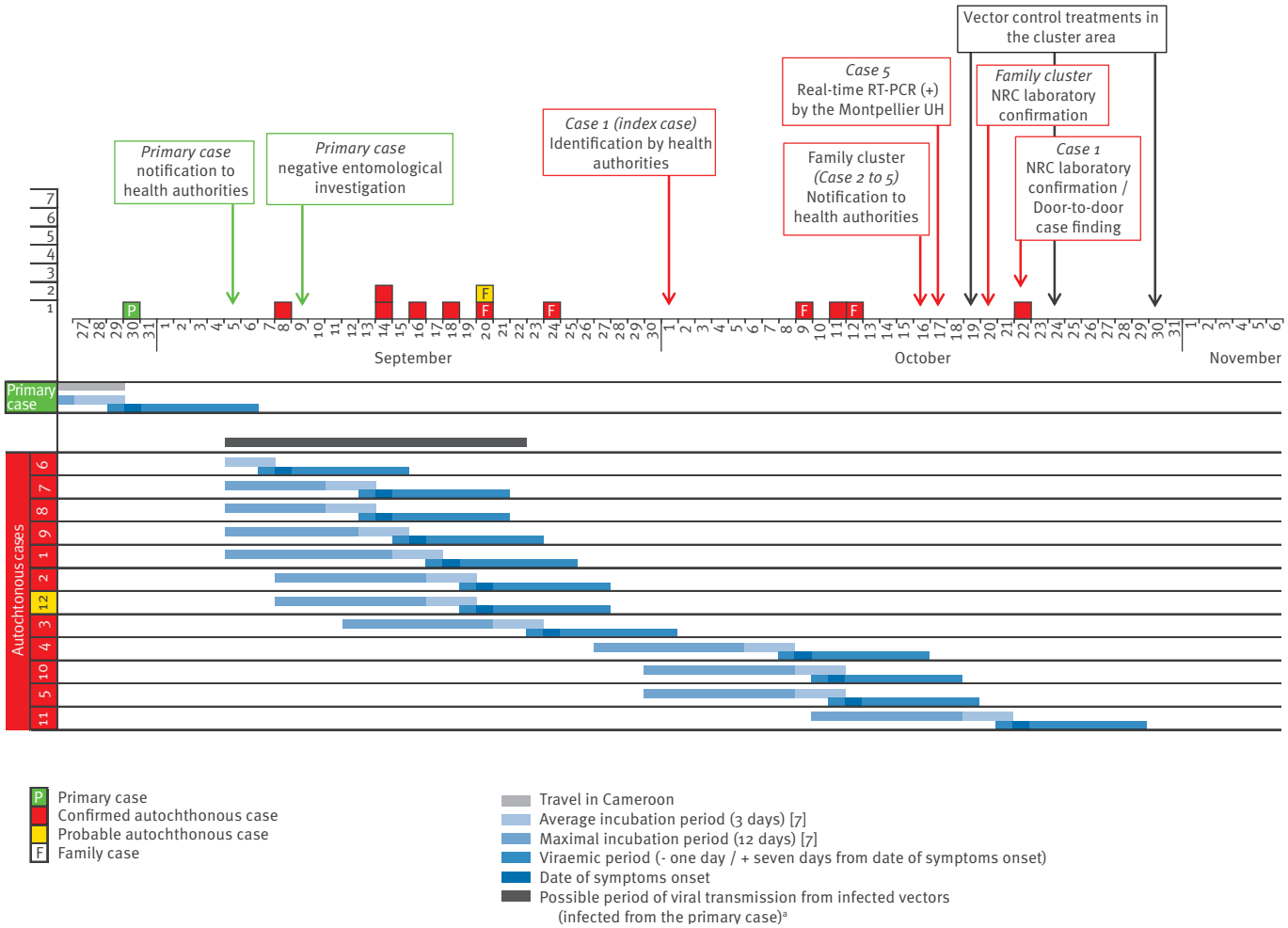
For each new suspected case identified, blood samples were collected and analysed by the NRC. Real-time RT-PCR was performed on samples collected within eight days after symptoms onset and serology on samples collected after day 5 of symptoms onset.

Entomological investigations

Entomological investigations were carried out inside the primary case's property, as soon as this case was identified, and in the affected area, in order to estimate vector densities, using BG Sentinel adult mosquito traps. Prospection of mosquito breeding sites was conducted concomitantly to the door-to-door case finding.

Figure

Timeline of symptoms onset for imported and autochthonous cases of chikungunya and epidemiological features, Montpellier, France, September–October 2014 (n = 13)



NRC: National Reference Center; RT-PCR: reverse transcriptase-PCR; UH: University Hospital.

Cases numbered by order of identification.

Source : French Institute for Public Health Surveillance (Institut de veille sanitaire), 2014.

[a] Possible period of viral transmission from infected vector (infected from the primary case):- mosquitoes biting the primary case between the first day and the last day of his viraemic period, extrinsic incubation period: seven days [8], mosquito lifespan: 10 days [9].

Results

Epidemiological investigations

Primary case

An imported case living in the same area as the five autochthonous cases was identified in the enhanced surveillance database. The patient, who had returned from Cameroon on 29 August, had symptoms onset two days later. He consulted at the Montpellier UH and was diagnosed on 5 September by the laboratory of virology via chikungunya-specific real-time RT-PCR on a serum sample at day 3 post symptoms onset (Figure, Table 1). He was immediately notified to the regional health authority. Entomological investigators, responsible for interventions at that time, could not enter his property and did not identify the vector in the vicinity of the case's residence. No mosquito-control treatment was performed then.

Autochthonous cases

The door-to-door case finding and notification by health professionals of suspected autochthonous cases enabled the detection of 20 autochthonous suspected cases of chikungunya in the neighbourhood of Cases 1 to 5 (200m radius). Six cases were laboratory-confirmed by the NRC (Table 1) and the results were negative for 14. All six confirmed cases had been identified through the door-to-door investigations. At the time of investigation, five had fully recovered and for sixth case, the symptoms started on the very day of the investigation (Figure). Moreover, epidemiological

Table 1

Laboratory investigation of imported and autochthonous cases of chikungunya by the National Reference Centre, Montpellier, France, September–October 2014 (n = 13)

Case number	Date of symptoms onset	Date of sampling[a]	Serological tests for chikungunya		Chikungunya real-time RT- PCR
			IgM	IgG	
Imported[b]	30 Aug 2014	D3	ND		Positive
1[c]	18 Sep 2014	D5	Positive	Negative	Negative
		D29	Positive	Positive	ND
2	20 Sep 2014	D28	Positive	Positive	ND
3	24 Sep 2014	D24	Positive	Positive	ND
4	09 Oct 2014	D7	Positive	Positive	Negative
5	12 Oct 2014	D4	ND		Positive
6	08 Sep 2014	D4	ND		Positive
7	14 Sep 2014	D51	Positive	Positive	ND
8	14 Sep 2014	D51	Positive	Positive	ND
9	16 Sep 2014	D6	Positive	Negative	Negative
10	11 Oct 2014	D5	Positive	Negative	Negative
		D23	Positive	Positive	ND
11	22 Oct 2014	Do	ND		Positive
12	20 Sep 2014	ND	ND		ND

ND: not done; RT-PCR: reverse transcriptase-PCR.

[a] Day post symptom onset.

[b] Primary case.

[c] Index case.

investigations conducted among the family cases (cases 2 to 5) identified an additional family member as a probable case, but this patient did not consent to being tested.

Altogether, 12 autochthonous chikungunya cases were identified: 11 confirmed and one probable. For three confirmed cases, the CHIKV was detected (real-time RT-PCR positive) and for eight, IgM with or without IgG antibodies against CHIKV were detected (Table 1). The CHIKV sequence data obtained from sera of two autochthonous cases indicated that the CHIKV infection was due to a strain belonging to the East Central South African (ECSA) genotype harbouring the E1-A226V adaptive mutation (data not shown). Sequencing performed from the primary case serum showed same results.

All cases lived in or had visited the same area of Montpellier, a square of side 250m enclosing small buildings and individual houses with many gardens. The median age was 59 years (range 22–80). Half of the cases (6/12) were women. The date of symptoms onset ranged from 8 September 2014 to 22 October 2014. All cases presented with fever and incapacitating arthralgia, mainly in the hands or feet. A rash was present for 10/12 (Table 2), which appeared mainly after fever onset (median two days; range 0–5). Nine of the 12 cases were free of general symptoms (fever, rash, myalgia etc.) within eight days (range 4–21) but seven cases still suffered from persistent joint pains two months after symptoms onset. All cases consulted their GPs while symptomatic but chikungunya diagnosis was suspected only for one (Case 1, index case). Five were referred to the Montpellier UH where the diagnosis was suspected for four of them (Cases 2 to 5) and laboratory tests carried out.

Entomological investigation and control measures

Following the identification of Case 1 (index case) and immediately after the primary case had been identified, entomological investigations were repeated in mid-October in the neighbourhood of the primary case residence. They showed very high densities of *Aedes albopictus* larvae and adults (average of 30 mosquitoes per BG-Sentinel trap per day), promoted by numerous gardens located in the area providing mosquito breeding and resting sites.

A mosquito-control treatment was therefore performed three days later (Figure) in the outbreak area (±150m radius circle around the residences of the primary case, of the family cases and the place visited by case 1), with Ultra Low Volume spraying of deltamethrin (Cerathrin and Aqua-KOthrine 2 and 1g of active substance.ha^{-1}, respectively). This operation was repeated twice, five days and 11 days later, within a larger area (±250m radius circle around the case's residences), with consideration to sensitive persons and activities as well as respective institutions in the vicinity such as nursery, kindergarten, elementary and high schools. Following the first insecticide treatment, the vector population declined drastically in the area: from 243

Table 2

Distribution of symptoms among autochthonous cases of chikungunya, Montpellier, France, September–October 2014 (n = 12)

Symptoms	Number of cases
Fever › 38.5 °C	12
Arthralgia	12
feet / ankles	10
hands / wrists	9
Rash	10
Myalgia	7
Diarrhoea	3
Headache	2
Back pain	1

Ae. albopictus collected per day with 8 BG Sentinel traps 24h before the treatment to eight per day after the treatment. Mosquito-control treatment was also performed around places (±150m radius circle) frequented by each case during viraemia.

The numerous mosquito breeding sites identified in the neighbourhood houses during the door-to-door inspection were eliminated by the Entente Interdépartementale pour la Démoustication (EID) vector control professionals, involving each inhabitant for pedagogical purposes.

Before the fogging of insecticide, an information leaflet was distributed in all mail-boxes inside the treatment area, concerning the epidemiological situation, the aim of the treatment and with explanation how to limit contact with insecticides, and giving recommendations about community mosquito control and protection i.e. maintaining gardens regularly and drying containers filled with water, using repellent, wearing long-sleeved clothes, etc.

Discussion

This outbreak involving 12 autochthonous chikungunya cases is the first significant outbreak in Europe since the 2007 epidemic in Emilia Romagna, Italy [5]. It started as all the conditions for autochthonous transmission of CHIKV were met in a densely populated neighbourhood of Montpellier with high densities of *Aedes albopictus*.

The absence of immediate vector control treatment around the primary case's residence where the vector was initially not identified, and the delay in identifying the first autochthonous cases enabled the establishment of a CHIKV transmission cycle, which involves several generations of mosquitoes (incubation 2–12 days [7], extrinsic incubation period 4–13 days [8], mosquito lifespan 10-30 days [9]). This delay is due to the fact that chikungunya is rare in mainland France and except for case 1, none of the GPs consulted by cases suspected the disease.

However, there was a prompt response following the alert. The epidemiological investigations and the repeated vector control treatments performed in the area and around places frequented by cases helped to contain the outbreak: the number of cases was probably curtailed and no spread beyond the affected area was identified. Only one case (case 11) presented with an onset of symptoms three days after the first treatment in the area. The effectiveness of vector control measures was also suggested by the results of trapping before and after treatments. The beginning of autumn and weather conditions in October might also have contributed to the end of the outbreak: the cooler temperatures were unfavourable to virus transmission and vector activity, the shorter daylight period induced egg diapauses and a decrease in vector density. In this outbreak involving a few cases, no socioeconomic factor was identified as favouring the transmission.

The outbreak occurred as the ongoing chikungunya epidemic in the Caribbean Islands [10] alerted the French public health authorities and challenged the national surveillance system [3,11,12]. Four hundred and five of the 449 (90%) imported cases of chikungunya identified during the 2014 season in the French vector-colonised districts were returning from the French West Indies, where the Asian genotype of CHIKV circulates [13]. No autochthonous transmission had been identified from those imported cases. The primary case in the outbreak, however, was a viraemic traveller returning from Cameroon, infected by a strain belonging to the ECSA genotype with the E1-A226V adaptive mutation. These observations raise questions about the adaptation of the Asian genotype CHIKV strain circulating in the Caribbean to *Aedes albopictus* [14].

This outbreak, like the previous autochthonous cases of chikungunya and dengue which occurred in southern France since 2010 [4,15-17], highlights that autochthonous transmission of vector-borne diseases is possible and can lead to outbreaks in France, under favourable climatic and entomological conditions. Repeated episodes of transmission of chikungunya or dengue viruses are likely to occur in the future, especially as the vector is spreading further each year: in 2014, in France, more than 14 million residents live in areas colonised by *Aedes albopictus*, including many densely-populated cities.

This episode, detected and contained early when the number of cases was still limited, shows the importance of the French contingency plan, whose purpose is, more than to avoid autochthonous transmission, to contain them. The plan organises epidemiological and entomological surveillances, facilitates the coordination of investigations and mosquito control activities, allows the anticipation of necessary resources in case of an outbreak and provides regulatory tools. This plan is evaluated and adapted every year in order to

maintain its efficiency. However, the involvement of the population and health professionals is a key point for success. Thus, given the observations stated during the investigations, the following actions need to be pursued in the French vector- colonised areas to improve public health response: (i) increasing population awareness regarding the risk of chikungunya and dengue and consequently improving prevention (from individual protection to breeding sites control) in order to limit vector-borne transmission; (ii) increasing awareness of physicians and laboratories regarding the possibility of autochthonous transmission, the appropriate laboratory diagnosis tools and the notification of cases; (iii) maintaining a coordinated approach and a close collaboration between epidemiological and entomological surveillance, as well as a concerted preparedness of the various parties such as national and local health authorities, vector control professionals, national laboratory before the start of the season in order to facilitate rapid response.

Conclusion

This outbreak, following importation of an ECSA CHIKV strain by a traveller returning from Cameroon, is the first significant one in mainland France. Such a local circulation of the virus was not unexpected and the national contingency plan showed its effectiveness in controlling the outbreak. However, some weaknesses, in vector control measures around the primary case and awareness of health professionals, facilitated the spread of the virus. Contingency plan and epidemiological and entomological surveillance stakeholder preparedness is necessary to implement rapid and proportionate measures of surveillance and control. Awareness of health professionals and the community about vector control and disease symptoms need to be strengthened.

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Conflict of interest

None declared.

Authors' contributions

Contribution to the epidemiological investigations: E Delisle, C Rousseau, MC Paty, A Cochet, O Catelinois, F Golliot, A Septfons, A Mendy, MB Moyano, L Laporte, J Maurel. Interviews of the patients: A Mendy, MB Moyano, L Laporte, J Maurel, E Delisle. Diagnosis of the family cases and notification to health authorities: E Tchernonog, J Reynes, V Foulogne. Implementation of the control measures: B Broche. A Wiegandt, I Estève-Moussion. Coordination of the control measures: B Broche. Entomological investigations and coordination of vector control treatment: G L'Ambert, JB Ferré. Laboratory investigations: I Leparc-Goffart, C Prat, O Flusin. Coordination of the epidemiological investigations: E Delisle, C Rousseau (regional level), MC Paty (national level). Supervisor of the investigations: F Golliot. Drafted the manuscript: E Delisle, C Rousseau, MC Paty. Contribution to the writing of the paper: A Cochet, O Catelinois, G L'Ambert, F Jourdain, I Leparc-Goffart, A Wiegandt.

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Cheese-related listeriosis outbreak, Portugal, March 2009 to February 2012

R Magalhães[1], G Almeida[1], V Ferreira[1], I Santos[1], J Silva[1], M M Mendes[2], J Pita[2], G Mariano[2], I Mâncio[2], M M Sousa[3], J Farber[4], F Pagotto[4], P Teixeira (pcteixeira@porto.ucp.pt)[1]

1. Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Porto, Portugal
2. Autoridade de Segurança Alimentar e Económica, Lisbon, Portugal
3. Administração Regional de Saúde de Lisboa e Vale do Tejo, Lisbon, Portugal
4. Listeriosis Reference Centre for Canada, Bureau of Microbial Hazards, Health Canada, Ottawa, Ontario, Canada

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In Portugal, listeriosis has been notifiable since April 2014, but there is no active surveillance programme for the disease. A retrospective study involving 25 national hospitals led to the detection of an outbreak that occurred between March 2009 and February 2012. The amount of time between the start of the outbreak and its detection was 16 months. Of the 30 cases of listeriosis reported, 27 were in the Lisbon and Vale do Tejo region. Two cases were maternal/neonatal infections and one resulted in fetal loss. The mean age of the non-maternal/neonatal cases was 59 years (standard deviation: 17); 13 cases were more than 65 years-old. The case fatality rate was 36.7%. All cases were caused by molecular serogroup IVb isolates indistinguishable by pulsed-field gel electrophoresis and ribotype profiles. Collaborative investigations with the national health and food safety authorities identified cheese as the probable source of infection, traced to a processing plant. The magnitude of this outbreak, the first reported food-borne listeriosis outbreak in Portugal, highlights the importance of having an effective listeriosis surveillance system in place for early detection and resolution of outbreaks, as well as the need for a process for the prompt submission of *Listeria monocytogenes* isolates for routine laboratory typing.

Introduction

Listeria monocytogenes is an intracellular bacterial pathogen of humans and a variety of animal species. In humans, *L. monocytogenes* infections are mainly food-borne and can cause an invasive and often fatal disease in pregnant women and their fetuses, newborns, elderly people and immunocompromised individuals, with a case fatality rate of up to 30% [1]. The incidence of listeriosis increased in several European countries between 2009 and 2013 (such as Germany, the Netherlands, Spain and the United Kingdom [1,2]) and, was the most frequent cause of hospitalisation and death (15.6%) due to the consumption of contaminated food in Europe in 2013 [2]. This increase reinforces the need for each country to establish enhanced molecular surveillance of listeriosis for efficient outbreak detection, investigation and control, as carried out by PulseNet USA or the Centre National de Référence des Listeria, Institut Pasteur, Paris, for example [3,4]. A similar programme for listeriosis surveillance at European Union level by harmonising methodological variables such as case definition, laboratory procedures and reporting systems is crucial. A pilot project was conducted by the European Centre for Disease Prevention and Control (ECDC) between January and March 2013 aiming to evaluate a *Listeria* external quality assurance scheme for the typing of *L. monocytogenes* that covered pulsed-field gel electrophoresis (PFGE) method and serological typing (both as a phenotypic and a multiplex polymerase chain reaction (PCR)-based method) [5]. Results demonstrated that the majority (59%) of the participating laboratories were able to produce a PFGE gel of sufficiently high quality and the average score for serotyping among the participants was 94% and 97% for traditional and multiplex PCR based methods, respectively; however, higher quality could be achieved through trouble-shooting assistance and training.

In the absence of an active surveillance system for listeriosis at a national level, a collaborative study between the *Listeria* Research Centre of Escola Superior de Biotecnologia (LRCESB) and 25 of the major national hospitals (on a voluntary basis), covering about 90% of the population, was established in 2003 with the aim of obtaining epidemiological data on human listeriosis cases in Portugal and characterising clinical isolates of *L. monocytogenes* both phenotypically and genetically. In 2003, the incidence of listeriosis was 0.14 cases per 100,000 population [6]. An increase was reported between 2003 and 2007, i.e. it was 0.23 cases per 100,000 inhabitants for the year 2007 [7]. As a result of this study, an increase in the number of listeriosis cases was detected between January and July 2010, particularly in the Lisbon and Vale do Tejo region

that corresponds to 13% of the total area of mainland Portugal and 34% of the total population (3.6 million inhabitants) [8], representing the first detected outbreak of listeriosis in Portugal. Here we describe the outbreak, as well as give details of the investigations carried out in order to determine the source of infection.

Methods

Case definition

A listeriosis case was defined as a non-maternal/neonatal (non-MN) patient who met the laboratory criteria or a mother with a laboratory-confirmed listeriosis infection in her fetus, stillborn or newborn, as described in the Commission Decision of 28/IV/2008 [9]. Cases (laboratory confirmed with unknown clinical criteria) were detected through voluntary reporting by hospitals to the LRCESB in Porto.

If the pathogen was isolated from a pregnant woman and her newborn, stillborn or fetus, this was counted as a single case. Information regarding the sex and age of the patient, underlying pathology (if present), the tissue or fluid from which the bacteria were isolated and the year of isolation was reported.

Culture collection

Hospitals sent isolates of *L. monocytogenes* to LRCESB for species confirmation and typing. Species confirmation was performed by carbohydrate fermentation (rhamnose, xylose and mannitol) and Christie Atkins Munch-Petersen (CAMP) test [10]. Confirmed isolates of *L. monocytogenes* were stored in tryptic soy broth with 30% (v/v) glycerol at –80 °C in the culture collection of the LRCESB.

Molecular-serotyping

Molecular serotype of *L. monocytogenes* isolates was determined by multiplex PCR according to Doumith et al. [11]. This assay differentiates five major subtypes, each representing more than one serotype: geno-serogroup IVb (serotypes 4b, 4d and 4e), geno-serogroup IIa (serotypes 1/2a and 3a), geno-serogroup IIb (serotypes 1/2b, 3b and 7), geno-serogroup IIc (serotypes 1/2c and 3c) and geno-serogroup IVa (serotypes 4a and 4c).

Pulsed-field gel electrophoresis

PFGE typing was performed according to the standard CDC PulseNet protocol [12] using the restriction enzymes *Asc*I and *Apa*I and gel run in CHEF III DR System (Bio-Rad, Laboratories, Hercules, CA, United States). *Salmonella enterica* serovar Braenderup H9812 (ATCC) DNA digested with *Xba*I was used as a reference size standard. Cluster analysis of the PFGE types was performed with the GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium) by the unweighted pair group method with average linkages (UPGMA), using the Dice coefficient, and visually validated.

Ribotyping

Automated ribotyping was performed using the restriction enzyme *Eco*RI and the RiboPrinter microbial characterisation system (Qualicon Inc., Wilmington, DE, United States), as previously described [13,14].

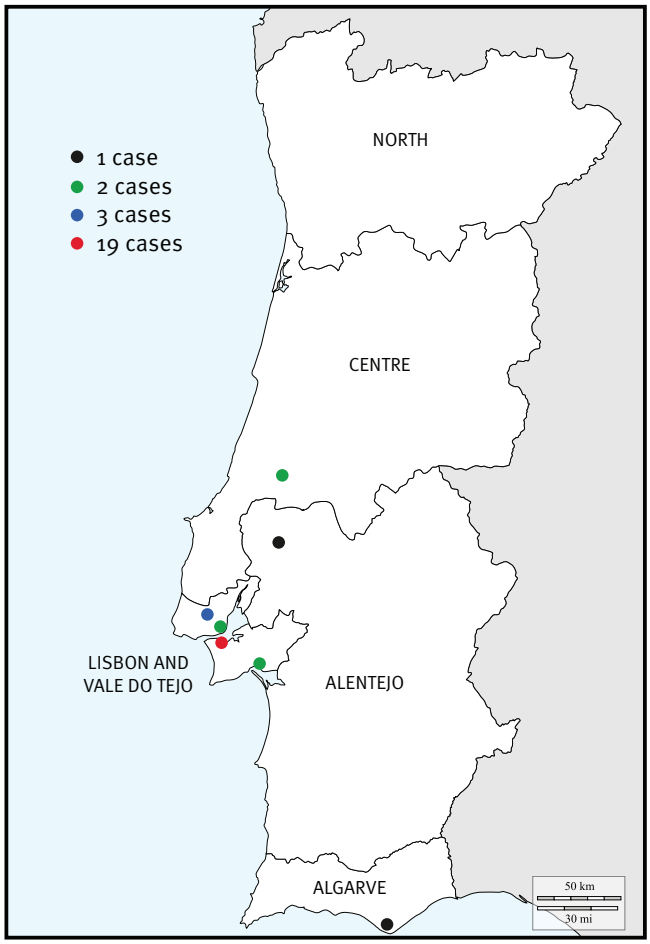
Outbreak investigation

The outbreak was investigated by the national health (Direção Geral de Saúde and Administração Regional de Saúde de Lisboa e Vale do Tejo) and food safety (Autoridade de Segurança Alimentar e Económica) authorities in collaboration with LRCESB.

A standardised questionnaire (adapted from a Canadian listeriosis outbreak, kindly supplied by Dr Jeff Farber of the Public Health Agency of Canada) was administered by the national health authority to patients diagnosed with listeriosis or their families (face-to-face interview) concerning their diet histories in the two months before symptom onset, with reference to the type of food consumed and household shopping patterns.

Figure 1

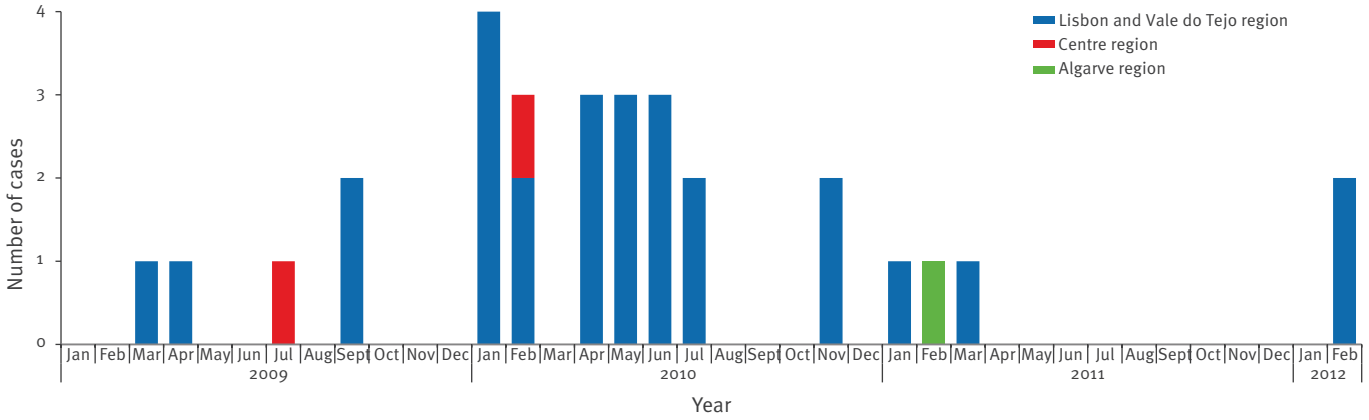
Distribution of human listeriosis cases in Portugal with the outbreak *Listeria monocytogenes* strain, March 2009–February 2012 (n = 30)



Map adapted from www.d-maps.com/m/europa/portugal/portugal19.pdf.

Figure 2

Human listeriosis cases with the 2010 *Listeria monocytogenes* outbreak strain, Portugal, March 2009–February 2012 (n = 30)



All except three cases were in the Lisbon and Vale do Tejo region (two were in Centre region, one case occurred in the Algarve region). Information on the number of listeriosis cases caused by *Listeria monocytogenes* strains with non-outbreak pulsed-field gel electrophoresis types for each year is available from Magalhães et al. [15].

Analysis of food products and environmental samples was conducted by the food safety authority. *L. monocytogenes* isolates from food and environmental samples were sent to LRCESB for typing.

International enquiry

To determine if the outbreak-associated strain of *L. monocytogenes* had been recovered from clinical or food samples from other countries, the PFGE type was communicated and compared with those of *L. monocytogenes* isolates in databases in France (Centre National de Référence des Listeria, Institut Pasteur), Canada (Listeriosis Reference Centre, Health Canada) and United States (Food Microbe Tracker, Food Safety Laboratory, Cornell University).

Results

Recognition of the outbreak

Between January and July 2010, a high number of listeriosis cases was observed (40 cases compared with 20 cases observed during all of 2009) [15], particularly in the Lisbon and Vale do Tejo region, where the majority of the cases were reported. Molecular typing of the 40 *L. monocytogenes* clinical isolates revealed that 18 serotype IVb isolates presented the same PFGE type and ribotype, which had been observed for five isolates recovered in 2009, four of which were in the Lisbon and Vale do Tejo region (in March, April and September) and one in the Centre region (in July) (Figures 1 and 2). This PFGE type was not found in the databases searched.

In July 2010, the national health and food safety authorities were alerted to the increased number of cases and an outbreak investigation was initiated. A public health alert was issue to national hospitals requesting prompt notification and reporting of cases. LRCESB continued to receive clinical isolates for typing. Continued monitoring detected two more cases with the outbreak strain in November 2010, and three more cases in January, February and March 2011 (two in the Lisbon and Vale do Tejo region and one in the Algarve). Thereafter, in February 2012, there were two new cases with the same strain in the Lisbon and Vale do Tejo region. The total number of outbreak cases between March 2009 and February 2012 was 30.

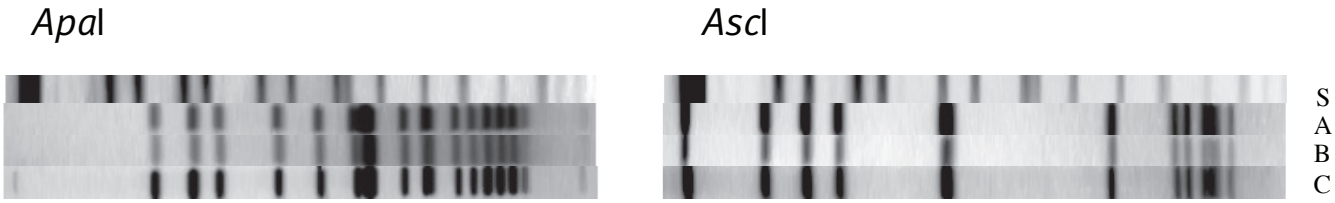
Trace-back and investigation of the food source

Analysis of the epidemiological questionnaires pointed to different types and sizes of food retailers and identified the following as possible sources of infection: cheeses (cured cheese and queijo fresco, made from pasteurised cow and goat milk), ice cream, ham and fermented sausages. On the basis of data gathered concerning the type of establishments where the food products were purchased, as well as the geographical location of the cases, suspected foods and foods commonly associated with listeriosis, the food safety authority inspected 42 food retailers and collected 103 samples for analysis (51 meat products, 24 dairy products, 13 ready-to-eat foods and 15 environmental swabs). *L. monocytogenes* was detected in four samples collected at a retailer: three from queijo fresco and one from a swab taken from a ham slicing-machine; one queijo fresco sample contained counts of *L. monocytogenes* greater than 100 colony-forming units/g.

PFGE typing revealed that isolates recovered from two queijo fresco samples of different brands from the same retailer showed the same PFGE type as the clinical isolates with the outbreak strain. Further investigation of the processing plants where these cheeses had been produced involved collecting and testing environmental and cheese samples. The outbreak strain was

Figure 3

Pulsed-field gel electrophoresis type of the *Listeria monocytogenes* outbreak strain from 2010 in Portugal, using *Apa*I and *Asc*I restriction enzymes



A: *L. monocytogenes* isolate from a listeriosis case from a hospital in the Lisbon and Vale do Tejo region (February 2010); B: *L. monocytogenes* isolate from a cheese sample collected at a retailer selected on the basis of the results of the epidemiological questionnaires (October 2010), C: *L. monocytogenes* isolate from a contaminated cheese sample collected at a processing plant located in the Alentejo region and identified by trace-back investigations (March 2011); S: *Salmonella* Braenderup size control.

detected in *L. monocytogenes* isolates from cheese samples from one of the two processing plants investigated (located in the Alentejo region) (Figure 3). Thus, cheeses produced by this plant were considered the probable source of the outbreak; cross-contamination between products or contamination from the environment, or both, may have occurred at retail level, as both suspected brands of queijo fresco were sold in the same market. As a result of these findings, the food safety authority recalled both products and more samples from the processing plant were analysed. Cheeses made with pasteurised cow and goat milk collected at the processing plant tested positive for *L. monocytogenes* and the collected isolates had the same PFGE pattern as the outbreak strain. Subsequently, in March 2011 the processing plant voluntarily suspended its activities during 15 days. After appropriate cleaning and disinfection measures, intensified product and environmental sampling was carried out. No positive samples were detected and products were allowed to be sold in the marketplace. Samples were then collected monthly by the food safety authority and no further positive samples have been detected.

Characteristics of listeriosis outbreak-associated cases

Of the 30 cases, two were MN cases, both of which occurred in 2010 (Table).

One MN case resulted in stillbirth and the other MN case involved a newborn with unknown outcome. For the 28 non-MN cases, isolates were collected from blood (n = 16), cerebrospinal fluid (n = 10) and from both blood and cerebrospinal fluid (n = 2). The mean age of the 27 non-MN cases with a reported age was 58.9 years (standard deviation: 17); the median was 64 years (range: 15–83); 13 non-MN cases were older than 65 years. The ratio of male:female non-MN cases was 22:6. Information was available for 20 non-MN patients with underlying conditions (e.g. diabetes mellitus, cancer, hepatitis, human immunodeficiency syndrome (HIV) infection/acquired immunodeficiency syndrome (AIDS)). For seven non-MN cases, no such information was available. The absence of known predisposing condition was reported for one 15 year-old patient. The overall case fatality rate, for MN and non-MN cases, was 37% (11/30).

Discussion

As there is no active surveillance programme for listeriosis in Portugal, outbreak detection is extremely difficult. The incubation period of the infection can be very long, up to 70 days, which makes it difficult to find a link between cases [1]. Detection of the outbreak

Table

Listeriosis outbreak-associated cases, Portugal, March 2009–February 2012 (n = 30)

Data	Number of cases				
	2009	2010	2011	2012	Totals
Clinical form					
Non-MN	5	18	3	2	28
MN	0	2	0	0	2
Sex					
Female	2	5	1	0	8
Male	3	15	2	2	22
Age of non-MN cases (years)					
<65	3	8	2	1	14
≥65	2	10	1	0	13
Age unknown	0	0	0	1	1
Clinical sample of non-MN cases					
Blood	4	10	1	1	16
CSF	1	6	2	1	10
Blood and CSF	0	2	0	0	2
Fatal outcome					
Non-MN	3	7	NA	NA	10
MN	NA	1	NA	NA	1

CSF: cerebrospinal fluid; MN: maternal/neonatal cases; NA: not available; non-MN: non-maternal/neonatal cases.

reported here was due mainly to retrospective investigations. The amount of time between the presumed onset of the outbreak (March 2009) and its recognition was extremely long (16 months). This long delay amplified the magnitude of the outbreak, leading to a high number of cases (n = 30) and a high case-fatality rate (36.7%). Underestimation of the number of cases is likely, as many cases usually go unreported and unrecognised, since patient data and strains are voluntarily reported and listeriosis is an infrequent disease [2], with clinical symptoms that are difficult to identify [16].

Typing of clinical and food isolates of *L. monocytogenes* by molecular techniques, such as PFGE, was essential for the identification of cheese of a specific brand as being the most probable source of contamination. Although a cheese from another producer was contaminated at retail by a strain with the outbreak-associated PFGE type, this was probably a result of cross-contamination since no positive samples were detected in the processing plant. Increased risk of cross-contamination of ready-to-eat foods by *L. monocytogenes* in a retail environment has been demonstrated in several studies [17-20]. For example, a quantitative risk assessment conducted by Endrikat et al. suggested that ready-to-eat deli meats sliced at a retailer are five times more likely to cause listeriosis than pre-packaged products (per annum basis) [21].

Additional information is needed for a better understanding of the risk factors and for the development of improved strategies for controlling *L. monocytogenes* in these environments.

The long duration of this outbreak (March 2009 to February 2012) is noteworthy and reinforces the importance of setting up an effective multidisciplinary team able to help ensure rapid notification of cases and the prompt submission of *L. monocytogenes* isolates for routine laboratory typing.

Of the 28 non-MN cases, 13 were 65 years of age or older and at least 20 cases presented an underlying condition. In European countries with established surveillance programmes, such as France, Germany and the United Kingdom, the incidence of listeriosis is reported to be increasing and the distribution of cases is shifting, primarily affecting elderly persons and those with predisposing medical conditions, leading to a high case fatality rate [1,2]. This is of concern as life expectancy increases, including for those who are immunocompromised (e.g. those with AIDS, under immunosuppressive therapy for cancer) [22]. In addition, food habits are changing worldwide, with an increasing demand for processed ready-to-eat foods [23]. Therefore, it is likely that there will be an increased risk of food-borne listeriosis.

Data gathered from the surveillance of human disease and also from all stages in the food production chain should be continuously collected and analysed to understand the ecology of *L. monocytogenes* and its routes of transmission. This will be crucial for developing enhanced strategies to control this organism and contribute to a decrease in the incidence of food-borne listeriosis in Portugal.

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Conflict of interest

None declared.

Authors' contributions

Maria Manuela Sousa was responsible for the collection of epidemiological data and for the link between hospitals and Centro de Biotecnologia e Química Fina (CBQF). Autoridade de Segurança Alimentar e Económica (ASAE) team was in charge of collection and analysis of food products and environmental samples. CBQF team was responsible for collecting clinical isolates supplied by the hospitals and food isolates supplied by ASAE. CBQF and Listeriosis Reference Centre for Canada teams were responsible for characterisation of the isolates. All authors participated in the analysis and interpretation of data. Rui Magalhães, Gonçalo Almeida, Vânia Ferreira and Paula Teixeira drafted the manuscript. Jeffrey Farber and Franco Pagotto critically reviewed the draft manuscript and provided substantive input. All authors approved the final version.

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Monitoring meticillin resistant *Staphylococcus aureus* and its spread in Copenhagen, Denmark, 2013, through routine whole genome sequencing

M D Bartels (mette.damkjaer@dadlnet.dk)[1,2], H Larner-Svensson[2,3], H Meiniche[3], K Kristoffersen[3], K Schønning[1,4], J B Nielsen[1,3], S M Rohde[3], L B Christensen[3], A W Skibsted[3], J O Jarløv[5], H K Johansen[6], L P Andersen[7], I S Petersen[5], D W Crook[8], R Bowden[9], K Boye[1], P Worning[3], H Westh[1,3,4]

1. Department of Clinical Microbiology, Hvidovre University Hospital, Denmark
2. These authors contributed equally to this work
3. MRSA Knowledge Center, Hvidovre University Hospital, Denmark
4. Institute of Clinical Medicine, University of Copenhagen, Denmark
5. Department of Clinical Microbiology, Herlev University Hospital, Denmark
6. Department of Clinical Microbiology, Rigshospitalet, Denmark
7. Department of Infection control, Rigshospitalet, Denmark
8. Infectious Diseases and Microbiology Unit John Radcliffe Hospital, Oxford, United Kingdom
9. Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom

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Typing of meticillin resistant *Staphylococcus aureus* (MRSA) by whole genome sequencing (WGS) is performed routinely in Copenhagen since January 2013. We describe the relatedness, based on WGS data and epidemiological data, of 341 MRSA isolates. These comprised all MRSA (n = 300) identified in Copenhagen in the first five months of 2013. Moreover, because MRSA of staphylococcal protein A (*spa*)-type 304 (t304), sequence type (ST) 6 had been associated with a continuous neonatal ward outbreak in Copenhagen starting in 2011, 41 t304 isolates collected in the city between 2010 and 2012 were also included. Isolates from 2013 found to be of t304, ST6 (n=14) were compared to the 41 earlier isolates. In the study, isolates of clonal complex (CC) 22 were examined in detail, as this CC has been shown to include the hospital-acquired epidemic MRSA (EMRSA-15) clone. Finally, all MRSA ST80 were also further analysed, as representatives of an important community-acquired MRSA in Europe. Overall the analysis identified 85 *spa*-types and 35 STs from 17 CCs. WGS confirmed the relatedness of epidemiologically linked t304 neonatal outbreak isolates. Several non-outbreak related patients had isolates closely related to the neonatal isolates suggesting unrecognised community chains of transmission and insufficient epidemiological data. Only four CC22 isolates were related to EMRSA-15. No community spread was observed among the 13 ST80 isolates. WGS successfully replaced conventional typing and added information to epidemiological surveillance. Creation of a MRSA database allows clustering of isolates based on single nucleotide polymorphism (SNP) calling and has improved our understanding of MRSA transmission.

Introduction

Whole genome sequencing (WGS) is expected to transform the practice of clinical microbiology and infection control [1,2]. Advances in WGS technology and extended multiplexing on desktop-based WGS machines has reduced sequencing cost to approximately EUR 100 per genome. While detailed bioinformatics analysis remains a challenge, commercial software and web-based solutions are now available for performing multilocus sequence typing and screening for gene-based antibiotic resistance on the WGS data [3].

The sequence-based typing method staphylococcal protein A (*spa*)-typing of meticillin-resistant *Staphylococcus aureus* (MRSA) has been performed since 2003 at the MRSA Knowledge Center, Department of Clinical Microbiology, at Hvidovre Hospital [4]. In January 2013, this Sanger sequencing method was replaced by WGS of all MRSA isolates and we routinely produce 24 full genomes twice a week. From WGS data, script-based bioinformatics programmes are used to identify *mec*A, *mec*C, *nuc*, *ccr* and Panton–Valentine leukocidin (PVL) genes as well as the arginine catabolic mobile element (ACME) genes (*arc*A to *arc*D). Bioinformatics is also used to determine direct repeat units *(dru)* types and multilocus sequence types (MLST). Furthermore, single nucleotide polymorphism (SNP) analysis is used routinely to compare relatedness of MRSA isolates.

Table

Typing information on meticillin resistant *Staphylococcus aureus* isolates derived from analysing whole genome sequence data and number and proportion of isolates obtained by screening, Copenhagen, Denmark, 2013[a] (n=341)

Clonal complex	Isolates N	Sequence types (N)	*Spa*-types t (N)	Panton–Valentine leukocidin N (%)	Obtained by screening N (%)
1	20	ST1 (12); ST1_SLV (1); ST772 (6); ST2689 (1)	t127 (10); t177 (1); t345 (1); t386 (1); t657(4); t5388 (1); t5608 (1); t7358 (1)	7 (35)	8 (40)
5	45	ST5 (30); ST5_SLV (1); ST105 (1); ST149 (6); ST149_SLV (2); ST225 (2); ST1457 (1); ST2626 (2)	to02 (33); to03 (2); to62 (3); to67 (1); t105 (1); t442 (1); t579 (2); t688 (1); t854 (1)	6 (13)	27 (60)
6	56[b]	ST6	t304 (55); t104 (1)	0 (0)	40 (71)
8	59	ST8	to08 (28); to24 (17); t118 (1); t121 (1); t304 (3); t801 (1); t596 (1); t1476 (1); t1774 (2); t2849 (1); t3240 (1); t6197 (1); t13119 (1)b	26 (44)	30 (51)
22	29	ST22 (20); ST22_SLV (1); ST1327 (6); ST2371 (2)	to05 (8); to22 (1); to32 (1)d; t223 (6); t670 (1); t852 (5); t1328 (1); t2834 (1); t4326 (2); t11808 (2); t12093(1)	10 (34)	19 (66)
30	42	ST30 (36); ST36 (1); ST1456 (4)	to19 (29); to21 (4); t253 (2); t685 (1); t964 (1); t1133 (2); t1752 (2); t2872 (1)	38 (90)	18 (43)
45	13	ST45 (9); ST46 (2); ST508 (1)	to15 (4); to26 (1); to50 (1); to69 (1); t116 (1); t230 (1); t583 (1); t728 (1); t2988 (2)	0 (0)	7 (54)
59	9	ST59 (8); ST59_SLV (1)	t437 (8); t3513 (1)	6 (67)	5 (56)
72	18	ST72 (16); ST72_SLV (2)	t148 (5); t324 (8); t791 (3); t2409 (1); t11917 (1)	2 (11)	14 (78)
80	17	ST80 (13); ST80_SLV (4)	to44 (11); t131 (1); t376 (3); t1028 (2)	17 (100)	14 (82)
88	10	ST88 (9); ST859 (1)	t325 (1): t690 (2); t729 (1); t1855 (1); t5147 (5)	2 (20)	2 (20)
97	1	ST97	t267	0 (0)	0 (0)
130	1	ST130	t1048	0 (0)	0 (0)
152	2	ST152	t355 (1); t454 (1)	1 (50)	1 (50)
228	1	ST111	to41	0 (0)	0 (0)
239	1	ST239	t138	0 (0)	1 (100)
398	5	ST398	to11 (1); to34 (4)	0 (0)	2 (40)
Not known	6	ST140 (2); ST1245 (2); ST1633_SLV; ST1943 (1)	t296 (2); t355 (1); t843 (2); t978 (1)	1 (17)	4 (67)
MLST[c] failure	7	MLST failure[c]	to24 (2); to62 (2); t304 (1); t437 (1)	1 (14)	2 (29)
Total	341	–	–	117 (34)	194 (57)

MLST: multilocus sequence type; SLV: single locus variant; *Spa*-type: staphylococcal protein A-type; ST: sequence type.

[a] Whole genome sequencing was conducted in 2013 on the 341 isolates. Of these, 300 had been obtained in 2013, while 41 were selected t304, ST6 isolates from the years 2010 to 2012.

[b] 41 of these isolates were from the years 2010 to 2012.

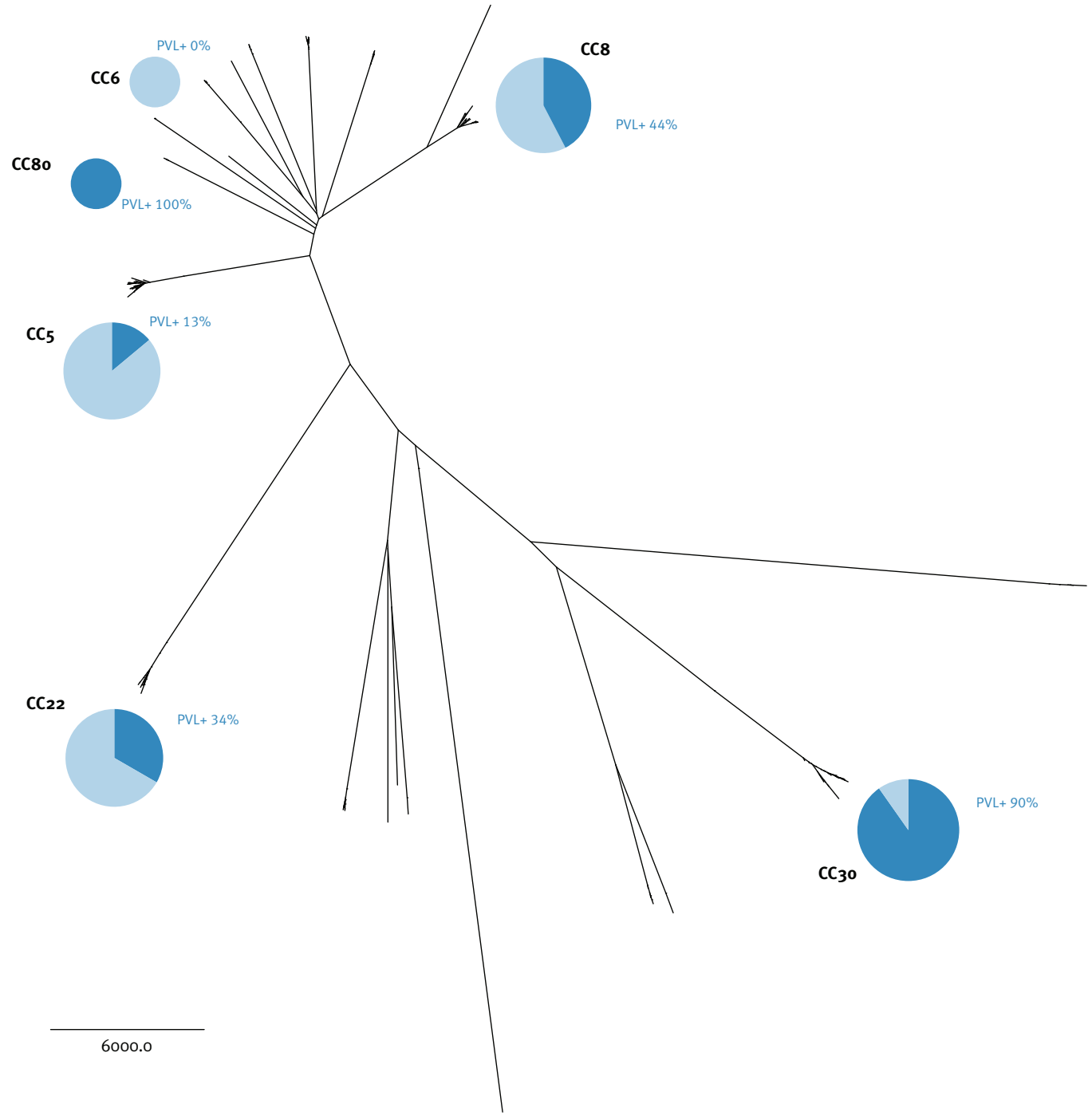
[c] MLST failure: In seven cases a ST could not be obtained due to a gene being in two contigs, the identification of a new allele or a new combination of alleles leading to a novel ST not listed in the MLST database.

[d] This isolate was included as a ST22 isolate having six of the alleles in ST22, but a missing *glpF* gene.

In this study, the overall relatedness of all consecutive MRSA isolates from the first five months of 2013 (n=300) in Copenhagen, Denmark, was assessed by SNP analysis. In addition, 41 isolates of *spa*-type (t) 304 (t304) from 2010 to 2012 were whole genome sequenced, and compared with 14 of the isolates from 2013, that were determined to be t304, ST6. A continuous outbreak has been caused by t304, ST6 in neonatal wards in Copenhagen since 2011, and the t304 isolates from prior to 2013 were included to study the t304, ST6 isolates both before and during the outbreak period in more detail. All clonal complex (CC) 22 isolates were selected for detailed analysis as this CC contains the globally important international hospital-acquired MRSA (HA-MRSA) clone epidemic MRSA-15 (EMRSA-15) [5]. Finally, all ST80 isolates (European clone) were described in more detail as representatives of an important community-acquired MRSA (CA-MRSA) in Europe [6].

Figure 1

Phylogenetic tree of all meticillin resistant *Staphylococcus aureus* isolates found in Copenhagen, Denmark, January–May 2013 (n=300)

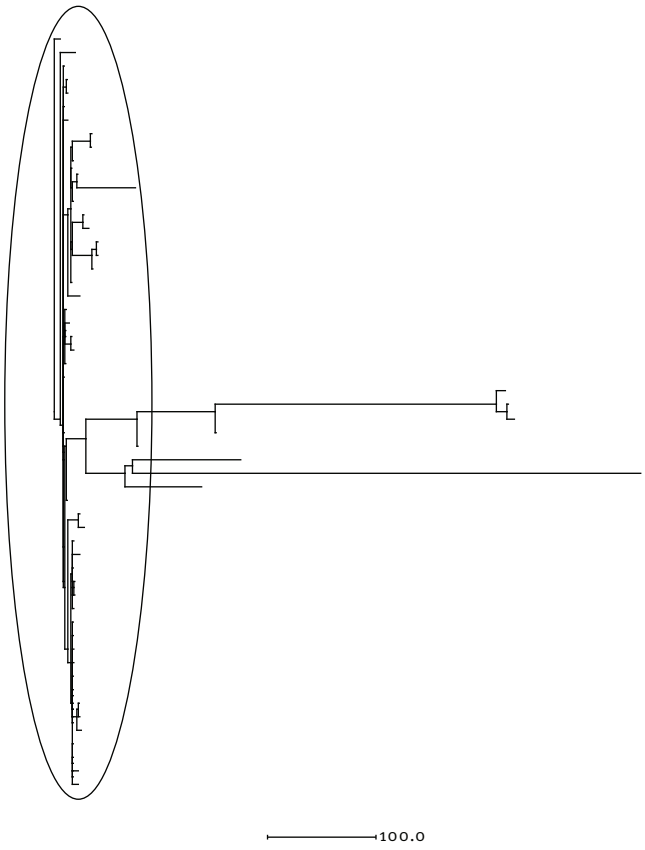


CC: clonal complex; PVL: Panton–Valentine leukocidin.

Six of the larger clonal complexes (CC) are indicated at the end of branches, as well as the percentage of PVL-positive isolates within each CC. The length of the scale bar corresponds to 6,000 single nucleotide polymorphisms (SNPs).

Figure 2

Unrooted neighbour joining tree of meticillin resistant *Staphylococcus aureus* isolates of staphylococcal protein A-type 304, sequence type 6, Copenhagen, Denmark, 2010–2013 (n=55)



The circle marks 49 isolates that include the 34 neonatal outbreak isolates and 15 isolates with between four and 36 single nucleotide polymorphisms (SNPs) to the closest neonatal isolates. These 49 isolates are further presented in Figure 3. The length of the scale bar corresponds to 100 SNPs.

Methods

Setting

The Capital Region of Denmark manages 12 hospitals and the general practice healthcare services of the region. This study covers the region's 1.73 million inhabitants. MRSA isolates are from individual patients. They are identified in clinical or screening samples submitted to the three Departments of Clinical Microbiology in the Capital Region. WGS is only performed at Hvidovre Hospital and all MRSA isolates from the two other Departments of Clinical Microbiology are sent to Hvidovre Hospital for WGS.

Isolates and patient data

Each cultured isolate is immediately confirmed to be MRSA with an in-house multiplex real-time polymerase chain reaction (PCR) that can detect the presence of *nuc*, *mec*A and *mec*C (data not shown). In this study, a total of 341 MRSA-confirmed isolates were whole genome sequenced. Three-hundred consecutive isolates were collected between 1 January 2013 and 31 May 2013, while 41 isolates of t304, ST6 were collected in the period from 2010 to 2012. The latter 41 isolates were originally *spa*-typed by Sanger sequencing and were whole genome sequenced in 2013.

According to the national MRSA guidelines all patients seen in a hospital are asked for MRSA risk factors. These include hospitalisation abroad within the last six months, previous MRSA positivity, contact to a MRSA positive person and contact with pigs within the last six months. In our internal microbiology database, a case report form is generated for each patient. The report contains information on date of sampling, submitting hospital unit or general practitioner, sample site, known MRSA contacts, household relations and antibiogram of the isolate. Sample types were categorised as being either from infection or screening. Screening samples were from hospitalised patients having risk factors as mentioned above, household contacts of MRSA patients and/or outbreak investigations in hospitals and nursing homes.

Whole genome sequencing procedures

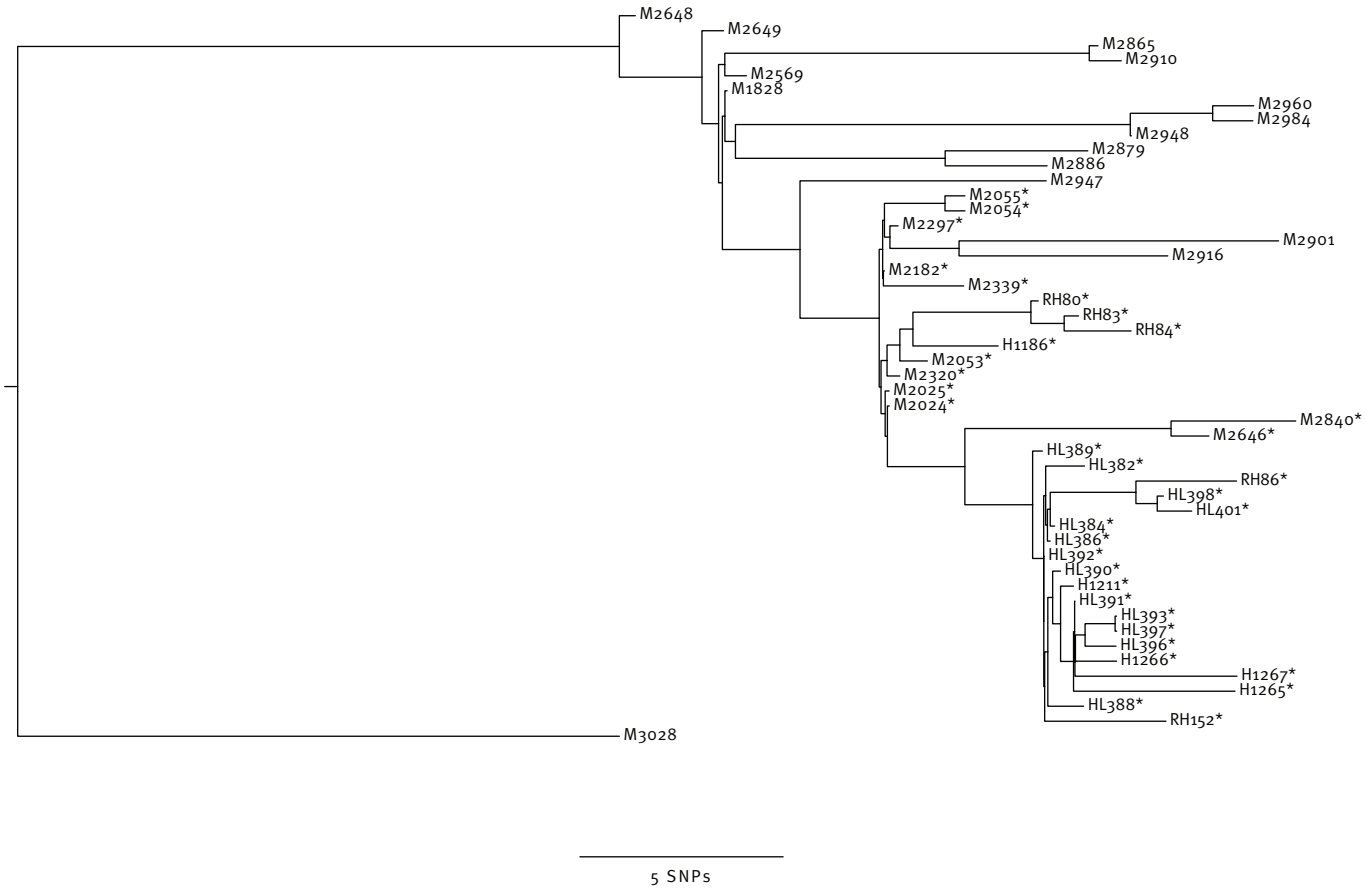
Each confirmed MRSA was whole genome sequenced on a MiSeq (Illumina, United States (US)). The current workload is a four-day set-up. DNA concentrations were normalised using a Qubit (Invitrogen, United Kingdom (UK)). Libraries were made with Nextera XT DNA sample preparation kit (Illumina, US), genomes multiplexed to 24 isolates per run and sequenced with 2 x 150 bp paired-end reads. For the detection of single nucleotide variants relative to the reference, we used a reference-based mapping approach via Stampy (Lunter G). Reads were mapped to a USA300 reference sequence (US300_TCH1516) using Stampy v1.0.11 with no BWA pre-mapping and an expected substitution rate of 0.01 [7]. Variants were called using SAMtools v0.1.12 mpileup command with options –M0 -Q30 –q30 –040 –e20 –h100 –m2 –D –S. The genome was assembled using Velvet v1.0.11 [8], with hash (kmer) size and coverage parameters optimised to give the highest number of bases in contigs with length greater than 1 kb. In cases were a *spa*-type could not be generated, WGS was repeated.

Staphylococcal protein A-types, sequence types and Panton–Valentine leukocidin genes

An additional script has been developed in-house to analyse MRSA genomes for *mec*A, *mec*C, *nuc*, *ccr* genes, *spa*-type, MLST, *dru* types and PVL. *Spa*-types and ST were called from the assembled contigs by comparison to the published types on the SpaServer [9] and the *S. aureus* MLST.net database [10]. A CC was assigned based on the MLST.net webpage. Each isolate was reported in the Laboratory Information System with confirmation of being an MRSA, *spa*-type, ST and presence or absence of PVL genes.

Figure 3

Unrooted neighbour joining tree of meticillin resistant *Staphylococcus aureus* isolates of staphylococcal protein A-type 304, sequence type 6, that were implicated in a neonatal outbreak (n=34) as well as closely related isolates (n=15), Copenhagen, Denmark, 2010–2013



The isolates depicted in the tree are those marked in Figure 2. The numbers at the end of branches are the internal isolate numbers. The 34 isolates epidemiologically related to the neonatal outbreak are marked with asterisks (*). The length of the scale bar corresponds to five single nucleotide polymorphisms (SNPs).

Phylogenetic analyses

Phylogenetic analyses were performed using a distance method, based on pairwise nucleotide sequence alignments for the *S. aureus* core genome, as defined by USA300, among all strains. Phylogeny was inferred by neighbour-joining analysis as implemented in SplitsTree v4.11.3 [11]. Tree drawing was managed with FigTree [12].

Staphylococcal protein A-type 304 analysis

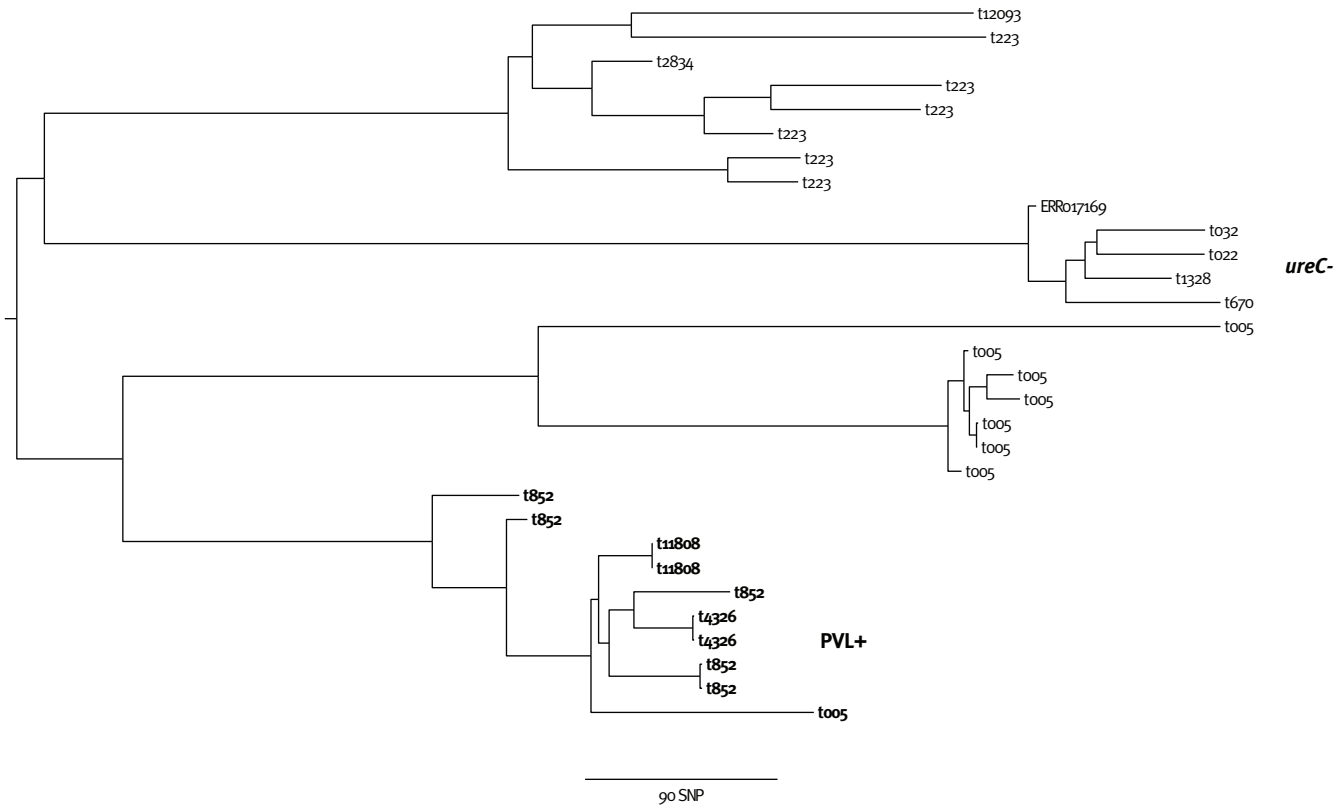
To illustrate the value of WGS in outbreak investigation 14 t304 isolates obtained in 2013 and 41 t304 isolates from 2010 to 2012 were sequenced. t304 has been associated with a neonatal intensive care unit outbreak spreading to several hospitals in Copenhagen mainly in 2011 and 2012. Both isolates from persons with and without known contact to the neonatal units were included. Epidemiological data for all persons were registered and compared with the findings in the SNP analysis.

Analysis of sequence type 80 and clonal complex 22 isolates

To study the import and spread of international well known MRSA clones, we analysed in depth CC22 isolates and their relationship to a representative of the international, HA-MRSA clone, EMRSA-15. The reference isolate ERR017169 of ST22-A2 was included as a quality control (QC) isolate for EMRSA-15 [5]. All CC22 isolates were also screened for a 1bp deletion in the *ure*C gene, an identifier of EMRSA-15 according to a recently published paper [5]. The European clone (ST80, PVL positive) was studied more in detail as a representative of a typical CA-MRSA. This clone is known to be very homogeneous and therefore a QC isolate was not included [13]. Epidemiological data for all persons were registered and the results of the analysis of these data were compared with the findings in the SNP analysis.

Figure 4

Unrooted neighbour joining tree with the ERR017169 strain representative of epidemic meticillin resistant *Staphylococcus aureus* (EMRSA)-15 and 29 MRSA isolates of clonal complex 22, Copenhagen, Denmark, January–May 2013



PVL: Panton–Valentine leukocidin; SNP: single nucleotide polymorphisms.

For each isolate the staphylococcal protein A-type (t) is displayed at the end of branches, and the 10 PVL-positive isolates are in bold. Isolates with a 1 bp deletion in the *ureC* gene characteristic of EMRSA-15 are also indicated. The length of the scale bar corresponds to 90 SNPs.

Results

Overall relatedness of the isolates

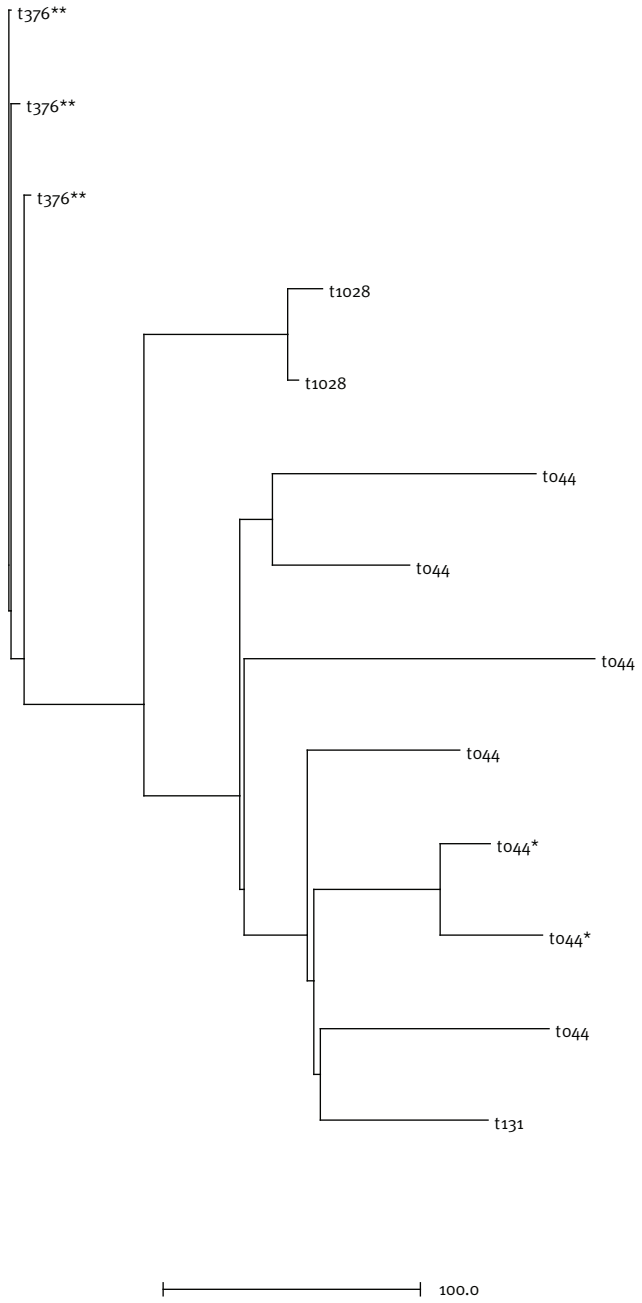
A total of 194 (57%) of the 341 isolates were obtained through MRSA screening (Table). *mec*A was found in 337 isolates while four isolates contained *mec*C. Based on WGS, a *spa*-type could be assigned for all 341 isolates. Of these, 15 obtained a *spa*-type after repeating WGS. A ST was generated by our script in 334 of 341 isolates (98%). A lacking ST (7 isolates) was caused by a gene being in two contigs, the identification of a new allele, or a new combination of alleles leading to a novel ST not listed in the MLST database. Eighty-five *spa*-types and 35 STs as well as eight single locus variants were identified (Table). Of the 334 isolates with a ST, 328 fell into 17 CCs. The CC for the remaining six isolates with an identified ST could not be determined using the MLST database.

Most of the 300 isolates from 2013 belonged to CC 8 (59 isolates), followed by CC5 (45 isolates) and CC30 (42 isolates). In the first five months of 2013, PVL genes were present in 117 of the 300 isolates (39%) and in 33 *spa*-types and 16 STs. Among the international well known MRSA clones, we found isolates that, based on *spa*-type, MLST/CC and PVL-positivity were similar to US300 (t008, ST8, PVL+; n=28), South-West Pacific clone (t019, ST30, PVL+; n=29), European clone (t044, ST80, PVL+; n=7), ST239 and EMRSA-15 (ST22; n=20). Only five of the 300 isolates belonged to the livestock associated ST398.

The neighbour joining tree of the 300 MRSA isolates from 2013 (Figure 1) reveals a picture of high genetic diversity of MRSA in Copenhagen. Major clones (clades) are easily seen as clusters at the end of branches (Figure 1). The larger CCs are marked, as is the amount of PVL-positive isolates within these CCs. Within each CC further discrimination can be obtained by comparing the isolates in an unrooted neighbour joining tree through SNP calling.

Figure 5

Unrooted neighbour joining tree of meticillin resistant *Staphylococcus aureus* isolates of sequence type 80 found in Copenhagen, Denmark, January–May 2013 (n=13)



*Same household, 59 SNPs between the two isolates.

**Father and two daughters.

SNPs: single nucleotide polymorphisms.

The staphylococcal protein A-type (t) of each isolate is displayed at the end of branches. The length of the scale bar corresponds to 100 SNPs.

Staphylococcal protein A-type 304 analysis

Healthcare-associated MRSA outbreaks are rare in Denmark, and the only clusters identified as HA-MRSA were t304, ST6, PVL-negative, associated with a neonatal outbreak spreading in neonatal wards in the Capital Region (Figure 2 and Figure 3) and t024, ST8, PVL-negative, a clone that has been healthcare associated since 2003 [14]. t304, ST6 in neonates and their relatives were, apart from one case in February 2013, all identified either in the last seven months of 2011 (n=7) or in 2012 (n=26) . Comparing the 55 t304, ST6 isolates from Copenhagen in the period from 2010 to 2013, the epidemiologically related outbreak isolates (n=34) were genetically closely related, having from 1 to 10 SNPs compared with the closest related outbreak isolates. t304 isolates from the same neonatal ward were in most cases even closer related with a maximum of five SNPs separating them. The t304 patients who were not epidemiologically related to the outbreak (n=21), were in six cases clearly different from the outbreak strains with between 67 and more than 200 SNPs separating them from the outbreak strains. Seven isolates had between 11 to 36 SNPs compared with the closest related of the outbreak isolates, while eight isolates only had from four to ten SNPs (Figure 2 and Figure 3).

Clonal complex 22 analysis

An expansion of the CC22 isolate tree is shown in Figure 4 and compared with a ST22 isolate representative of EMRSA-15 [5]. The 29 Danish CC22 isolates are diverse and include ten different *spa*-types. Ten of these isolates were from patients living in four different households. Based on SNP calling, the clusters of these isolates within households differed between 0 to 14 SNPs, while epidemiologically unrelated isolates differed at between 69 to 1,207 SNPs. PVL was found in ten of the 29 isolates (32%) including all t852, t4326, t11808 and one of eight t005 isolates. Four of the 29 isolates had the 1 bp deletion in *ure*C described as typical for EMRSA-15 and were the isolates closest related to the English prototype ST22 isolate ERR017169 based on SNP calling. These four patients had been hospitalised before the finding of MRSA, two of them in Italy and Spain and two in Denmark. Only one of the remaining 25 CC22 patients had been hospitalised before the finding of MRSA. Nineteen of the 29 patients were not of ethnic Danish origin, and one of the five ethnic Danes had recently travelled in India. We assume that most of these CC22 isolates were acquired through international travel.

Sequence type 80 analysis

A total of 13 isolates belonging to the European clone are presented in Figure 5. They were all PVL-positive and represented four different *spa*-types of which t044 accounted for seven isolates. Two of the t044 isolates differing with 59 SNPs were from two persons living in a three-generation household. SNP analysis revealed that the 59 SNPs were dispersed throughout the genome, and could therefore not be explained by a single recombination event. The rest of the t044 isolates were from patients not epidemiologically related, and they differed with 130 to 365 SNPs. There were three t376 isolates, which were from a father and his

two daughters and which had a maximum of 10 SNPs between them. Two isolates of t1028 had only 17 SNPs between them, but we have not identified an epidemiological link between these patients.

Discussion

The emergence of predominantly CA-MRSA was first recognised in Denmark in 2003 [14]. Since 2003 the number of new MRSA patients in Copenhagen has increased 20-fold from 33 to 674 cases in 2012 [4]. The genetic diversity that was already seen in the first years of the MRSA epidemic has increased over the years, and in 2012, 142 different *spa*-types were found in Copenhagen (data not shown). This diversity is mainly related to travel and tourism e.g. cases where Danish families visit relatives in countries where MRSA is prevalent in the community, and acquisition in persons working with healthcare abroad [15]. The high diversity based on *spa*-types indicates a low level of endemic transmission, and could be further differentiated in this study, based on WGS. Cases imported through hospitalisation abroad are routinely found through screening of these patients when they are transferred to Danish hospitals. In general, MRSA is first isolated in clinical samples and subsequent ring screening usually identifies carriers in their households. However, there is an under-identification of MRSA as a number of patients have had recurrent skin and soft tissue infections before they are sampled. This means that some patients who only have a single infection or short-term carriage state and then clear MRSA are not identified.

Livestock associated MRSA, ST398, accounted for less than 2% of MRSA in Copenhagen, a capital city surrounded by a large suburban area with little livestock production. However, ST398 was the most common clone in the whole of Denmark in 2012 accounting for 232 of 1,556 isolates (15%), predominantly seen in regions with a large pig production [4].

Two UK studies on neonatal MRSA outbreaks have confirmed the usefulness of WGS in outbreak investigations, and showed that the number of SNPs between outbreak related isolates were less than 15 [16,17], except in one case where a mutation in the *mutS* gene was explained as the reason for more divergence. Furthermore, Harris et al. [16] found additionally ten isolates that were initially not considered part of a particular outbreak, but were closely related to outbreak strains by WGS, and retrospective epidemiological links were found in most cases. In our study, WGS could confirm the relatedness between 34 t304 isolates that were epidemiologically related to the neonatal outbreak in Copenhagen. Six isolates were clearly different from the outbreak strains, and this was also consistent with patient data. Fifteen isolates, that were not considered outbreak-related based on epidemiological data, differed in eight cases with only four to 10 SNPs to the closest related outbreak strains, and in seven cases with 11 to 36 SNPs (Figure 3). Since 11 of these 15 isolates were obtained in 2013, we hypothesise that a community transmission chain might exist, but that the epidemiological link between patients is missing. WGS seems promising in outbreak investigations, and might add information on the spread of outbreak clones outside the hospital setting, where the connection between patients is frequently not clear.

The CC22 isolates found were diverse and only four isolates were similar to the English prototype CC22, EMRSA-15. Two of these four isolates were imported from Spain and Italy. In 2012, t032, CC22 accounted for 89 isolates and was the fifth most common MRSA type in Denmark representing almost 6% of new MRSA cases [4], but only seven of these 89 isolates were from the Capital Region [18].

The typical CA-MRSA ST80 was only found in 13 patients in the five month period. There is evidence of repeated introduction of this clone into Denmark with only one case of possible community spread, t1028, based on the number of SNPs (Figure 5). Although there is no consensus on how many SNPs define whether isolates are related or unrelated [19], it was surprising that two household isolates of ST80 had as many as 59 SNPs between them. A closer look at this household revealed that this family of three generations immigrated to Denmark five years prior, and presumably have been MRSA carriers for many years. According to Holden et al. [5], SNPs are estimated to occur in the core genome at a rate of 1.3×10^{-6} substitutions per nucleotide per year or three to four SNPs per year. Based on the relatively few closely related isolates in our study, we wonder if we will have a congruent SNP evolution for all MRSA clones.

Our policy until the end of 2012 has been to *spa*-type all MRSA using differences in *spa*-types to delineate different clones e.g. t024 different from t008 [20]. However, when less common *spa*-types were observed the link to international clones was often not seen. For example, the rare t1133, ST1456, PVL-positive, with only 11 isolates in the SpaServer database, was not easily associated to the South-West Pacific clone (t019, ST30, PVL+) before the neighbour joining tree clustered them together. Currently, the *spa*-type, ST and absence/presence of PVL are routinely found from the laboratory information system. The cost of WGS is steadily going down and today it would be more expensive for us to perform traditional Sanger sequencing for *spa*-typing and MLST. Furthermore, the WGS platform is universally applicable and is now routinely used in other healthcare-associated outbreak investigations.

A disadvantage is the slower turnaround time in our WGS-setup before typing results can be delivered. WGS is run in batches of 24 isolates, typically twice weekly. This means that from the date of sample to a WGS typing result can be up to 12 days or 4–8 days after the isolate is received for WGS. The current MLST *S. aureus* database contains fewer isolates and types than the *spa* server database and this leads to a number of

isolates only having six known alleles and one novel allele. In these cases our software calls all the STs with six matches, and we manually assign the ST as a single locus variant (SLV) of the most prevalent ST.

The data presented in this study show that after building up a local MRSA whole genome database, real time WGS gives additional information to the evolution and spread of MRSA. The method can be used to investigate outbreaks, but epidemiological data still need to be included. More insight into how to interpret the relatedness between isolates based on SNP analysis is needed as well as improved software designed for persons without bioinformatics skills.

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Conflict of interest

None declared.

Authors' contributions

MDB, HLS and HW: Contributed substantially to the design, the analysis and the interpretation of data. Drafted the work and revised it for important intellectual content. HM, KK, KS, JBN, SMR, LBC, AJWS, JOJ, HKJ, LPA, ISP, DWC, RB, KB, PW: Contributed substantially to the interpretation of data. Revised the work critically for important intellectual content.

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