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Acute flaccid paralysis following enterovirus D68 associated pneumonia, France, 2014

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Human enterovirus D68 (EV-D68) is known to be associated with mild to severe respiratory infections. Recent reports in the United States and Canada of acute flaccid paralysis (AFP) in children with detection of EV-D68 in respiratory samples have raised concerns about the aetiological role of this EV type in severe neurological disease. This case study is the first report of AFP following EV-D68 infection in Europe.

We report the first case of acute flaccid paralysis (AFP) following enterovirus-D68 (EV-D68) infection in Europe. The United States (US) and Canada are currently experiencing nationwide outbreaks of EV-D68 infections associated with severe respiratory diseases especially in children with underlying respiratory disease that began in mid-August 2014 [1,2]. Concomitantly, clusters of neurological illness characterised by AFP with anterior myelitis have been reported in the US and Canada [3,4]. The detection of EV-D68 in nasopharyngeal specimens of some affected children raises the question of a possible link between EV-D68 infections and severe neurological disease.

Human enterovirus D68 belongs to the enterovirus D species within the *Enterovirus* genus in the *Picornaviridae* family. Biologically close to rhinoviruses, EV-D68 has been mainly associated with acute respiratory infection with clinical presentation ranging from mild to severe disease requiring intensive care [5–11].

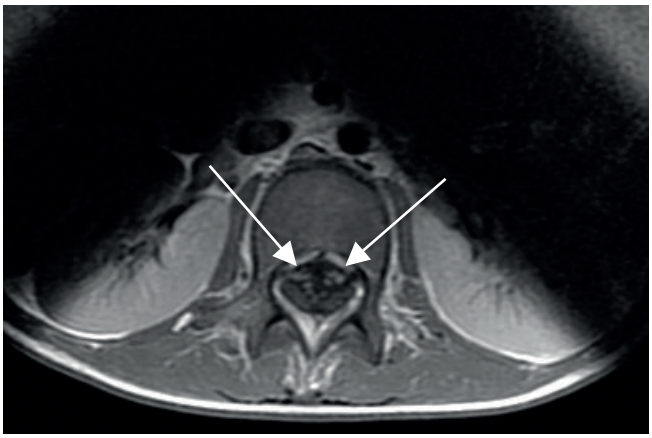
Case report

The patient was a previously healthy four year-old boy who was initially taken to his general practitioner's surgery for headache and vomiting on 20 September 2014. On 22 September 2014, he presented with a febrile meningeal syndrome without any sign of encephalitis.

Cerebrospinal fluid (CSF) showed pleocytosis (190 leucocytes: 92% of lymphocytes, norm<10 leucocytes) with normal protein and glucose levels, consistent with aseptic meningitis. On 26 September, he was transferred to the paediatric Intensive Care Unit for mechanical ventilation and fluid restoration because of acute respiratory distress and haemodynamic failure. Chest X-ray and thoracic tomodensitometry (TDM) confirmed bilateral pneumonia. Intravenous antibiotic therapy (ceftriaxone) was initiated. Acute myocarditis with apical hypokinesia was assessed by ultrasound examination. Levels of N-terminal of the prohormone brain natriuretic peptide (NT proBNP) and troponine Ic were up to 2,925 ng/L (norm:<450 ng/L) and 3.06µg/L (norm:<0.045 µg/L), respectively. Leucocytes were elevated two–three times above the upper limit of normal (20,630/mm^3, norm: 4,500–13,000/mm^3) with 16,880/mm^3 of polynuclear cells (norm: 1,500–8,000/mm^3). C-reactive protein level was elevated (75.8 mg/l, norm:<10 mg/l). The patient received 0.5 g/kg/day of

Figure 1

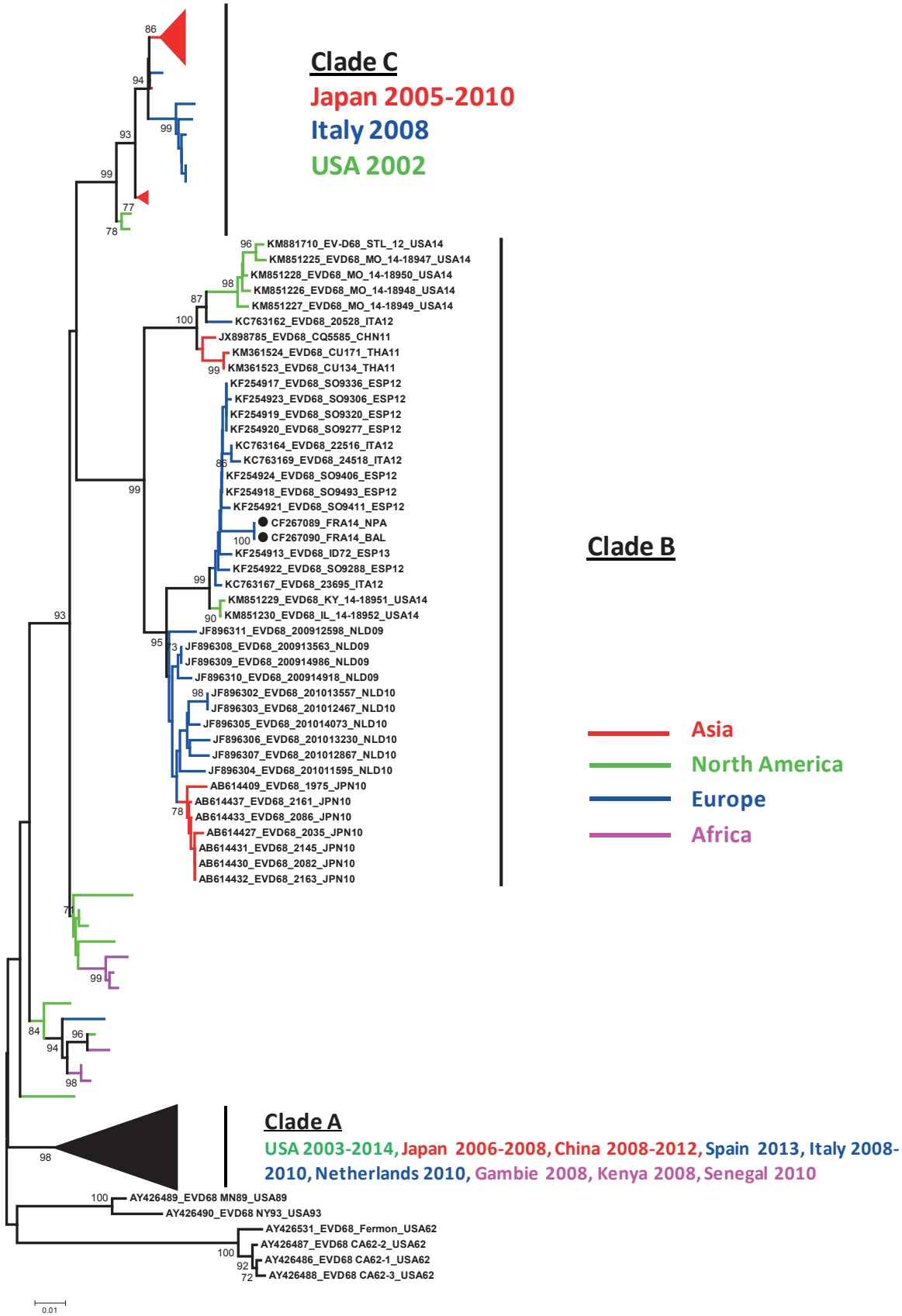
Spinal magnetic resonance image, acute flaccid paralysis case following enterovirus D68 infection, France, 2014



Gadolinium enhancement of the ventral nerve roots of the cauda equina is shown (arrows).

Figure 2

Phylogeny of enterovirus D68 sequences inferred with 190 VP1 sequences



The phylogenetic tree was constructed by the neighbor-joining method and evaluated with 1,000 bootstrap pseudoreplicates, using MEGA5. Only bootstrap values › 70 % are indicated. Genetic distances were calculated with Tamura-Nei's model of evolution and branch length is drawn to the indicated scale (proportion of nucleotide substitutions per site. Sequences were 740 pb long and started to nucleotide 1 relative to the VP1 gene of the Fermon prototype strain. For clarity, taxon names are not fully included in the tree except for clade B. The strains identified in the nasopharyngeal aspirate (CF267089_FRA14_NPA) and in the bronchoalveolar fluid (CF267089_FRA14_BAL) are labeled with a filled circle. Geographical origins and time of isolation of strains are indicated by the ISO-code abbreviation followed by the year of isolation.

intravenous immunoglobulin (IVIG) and milrinone for four days. On 27 September, he presented with flaccid tetraparalysis and dysphagia. Cerebral magnetic resonance imagery (MRI) was normal but spinal MRI showed gadolinium enhancement of the ventral nerve roots of the cauda equina (Figure 1).

Somatosensory evoked potentials confirmed that only the motor pathway was affected. Acute polyradiculoneuritis was excluded because there was no albumino-cytological dissociation and no antiganglioside antibodies in the CSF. There was no paraneoplasic syndrome (whole body-TDM and biological tumour markers were negative) and no inflammatory disease. Plasmapheresis and IVIG were implemented to shorten the recovery period. As of 6 november, the child has only recovered partial mobility of the extremities and of his left arm.

The child had up-to-date immunisation against poliomyelitis. He had neither underlying respiratory illness nor previous history of chronic disease, immunodeficiency or tick exposure. He had not travelled recently outside France and had had no contact with anyone arriving from North America. No family member presented with respiratory symptoms.

Blood, pre-IVIG serum, urine, respiratory and stool samples were screened. Bacteriological investigations including cultures, serology and genome detection in blood and CSF yielded negative results (Table).

Virological screening consisting in viral genome detection for numerous neurotropic viruses including EVs of three consecutive CSF was negative, as were all serological tests (Table). Viral cultures were negative. Rhinovirus-EV genome was detected in nasopharyngeal aspirates, bronchoalveolar fluid (BAL) and a stool sample using a one-step RT-PCR with previously described primers targeting the 1A and 1B regions encoding the VP4-VP2 capsid proteins [12]. To distinguish between rhinoviruses and EVs, amplified products were subjected to direct sequencing as previously described [13]. Blast analysis confirmed by phylogenetic analysis with VP4-VP2 sequences of rhinovirus and EV prototype strains assigned the strains to EV-D68. Partial 1D gene encoding the VP1 capsid protein was amplified by semi-nested RT-PCR using EV-D68 specific primers described by Tokarz et al. [14] in respiratory and stool samples, and subsequently sequenced (accession number LN626610). Phylogenetic investigation with all available sequences of EV-D68 (as of 9 October 2014) indicated that the strains belonged to clade B, according to the classification previously described [14]. The VP1 sequences were genetically close to sequences of some of the EV-D68 strains detected in 2014 in the United States (Figure 2).

Discussion

While EV-D68 has to date been almost exclusively associated with respiratory diseases, investigations are currently underway to determine its role in the acute neurological illnesses that have been reported in children in the US [3] and in Canada [4] since August 2014. Nine EV-D68-associated deaths are currently being investigated at the US Centers for Disease Control and Prevention (CDC) to confirm or refute EV-D68 as the cause of death [15]; as of 5 November, no information has been released about the death's preceding symptoms. The case reported here meets the definition given by CDC to identify similar neurological manifestations characterised by acute onset of focal limb weakness occurring on or after 1 August 2014 and MRI showing a spinal cord lesion largely restricted to grey matter [16]. Common features with the cases reported in the US include (i) respiratory illness preceding development of neurological symptoms, (ii) a local epidemiological context of EV-D68 detection among children admitted to hospital for respiratory infections leading to asthma crisis (data not shown) and (iii) EV-D68 detection in respiratory samples. By contrast, to our knowledge, neither meningeal syndrome nor myocarditis and acute respiratory distress syndrome had been reported in the days preceding the onset of paralysis in the US patients. The enterovirus genome was not detected in the CSF of this patient and we cannot assert that EV-D68 was associated with meningitis. There are two case reports in the literature of EV-D68 infection associated with severe neurological disease as evidenced by detection in the CSF [17,18]. As in recent reports, the significance of EV-D68 association with AFP is hampered by the fact that it was only detected in respiratory or stool samples, in which enteroviruses can be detected many weeks after infection. However, the absence of detection in CSF does not necessarily rule out this possibility since poliovirus and EV-A71, two recognised neurotropic EVs, are not frequently recovered [19]. Further physiopathological studies may be needed to assess the neurotropism of EV-D68.

There are increasingly numerous reports of polio-like illnesses in the US (64 cases as of 30 October 2014) [15]. Surveillance of AFP cases has already been implemented as a measure in the global initiative to eradicate poliomyelitis and should allow rapid identification of similar neurological manifestations in association with EV-D68 infection [20]. However, determination of AFP aetiologies can be challenging, because of the absence of pathogen detection in the CSF. Investigation of AFP cases should include both EV screening of two stool samples collected ≥ 24 hours apart and < 14 days after symptom onset [21] and early and quick testing of diverse samples, especially upper respiratory samples, for infectious agents including EVs, to increase the chance to identify a pathogen. In the case of EV-D68 infections, the detection capabilities of the EV-D68 genome of commercial and in-house molecular methods should be assessed.

Table

Bacteriological and virological investigations performed on clinical specimens collected in the first week of the course of the illness, acute flaccid paralysis case following enterovirus D68 infection, France, 2014

Collection date (2014)	Samples	Microbiological investigations	Methods	Results
22 September	CSF	Bacterial culture		Negative
		HSV1-2, EV, Rhinovirus-EV, VZV, ADV, CMV, EBV, HPeV	Real-time (RT)-PCR[a] and classic RT-PCR[b]	Negative
	Throat swab	EV	Real-time RT-PCR and EV-68 specific semi-nested RT-PCR	Negative
	Stool	EV	Real-time RT-PCR and EV-68 specific semi-nested RT-PCR	**EV-D68 positive**
24 September	Serum	*Borrelia, Mycoplasma pneumoniae, Chlamydia pneumoniae*	Serological tests	Negative
		EV, HSV1-2 / Rickettsia	Real-time (RT)-PCR and EV-68 specific semi-nested RT-PCR	Negative
		Human immunodeficiency virus (HIV), Parvovirus B19, HSV1-2 / Rickettsia	Serological tests	Negative
	Rectal swabs	EV, Rhinovirus-EV, HPeV	Real-time (RT)-PCR and EV-68 specific semi-nested RT-PCR	Negative
		Rotavirus, ADV	Immuno-chromatography	Negative
	BAL	Influenzae virus A-B, RSV, hMPV, EV, Rhinovirus-EV, ADV, BoV, hPIV1-4, hCoV, HSV1-2, CMV	Real-time (RT)-PCR and EV-68 specific semi-nested RT-PCR	**EV-D68 positive**
		Bacterial culture		Negative
	Nasopharyngeal aspirate	**Influenzae virus** A-B, RSV, hMPV, EV, Rhinovirus-EV, ADV, BoV, hPIV1-4, hCoV	Real-time (RT)-PCR and EV-68 specific semi-nested RT-PCR	**EV-D68 positive**
		Chlamydiae pneumonia, Mycoplasma pneumoniae	Real-time PCR	Negative
	Urine	Bacterial culture		Negative
25 September	Whole blood	EV, HHV6, CMV, EBV, ADV, VZV	Real-time (RT)-PCR	Negative
	Serum	Parvovirus B19	Real-time PCR	Negative
		HAV, HBV, HCV, Measles, Mumps, Rubella	Serological tests	Negative
		Rickettsia	Real time PCR	Negative
26 September	CSF	EV, Rhinovirus-EV, HSV1-2, VZV, ADV, CMV, EBV, HHV6	Real-time (RT)-PCR and EV-68 specific semi-nested RT-PCR	Negative
		Leptospira sp., *Escherichia coli, Listeria monocytogenes, Mycoplasma* sp., *Streptococcus agalactiae, Ureaplasma urealyticum, Bartonella, Borrelia, Rickettsia, Tropheryma Whipplei*	Real-time PCR	Negative

ADV: adenovirus; BAL: bronchoalveolar lavage; BoV: Bocavirus; CMV: cytomegalovirus; CSF: cerebrospinal fluid; EV: enterovirus; EBV: Epstein-Barr virus; HAV: hepatitis A virus; HBV: hepatitis B virus; hCoV: human coronavirus; HCV: hepatitis C virus; HHV: human herpes virus; hMPV: human metapneumovirus; HPeV: human parechoviruses; hPIV: human parainfluenzae virus; HSV: herpes simplex virus; RSV: respiratory syncytial virus; VZV: varicella zoster virus.

[a] For enteroviruses, a commercial pan-EV RT-PCR was used.

[b] Classic RT-PCR was used for rhinovirus-EV genome detection [12].

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Authors' contributions

Mathieu Lang, Nadia Savy, Sarah Maridet and André Labbé were involved in the clinical management of the patient. The virological investigations were performed by Audrey Mirand, Cécile Henquell and Hélène Peigue-Lafeuille. Magnetic resonance imagery was interpreted by Renan Pérignon. Audrey Mirand wrote the first draft of the paper. All authors reviewed the manuscript critically.

Conflict of interest

None declared.

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High-level azithromycin-resistant *Neisseria gonorrhoeae* clinical isolate in France, March 2014

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We report the first case in France of a high-level azithromycin-resistant *Neisseria gonorrhoeae* (minimum inhibitory concentration (MIC) = 96 mg/L) assigned to MLST7363 (NG-MAST ST6360), also resistant to ciprofloxacin and tetracycline but susceptible to ceftriaxone. The patient was a 51 year-old heterosexual man who returned following 1g azithromycin monotherapy. Mechanisms of azithromycin resistance were a C2599T mutation in the four copies of the *rrl* gene and a novel mutation in the promoter of the *mtrR* gene.

The surveillance of *Neisseria gonorrhoeae* (NG) susceptibility to antibiotics in France is based on a voluntary sentinel network of laboratories [1]. In March 2014, the first NG strain showing a high level of azithromycin resistance was isolated from the urine of a 51 year-old man living in the south of France. He had a history of a *Chlamydia trachomatis* infection 20 years ago. The patient was heterosexual and declared having sex with only two regular female partners (last sexual contact four and ten days before symptoms).

Case description

At the first visit, the patient presented with mild symptoms of urethritis. He was in good general state of health and his HIV test was negative. No microbiological analyses were performed and the patient was treated empirically with a single dose of azithromycin (1 g orally) for suspected *C. trachomatis* infection. The patient presented again with persisting symptoms 48 hours later. At that time, he was treated empirically with a single dose of spectinomycin (2 g intramuscularly) and bacteriological examination was performed with direct microscopic examination and culture. Culture results indicated the presence of an azithromycin–resistant NG isolate. The absence of *C. trachomatis* infection was determined retrospectively by negative nucleic acid amplification test. At a third visit, one week later the patient was successfully cured and free of clinical symptoms.

In France, the first-line treatment of uncomplicated urogenital gonorrhoea is ceftriaxone (500 mg intramuscularly), whereas spectinomycin is used in cases of contraindication to beta-lactams. For our patient, the clinician chose spectinomycin because of his own prescribing practices. Infection with the azithromycin–resistant-strain was presumably acquired in France since neither the patient nor his sexual contacts had travelled in the past six months or had other known sexual contacts. In addition, they had not received any antibiotic treatment in the preceding six months.

Microbiological investigation

The isolate was sent to the French National Reference Centre for gonococci (Paris, France), which confirmed the identification of NG by conventional biochemical criteria and by mass spectrometer laser Maldi-Tof (MicroFlex platform, Bruker Daltonik, Bremen, Germany). The antimicrobial susceptibility profile of the isolate was determined via gradient minimum inhibitory concentrations (MIC) (Etest bioMérieux, Marcy-l'Etoile, France). The isolate was resistant to azithromycin at a high level (MIC = 96 mg/L), resistant to ciprofloxacin (MIC > 32 mg/L) and to tetracycline (MIC = 2 mg/L) and had intermediate susceptibility to penicillin (MIC = 0.25 mg/L) without production of beta-lactamase enzyme. It was susceptible to cefixime (MIC = 0.016 mg/L), to ceftriaxone (MIC = 0.032 mg/L), to imipenem (MIC = 0.19 mg/L), to ertapenem (MIC = 0.023 mg/L), to gentamicin (MIC = 8 mg/L) and to spectinomycin (MIC = 8 mg/L) according to interpretive criteria from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [2].

Molecular investigation

Investigation of the mechanism of azithromycin resistance was further performed at the Associated Laboratory for the French National Reference Centre for gonococci (Paris, France). To fully determine the role of mutations in the peptidyltransferase loop of domain V

of the *rrl* gene encoding the 23S rRNA, all four alleles were amplified individually by PCR as described previously [3]. All four copies of the *rrl* gene harboured the substitution C2599T in the NG numbering system, which corresponds to C2611T in *Escherichia coli* numbering. This mutation can explain in part, but not fully, the high level of resistance to azithromycin.

The isolate was also screened by PCR for acquisition of rRNA methylase genes (*ermA, ermB, ermC* and *ermF*) and other genes involved in macrolide resistance *(ereA, ereB, mefA/E, mphA*) using methods previously described [4]. None of these genes were detected. No mutations were detected in the *rplD* and *rplV* genes encoding the ribosomal proteins L4 and L22, known to be involved in azithromycin resistance [4].

Interestingly, when screening the *mtrR* promoter region for mutations associated with upregulation of the MtrCDE efflux pump [4], we found one A to C mutation (underlined) resulting in a novel promoter of the *mtrR* gene: 5'TTGCACGGATACAAAGTCTTTTTTATAAT3' (nucleotides in bold indicate the -35 and -10 boxes). This mutation could contribute to the high level of resistance to azithromycin.

Molecular epidemiology typing was performed using the reference method of multi-antigen sequence typing (NG-MAST) [5]. Comparing the sequences determined for the *tpbB* and *porB* genes to those of the NG-MAST database, the sequence type ST6360 was assigned to the NG isolate via the on line NG-MAST data base (http://www.ng-mast.net). Moreover, using the multilocus sequence typing method previously described [6], the isolate was identified as sequence type ST7363.

Discussion

Sporadic infections with highly azithromycin-resistant *N. gonorrhoeae* isolates have been reported since 2001 in Argentina [7], in Scotland (2004) [8], in Wales (2007) [9], in Italy (2007) [10], in the United States (2011) [11] and in Sweden in 2013 [12]. Here we described the first isolate identified and presumably acquired in France. In previous publications [7,9,11,12], high-level azithromycin resistance was shown to result from the accumulation of mutations in the four *rrl* alleles at positions 2143 (G instead of A) or 2599 (T instead of C) in the domain V of the 23S rRNA involved in the binding site of azithromycin (NG numbering). In this report, our isolate harboured the C2599T mutation in the four *rrl* alleles. However, our isolate was more resistant to azithromycin that those previously described with these mutations (MIC ranging from 2 to 16 mg/L) [9], arguing that some additional mechanisms of resistance to azithromycin were active in this isolate.

In European countries, the decrease in sensitivity to third generation cephalosporin and to azithromycin has been linked to the spread of clone ST1407 [13]. The ST1407 is the most frequently observed sequence type in Europe. In contrast, it was comparatively uncommon in France where ST2992 and ST2 were the most frequent sequence types observed in 2010 [13]. Our NG isolate was assigned to MLST7363 (NG-MAST ST6360), never described in France but one of the four most prevalent sequence types found in Japan and associated with decreased susceptibility to cefixime [14]. However it has not been reported in connection with azithromycin resistance. Here, the NG strain was isolated after treatment with 1 g azithromycin monotherapy. We do not know if the patient had acquired a high-level azithromycin-resistant isolate from one of his partners or if an azithromycin-resistant mutant with a higher level of resistance was selected under treatment. However, this latter hypothesis is unlikely since the delay of 48 h between the antibiotic treatment and taking of the bacteriological sample seems too short for the acquisition of the observed mutations.

Azithromycin is not recommended as monotherapy for gonorrhoea [15,16], but it is recommended as co-treatment in combination with ceftriaxone at a dose of either 1 g or 2 g [15]. In addition, azithromycin is commonly administered empirically in monotherapy to treat urethritis that is presumed to be due to *C. trachomatis* or *Mycoplasma genitalium*. This practice can pose a risk of selecting azithromycin-resistant NG isolates [17,18].

Conclusion

In conclusion, the discovery of the first high-level azithromycin-resistant NG isolate in France argues for antimicrobial resistance surveillance to be continued and reinforces the need for cultivating bacteria to better survey multidrug resistant NG isolates. This finding highlights the importance of enhancing and strengthening measures to ensure adequate treatment for the patient and prevent further spread of azithromycin-resistant NG isolate.

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Conflict of interest

None declared.

Authors' contributions

BB, EC AG, GL and PS designed and initiated a surveillance of azithromycin resistance for all NG isolates collected in the sentinel network of laboratories. AB and FM performed and analysed all the laboratory work. AB, GL, AG and PS collected clinical information. BB and EC wrote the first draft of the paper and all co-authors were involved in finalising the paper.

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Did introduction of pneumococcal vaccines in the Netherlands decrease the need for respiratory antibiotics in children? Analysis of 2002 to 2013 data

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To estimate the effect of the introduction of the 7- and 10-valentpneumococcal vaccines in 2006 and 2011, respectively in the Netherlands, we assessed respiratory antibiotic use in one to nine year-old children between 2002 and 2013. Seasonal autoregressive integrated moving-average models were applied to estimate the percentage reduction in respiratory antibiotic use. When compared with the pre-vaccination period, the proportion of respiratory antibiotic prescriptions fell by 4.94% (95% CI: 4.63 to 5.26) and 9.02% (95% CI: 2.83 to 14.82) after the introduction of the 7-valent vaccine in children aged three and four years, respectively. After the introduction of the 10-valent vaccine, we observed a reduction of 13.04% (95% CI: 2.76 to 22.23), 20.31% (95% CI: 13.50 to 26.58), 16.92% (95% CI: 3.07 to 28.80), 22.34% (95% CI: 3.73 to 37.35), 23.75% (95% CI: 2.37 to 40.44) in two, three, four, six and seven year-old children, respectively. Thus, our results indicate a reduction in respiratory antibiotic prescriptions in young children after introduction of the pneumococcal vaccines. As only children in our study population aged one and two years born after March 2011 had received the 10-valent vaccine, the effects of the 10-valent vaccine in children aged three to nine years likely reflect the effects of the 7-valent vaccine and herd immunity.

Introduction

In 2001 in the Netherlands, 45% and 20% of all antibiotics in children were prescribed for respiratory tract and ear infections, respectively, with acute otitis media being the leading cause [1]. One of the common pathogens responsible for these infections, especially in young children, is *Streptococcus pneumoniae*. In the United States (US), it has been found in 44% of children hospitalised in 1999 to 2000 with community-acquired lower respiratory tract infections [2]. Due to substantial use of antibiotics for infections caused by *S. pneumoniae* [1], preventing these infections should remain an important public health goal.

In June 2006, 7-valent pneumococcal vaccination was introduced in the Netherlands as part of the national Dutch immunisation programme and was provided to all infants free of charge [3]. Prescription rates of oral antibiotics for children seemed to decrease after introduction of the vaccine [4], but no decline in ear, nose and throat problems has been observed [5]. The latter finding might be explained by replacement of pneumococcal serotypes. Despite an overall decrease in invasive pneumococcal disease (IPD) after the implementation of the 7-valent vaccination campaign in the Netherlands, a decrease of vaccine-serotype IPD was followed by an increase in IPD caused by non-vaccine serotypes [6,7]. There has been no information, however, on any changes in use of antibiotics that are usually used for acute otitis media and pneumonia in young children (one to nine year-olds) in the Netherlands after the 7-valent pneumococcal vaccine was introduced. Moreover, in 2011, the 7-valent pneumococcal vaccine was replaced by a 10-valent vaccine [8], whose effects have not yet been assessed in observational studies. Vaccinations were provided at two, three, four months and a booster dose at 11 months of age, with vaccination uptake rates of 94–95% [3].

To assess the patterns of respiratory antibiotic prescriptions in young children before and after the introduction of the pneumococcal vaccines in the Netherlands, we analysed the use of amoxicillin, azithromycin and sulfamethoxazole and trimethoprim from 2002 to 2013. In the Netherlands, antibiotics are available by prescription only. On the basis of Dutch general practitioner guidelines, these antibiotics are commonly prescribed for acute otitis media and pneumonia in children up to nine years of age in general practice [9,10]. We performed descriptive and time-series analyses to assess whether the introduction of the pneumococcal vaccines nationally reduced the proportion of respiratory antibiotic prescriptions in children one to nine years of age.

Methods

The study population consisted of one to nine year-old children identified from the IADB.nl database, which contains pharmacy-dispensing data from community pharmacies in the Netherlands. A detailed description of this database is available elsewhere [11]).

The main outcome of our study was the proportion of monthly respiratory antibiotic prescriptions in a particular age group (the number of monthly prescriptions in the age group per month divided by the number of children in that age group in that month). The aggregated measure of respiratory antibiotic prescriptions per year (the number of monthly prescriptions in the age group per year divided by the number of children in that age group in that year) was calculated as well. The outcome measure was based on prescriptions for amoxicillin (Anatomical Therapeutical Chemical (ATC) code J01CA04), azithromycin (J01FA10) and/or sulfamethoxazole and trimethoprim (ATC code J01EE01), as described above. The name of the antibiotic dispensed, ATC code [12], date of prescription and birthdate of the children included in the study were extracted from the IADB.nl database.

The first intervention studied was the introduction of the 7-valent pneumococcal vaccine in the Netherlands in June 2006 for all infants born after 1 April 2006 [3]. We also assessed the effects of the introduction of a 10-valent pneumococcal vaccine for infants born after 1 March 2011 [8].

The study period was chosen based on some preliminary analyses: we excluded data before 2002 as we observed a decrease in respiratory antibiotic use between 1995 and 2002, which might have been due to policies and interventions targeted at decreasing antibiotic use, and it was not our aim to assess these interventions in this study. Given that, the pre-vaccination period was defined as 1 January 2002 to 31 March 2007, i.e. the time before the 7-valent introduction of the pneumococcal vaccine was assumed to start having an effect. We considered that an effect of the introduction of the 7-valent pneumococcal vaccine could first be seen from 1 April 2007, as we anticipated that all four doses of the vaccine, including the booster dose, would have been administered and had an effect within a year after birth. The end of the period to assess the effect of the 7-valent vaccine was 30 April 2011, i.e. before the 10-valent vaccine replaced the 7-valent vaccine.

To study any additional effect of the introduction of the 10-valent pneumococcal vaccine, the end of the study period was set to 31 December 2013. We considered that an effect of the introduction of the 10-valent vaccine could first be seen from 1 March 2012, assuming that the full vaccination schedule would have been administered within a year after birth. The end of the study was 31 December 2013.

We assessed the effect of the 10-valent vaccine in two ways. We first ran the model including the introduction of both vaccines. Secondly, we assessed the effectiveness of the introduction of the 10-valent vaccination campaign as compared to pre-vaccination period, when data points following introduction of the 7-valent vaccine (1 June 2006 to 29 February 2012) were excluded.

Children born between 1 April 2006 and 28 February 2011 were assumed to have received the 7-valent vaccine; children born before 1 April 2006 were assumed to have not been vaccinated, but potentially indirectly protected by vaccination of younger age groups. Children born after 1 March 2011 were assumed to have received the 10-valent pneumococcal vaccine.

Statistical analysis

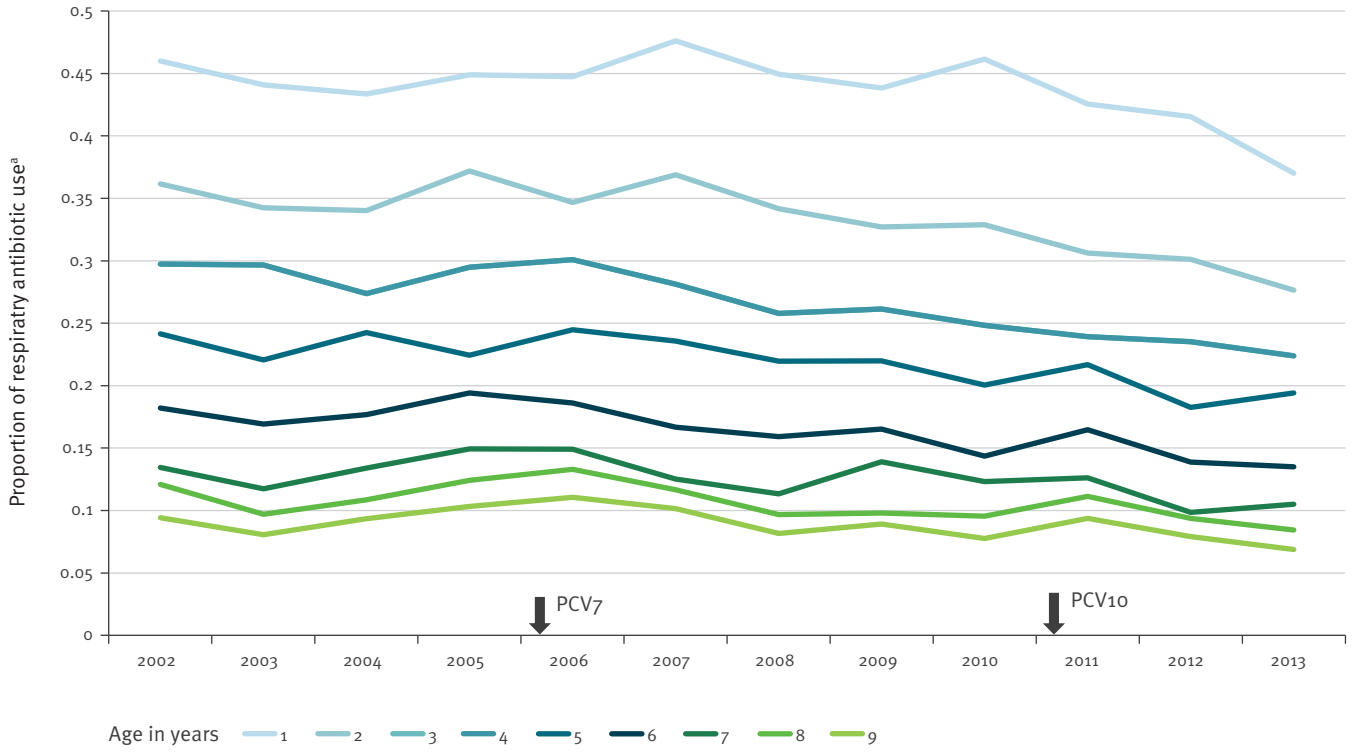
We first assessed the aggregated yearly respiratory antibiotic prescription proportions from 2002 to 2013 for each age group separately by plotting the data. We then assessed monthly antibiotic prescription proportions data using multiplicative decomposition [13] that shows the observed trend of the outcome as well as seasonal and random patterns, and the trend after removing the seasonal and random components.

To assess the effectiveness of the introduction of the pneumococcal vaccines, we used seasonal autoregressive integrated moving average (SARIMA(p,d,q)(P,D,Q)$_s$) time series models [14] with intervention analysis [15], where p and P is the number of auto-regressive components, d and D stands for differencing applied in the series, q and Q indicates the number of moving average components, and s is equal to the number of units of seasonal periods that are used in the model to remove additive seasonal effects. SARIMA allows us to estimate the effect of an intervention of interest by taking into account seasonal patterns. As pneumococcal illness tends to occur during the winter months, we assumed seasonal patterns occurring every 12 months, and therefore s was set to 12. We estimated the level (the abrupt change) of respiratory antibiotic prescription proportions and the change in trend (the slope) after, as compared with before, the introduction of the pneumococcal vaccines. The intervention variables were coded as 0 before the intervention and1 after the intervention. To assess the change in trends after the interventions were introduced, slope change variables denoting time were introduced. It was coded as 0 before the interventions, and afterwards counted the number of months after the introduction of the intervention of interest [16,17].

The best SARIMA models were identified based on the Akaike Information Criterion (AIC) during the pre-vaccination period for each age group separately [18]. They were then applied to estimate the effects of the introduction of the7- and 10-valent vaccines throughout each part of the study period, as described above. When both interventions were included in the same model, the best model (the model with only the first intervention

Figure

Proportion of yearly respiratory antibiotic prescriptions in children aged one to nine years, the Netherlands, 2002–13



[a] Number of users per year/number of children in the particular age group in that year.
The children's ages shown are the age at which the antibiotic was prescribed.

(introduction of the 7-valent vaccine) versus the model with both interventions) were selected based on a likelihood ratio test. The coefficients and their standard errors were estimated using maximum likelihood estimation. The percentage of change and its confidence intervals were calculated as (exp(coefficient)−1)×100% and (exp(coefficient+/−1.96 ×standard error)−1)×100%. The adequacy of each model was verified by visually assessing the correlograms (there should be negligible residual autocorrelation) and the plots of the residuals (the residuals of the model should be randomly scattered). The analysis was performed with RStudio 0.97.551 statistical software [19].

Results

Aggregated yearly estimates revealed that very young children had the most prescriptions of respiratory antibiotics: this decreased with age (Figure). We observed a slight decrease in respiratory antibiotic prescriptions after the introduction of the pneumococcal vaccines in 2006 and 2011 (Figure).We observed similar patterns when we inspected decomposed monthly trends of antibiotic prescriptions (data not shown).

To reveal the effects of the introduction of the pneumococcal vaccines on respiratory antibiotic prescriptions, we performed a time series analysis from 2002 to 2013. The best time series SARIMA models were identified based on AIC during the pre-vaccination period (see Table 1for the best model for each age group) and the likelihood ratio test when the additional effect of the introduction of the 10-valent vaccine was assessed in the model, including both interventions at the same time. The final models did not show evidence of autocorrelation and we could not detect clear patterns in the residual autocorrelation for most of the age groups. Only the models for the eight and nine year-old children showed significant autocorrelation at lag 12, indicating a remaining seasonal effect. However, due to low levels of autocorrelation, it is unlikely that this would have had a strong effect on the overall results.

The level of respiratory antibiotic prescription proportions decreased after the introduction of the 7-valent vaccine in most of the age groups (Table 1). The reduction was, however, only statistically significant in three and four year-old children, −4.94% (95% CI: −5.26 to −4.63) and −9.02% (95% CI: −14.82 to −2.83), respectively.

When we performed a likelihood ratio test, the model including both interventions was better than the model including the 7-valent vaccine intervention alone for a few age groups, namely in one, five and six year-olds.

Table 1

Effectiveness of introduction of 7-valent pneumococcal vaccine by age: change in level of respiratory antibiotic prescriptions and in trend of respiratory antibiotic prescription proportions, the Netherlands, 2002–13

Children's age in years	Change in level Percentage (95% CI)	Change in trend Percentage (95% CI)	Best SARIMA (p,d,q)(P,D,Q)12 model
1	1.20 (−3.51 to 6.13)	0.01 (−0.13 to 0.15)	(6,0,5)(8,1,9)12
2	2.55 (−1.85 to 7.15)	−0.26 (−0.42 to −0.10)	(8,0,8)(5,0,5)12
3	−4.94 (−5.26 to −4.63)	−0.16 (NA)[a]	(8,0,3)(7,0,7)12
4	−9.02 (−14.82 to −2.83)	−0.11 (−0.33 to 0.11)	(8,0,4)(4,1,3)12
5	−3.26 (−7.39 to 1.07)	−0.28 (−0.47 to −0.08)	(5,0,0)(9,0,9)12
6	−10.10 (−21.64 to 3.14)	−0.11 (−0.56 to 0.34)	(2,0,3)(6,1,8)12
7	−14.14 (−35.28 to 13.91)	0.15 (−0.75 to 1.06)	(0,0,1)(8,1,8)12
8	−7.93 (−24.17 to 11.79)	−0.23 (−0.85 to 0.40)	(2,0,5)(3,1,0)12
9	−12.72 (−31.47 to 11.17)	−0.20 (−1.11 to 0.72)	(0,0,0)(8,1,9)12

CI: confidence interval; NA: not applicable; SARIMA(p,d,q)(P,D,Q)s: seasonal autoregressive integrated moving average model with intervention analysis, where p and P is the number of auto-regressive components, d and D stands for differencing applied in the series, q and Q indicates the number of moving average components and s is equal to the number of units of seasonal period that are used in the model.

[a] The standard error could not be approximated using the maximum likelihood algorithm.

However, estimates of the effect of the introduction of the 10-valent vaccine were inconsistent: the proportions of antibiotic prescriptions decreased and/or increased after the introduction of 7- and/or 10-valent vaccination and the results were not statistically significant (Table 2).

When we assessed the effectiveness of the introduction of the 10-valent vaccine as compared with the pre-vaccination period, we observed a reduction in respiratory antibiotic prescriptions of −13.04% (95% CI: −22.23 to −2.76), −20.31% (95% CI: −26.58 to −13.50), −16.92% (95% CI: −28.80 to −3.07), −22.34% (95% CI: −37.35 to −3.73), −23.75% (95% CI: −40.44 to −2.37) in two, three, four, six and seven year-olds, respectively (Table 3).

The trends of antibiotic prescription proportions were similar before and after the interventions in most age groups (Tables 1–3).

Discussion

We found a decrease in antibiotic prescriptions for respiratory infections in three and four year-old children after the introduction of the 7-valent pneumococcal vaccine. Furthermore, we demonstrated that the introduction of the 10-valent pneumococcal vaccine continued to add benefit in terms of fewer respiratory antibiotic prescriptions. It is important to note, however, that the effect estimates of the latter intervention most likely include the effects of the 7-valent vaccinations as well as herd immunity, as in our study, only children aged one and two years born after 1 March 2011 received the 10-valent vaccine.

Our results are in line with the results from a recently conducted study on the effect of a 13-valent pneumococcal conjugate vaccine on admissions to hospital in the US: in children up to five years-old, introduction of the vaccine led to the reduction of all-cause, invasive pneumococcal and non-invasive pneumococcal or lobar pneumonia hospitalisation [20]. Such a decrease in outcomes, measured only a couple of years after

Table 2

Added effectiveness of introduction of 10-valent pneumococcal vaccine for different ages[a]: change in level and trend of antibiotic prescription proportions, the Netherlands, 2002–13

Children's age in years	7-valent vaccine		10-valent vaccine	
	Change in level Percentage (95% CI)	Change in trend Percentage (95% CI)	Change in level Percentage (95% CI)	Change in trend Percentage (95% CI)
1	5.54 (2.74 to 8.41)	−0.13 (−0.21 to −0.05)	−6.27 (−17.57 to 6.56)	−0.63 (−1.52 to 0.27)
5	2.72 (−3.92 to 9.82)	−0.29 (−0.50 to −0.07)	1.35 (−16.03 to 22.33)	0.32 (−0.87 to 1.53)
6	−10.32 (−21.03 to 1.85)	−0.08 (−0.41 to 0.25)	−0.08 (−0.41 to 0.25)	−9.72 (−28.15 to 13.44)

CI: confidence interval.

[a] Included are only the ages for which an added effect of the introduction of the 10-valent vaccine was demonstrated, based on a likelihood ratio test.

Table 3

Effectiveness of introduction of 10-valent pneumococcal vaccine for children aged one to nine years: change in level and trend of antibiotic prescription proportions as compared with the pre-vaccination period[a], the Netherlands

Children's age in years	Change in level Percentage (95% CI)	Change in trend Percentage (95% CI)
1	0.22 (–8.92 to 10.28)	–1.43 (–2.16 to –0.69)
2	–13.04 (–22.23 to –2.76)	–0.80 (–1.69 to 0.10)
3	–20.31 (–26.58 to –13.50)	–0.04 (–0.94 to 0.87)
4	–16.92 (–28.80 to –3.07)	–0.66 (–1.70 to 0.40)
5	–11.94 (–24.26 to 2.38)	–0.47 (–1.73 to 0.81)
6	–22.34 (–37.35 to –3.73)	–0.56 (–2.14 to 1.05)
7	–23.75 (–40.44 to –2.37)	–0.51 (–2.29 to 1.30)
8	–15.11 (–41.20 to 22.57)	–1.75 (–3.81 to 0.35)
9	–29.29 (–51.52 to 3.14)	–0.63 (–2.94 to 1.74)

CI: confidence interval.

[a] Pre-vaccination period: 1 January 2002 to 31 May 2006.

introduction of the new vaccine covering more serotypes and with vaccination coverage in children up to five years-old of 54%in the US study indicates not only direct, but also indirect protection. We also found that, although it was not statistically significant, there was an indication of a decrease in prescriptions of respiratory antibiotics in unvaccinated children aged eight and nine years born before the introduction of pneumococcal vaccination.

Our point estimates of the effect of the introduction of the pneumococcal vaccines showed a decrease, but it was not always statistically significant. Even though overall antibiotic prescription trends had a decreasing pattern, after the introduction of the vaccines, there were some fluctuations. The effects of the pneumococcal vaccines might therefore have been not significant due to quite low overall use of antibiotics in the Netherlands [1,21], thus making the data more sensitive to fluctuations. Moreover, it has been documented that after the introduction of the 7-valent pneumococcal vaccine in the Netherlands, invasive pneumococcal disease rates caused by non-vaccine serotypes increased [6,7]. This might also partly explain the observed fluctuations, even after the introduction of the vaccines. However, in our study, we were unable to explore the effect of serotype replacement, since serotype-specific clinical outcome data were not part of the dataset.

We may not have been able to show the benefits of the 10-valent pneumococcal vaccination in addition to the 7-valent campaign due to several reasons. First, the 10-valent vaccine covers additional three serotypes, meaning that the relative benefit of this campaign might be too small to detect when compared with the 7-valent vaccine. Additionally, as mentioned above, we observed some fluctuation (small increases and decreases) in the data following the introduction of the vaccines. These factors might explain why there was no statistically significant added benefit of the 10-valent vaccine and that for several ages, the model including the 7-valent vaccine alone appeared to be better than the model including both campaigns. These explanations seem plausible because when we assessed the effect of the 10-valent vaccine as compared with the pre-vaccination period, the observed reduction in respiratory antibiotic prescription proportions was large, reaching for some ages above 20%.

Because the pneumococcal vaccines targeted well-defined population groups at the national level at well-defined time points and vaccination uptake rates were high (94–95%) [3,8], we were able to study the effects of the interventions at the population rather than individual level. This is advantageous as large population-based databases, such as IADB.nl, which do not include individual vaccination information, can still be used to assess the impact of population-based interventions. By using SARIMA time-series models, we were able to estimate direct (among children age done to seven years) and indirect (among eight to nine year-olds) effects of the introduction of the pneumococcal vaccines. We were able to take seasonal effects into account as well as assess the introduction of both the 7-valent and 10-valent pneumococcal vaccinations by using different approaches.

Although we did not have information about the reason for the antibiotic prescriptions, the antibiotics that we looked at are specifically recommended to treat acute otitis media and pneumonia in young children in the Netherlands [9,10]. As *S. pneumoniae* is one of the leading causes of mucosal infections [22], our results are likely to indicate an effect of the introduction of the pneumococcal vaccines on health problems caused by *S. pneumoniae*.

In conclusion, our study provides evidence that introduction of the 7- and 10-valent pneumococcal vaccines were effective in reducing respiratory antibiotic prescriptions in young children, with a reduction of about 5–24% in antibiotic prescriptions for mucosal infections likely due to the introduction of the pneumococcal vaccines. Nevertheless, it is important to keep in mind that due to its recent introduction, the effect of the introduction of the 10-valent vaccine is likely in part due to the continuing effects of the 7-valent vaccine and herd immunity. Future studies are needed to further assess the effects of the pneumococcal vaccines on different health outcomes as well on populations other than children.

Conflict of interest

None declared.

Authors' contributions

Designed the study: GG. Prepared and analysed data: GG, MJB, JB. Interpreted the results: GG, MJB. Wrote the first draft: GG. Revised the article: GG, MJB, JB, EH. All authors read and approved the final manuscript.

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The experience of West Nile virus integrated surveillance system in the Emilia-Romagna region: five years of implementation, Italy, 2009 to 2013

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Predicting West Nile virus (WNV) circulation and the risk of WNV epidemics is difficult due to complex interactions of multiple factors involved. Surveillance systems that timely detect virus activity in targeted areas, and allow evidence-based risk assessments may therefore be necessary. Since 2009, a system integrating environmental (mosquitoes and birds) and human surveillance has been implemented and progressively improved in the Emilia-Romagna region, Italy. The objective is to increase knowledge of WNV circulation and to reduce the probability of virus transmission via blood, tissue and organ donation. As of 2013, the system has shown highly satisfactory results in terms of early detection capacity (the environmental surveillance component allowed detection of WNV circulation 3–4 weeks before human cases of West Nile neuroinvasive disease (WNND) occurred), sensitivity (capacity to detect virus circulation even at the enzootic level) and area specificity (capacity to indicate the spatial distribution of the risk for WNND). Strong correlations were observed between the vector index values and the number of human WNND cases registered at the province level. Taking into consideration two scenarios of surveillance, the first with environmental surveillance and the second without, the total costs for the period from 2009 to 2013 were reduced when environmental surveillance was considered (EUR 2.093 million for the first scenario vs EUR 2.560 million for the second). Environmental surveillance helped to reduce costs by enabling a more targeted blood unit testing strategy. The inclusion of environmental surveillance also increased the efficiency of detecting infected blood units and further allowed evidence-based adoption of preventative public health measures.

Introduction

West Nile virus (WNV) is a worldwide-distributed mosquito-transmitted flavivirus causing growing concern in Europe because of its ability to induce neuroinvasive disease (WNND) in humans [1]. In addition to the risk of vector-borne transmission, the high proportion of asymptomatic persons with the virus relative to those presenting with WNND, estimated at more than 100:1, poses a risk of WNV transmission via blood transfusion or organ transplantation [2].

WNV is maintained in the environment primarily by wild birds, in an enzootic cycle involving both migrating and residential species [3]. The virus may disappear or remain undetected for long periods, but during hot seasons and in places with suitable ecological conditions, the virus circulation may increase to affect humans and equids [3]. In Europe, based on the evidence to date, both the amplifying and the bridge vector roles are covered by *Culex pipiens sensu lato (s.l.)* [4-6], with *Cx. modestus* playing a bridge role in specific areas [7]. Generally, in temperate regions, it has been shown that the virus may overwinter in infected female mosquitoes as well as in residential birds, so there is no need for continuous re-introductions by migrating birds [8-11].

Although WNV circulation has been observed in several European Union (EU) Member States since the early 1950s, large WNV outbreaks have only been documented starting from the mid-1990s. The largest WNV outbreak in the EU, with over 390 confirmed cases, was reported in Romania in 1996 [4]. Greece experienced the second largest outbreak with 197 human cases in 2010 [12]. Sporadic human cases as well as outbreaks of various sizes have been reported

in Albania, Austria, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, the former Yugoslav Republic of Macedonia, Greece, Hungary, Italy, Kosovo under United Nation Security Council Resolution 1244, Montenegro, Romania, Russia, Serbia, Slovenia, Spain and Ukraine [13].

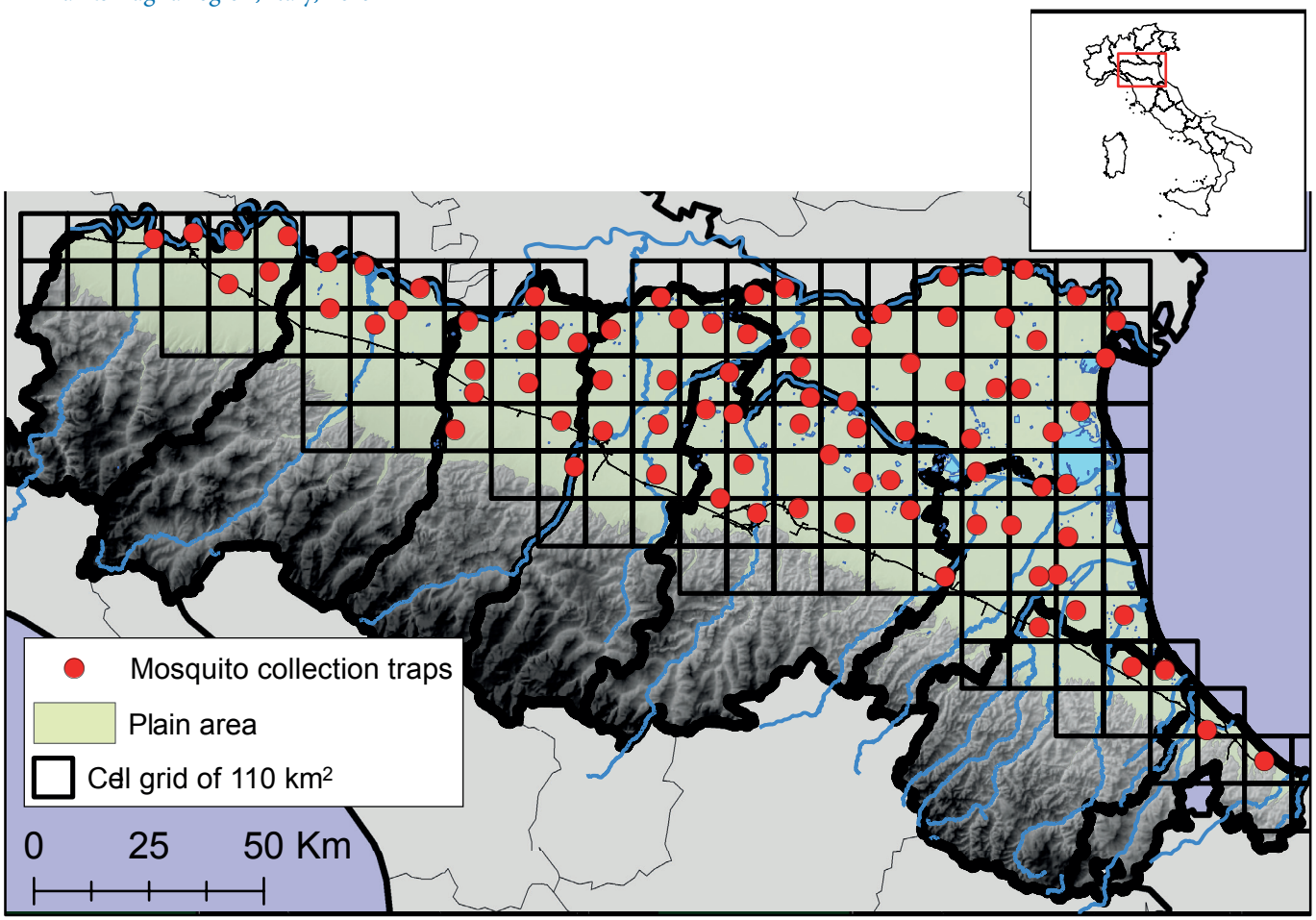
In Italy cases of WNV infection have been registered regularly since 2011 (with 69 in 2013, 50 in 2012 and 14 in 2011) and have been distributed in several regions, including the Emilia-Romagna region. Cases occur mostly in the summer and autumn following an annual seasonal pattern. To prevent WNV transmission by blood transfusion and organ donation a national surveillance plan has been in place since 2008. According to the last issue of this plan, screening of blood units and organs is performed between 1 July and 30 November in provinces where humans or equids infected with WNV have been registered in the previous season. For other provinces, blood unit and organ screening are initiated in the current season a week after the detection of an equine case of WNV infection or a human WNND case.

The prevention and control of WNV is complex and requires the implementation of a comprehensive surveillance system [14,15]. Environmental surveillance, based on mosquito and/or bird collection and subsequent screening for the pathogen, has been shown to perform well in detecting the virus circulation well before the occurrence of human cases in addition to allowing size estimations of human outbreaks of WNV and identification of affected areas [16-19]. Since 2009, an integrated surveillance targeting mosquitoes, birds, and humans, has been put into effect in the Emilia-Romagna region, northern Italy. The main goals of this regional WNV surveillance are to contain the spread of WNV infections in humans and to more effectively reduce the probability of virus transmission via blood, tissue and organ donation systems. The surveillance system, which was originally described by Angelini et al. [20] and Calzolari et al. [21], has undergone further adaptations over time.

In this report, we present the development of the surveillance plan in the five years following its first implementation. We also present a cost comparison between two scenarios for preventing WNV transmission by transfusion and/or organ transplantation: scenario A,

Figure 1

Distribution of mosquito collection stations in the target area for West Nile virus entomological surveillance, Emilia-Romagna region, Italy, 2013



which follows the WNV Italian national plan and scenario B, which is based on evidence from a surveillance integrating environmental observations. In scenario B, blood unit and organ screening within a season is initiated at the province level when the mosquito or the bird active surveillance show WNV circulation in this province. Should a WNND human case occur before the detection of WNV circulation by the entomological or ornithological surveillance in the province, the measures to be taken are as in the national surveillance plan for this particular situation.

Methods

Area under surveillance

The Emilia-Romagna region has a total surface area of ca 22,450 km² with a population of 4.47 million. The area under surveillance is ca 11,000 km² and is located in the Po valley plain, where more than 90% of the region's residents live, and where ecological conditions (such as *Cx. pipiens* breeding sites density and distribution, bird species population and environmental parameters) are considered suitable to WNV circulation.

Entomological surveillance

Following the detection of human cases of WNV infection in 2008 in the Emilia-Romagna region, a surveillance network was designed and operated in the summer period (June–October) [19-22]. In the 2009 season, mosquito collections were conducted partially in fixed stations and partially in occasional stations, with weekly to monthly periodicity. From the 2010 season the mosquito collections were standardised in fixed geo-referenced stations with fortnightly periodicity. Female mosquitoes were trapped using CO_2 baited traps (and gravid traps from 2012), activated one night per collection. The network was initially designed to cover the regional plain area using a grid with cells of ca 110 km² (Figure). The specific location of the station in each cell was chosen by skilled entomologists to optimise *Culex* collections. The surveillance plan was conducted regularly with slight modifications during the five years.

Collected mosquitoes were counted, identified at the species level and pooled according to date, location, and species, with a maximum number of 200 individuals per pool. In case of large collections a maximum number of 1,000 mosquitoes/trap/night/species (equals to 5 pools of 200) was submitted to the laboratory for analysis, while the remaining mosquitoes were determined to the species level and discharged. Pooled mosquitoes were stored in polypropylene cryotubes and frozen at -80 °C. For biomolecular analysis, samples were first ground by adding copper-plated round balls, phosphate buffer saline solution and vortexing, and then were subsequently centrifuged. To optimise costs of analysis, aliquots of small pools of mosquitoes (below 30 individuals) were combined with a super-pool approach [4].

Ornithological surveillance

According to the WNV national surveillance plan, an active surveillance was started since 2006 on corvid species, which are considered as agricultural pests and therefore the target of population control programmes. These include: Eurasian magpies (*Pica pica*), hooded crows (*Corvus cornix*), carrion crows (*Corvus corone*) and Eurasian jays (*Garrulus glandarius*). Birds were trapped in the plain and low hill areas (up to 600 m above sea level (a.s.l.)) in eight (Bologna, Ferrara, Forlì-Cesena, Modena, Parma, Piacenza, Ravenna, Reggio Emilia) of the nine provinces of the region. The culling programme was performed from May to October, dividing the surveyed area into quadrants sized 1,600 km² and collecting 15 to 20 specimens in each quadrant every month.

Birds' organ samples (brain, spleen, heart, and kidney) were pooled, ground, and submitted for biomolecular analysis. Samples from every bird were processed individually.

Human surveillance

The human surveillance system was based on the active identification of WNND human cases in a period defined every year by national guidelines (see results). According to the national case definition, every subject presenting fever (≥ 38.5 °C) and a neurologic manifestation such as acute flaccid paralysis, acute polyradiculoneuritis (Guillain–Barré syndrome), aseptic meningitis, or encephalitis was considered as a suspect case and therefore laboratory investigated.

Every suspect case was promptly reported to the Public Health Department and biological samples were transmitted to the Regional Reference Centre for Microbiological Emergency (CRREM) within the Unit of Clinical Microbiology of the St. Orsola University Hospital, Bologna. According to laboratory findings, the suspected cases were classified as confirmed cases following national guidelines (http://www.trovanorme.salute.gov.it/norme/dettaglioAtto?id=49423).

Blood donation system

The national strategy for prevention of WNV transmission by transfusion indicates that, in regions where no virus circulation was observed in the previous year, like was the case for the Emilia-Romagna in 2013, only blood donors with a minimum overnight stay in affected areas (defined at the province level) are to be tested for WNV RNA. Moreover a WNV RNA screening of all blood units in a given province shall start in a week following the first WNND human or equine WNV infection case detection.

In 2013, the integrated regional surveillance system requires that WNV nucleic acid testing (NAT) screening is applied to all blood donors in a province after reports of at least two positive mosquito pools by the entomological surveillance network, or one positive bird, within the limits of the province without waiting

Table 1

Main parameters and outputs of the surveillance activities, Emilia-Romagna region, Italy, 2009–2013

Year	Traps (n)[a]	Area around the trap (km²/trap)	Collection date of first WNV positive mosquito pool	Corvids collected (n)[b]	Collection date of first WNV positive corvid	Date of symptom onset of first WNND case	WNND cases (n)
2009	92	119	21 Jul	1,005	30 Jul	19 Aug	9
2010	102	98	26 Aug	806	1 Aug	ND[c]	0
2011	90	122	ND[c]	826	ND[c]	ND[c]	0
2012	96	114	ND[c]	1,204	ND[c]	ND[c]	0
2013	88	125	3 Jul	1,688	31 Jul	3 Aug	20

ND: none detected; WNND: West Nile neuroinvasive disease; WNV: West Nile virus.

[a] Mosquitoes are trapped from June to October every year.

[b] Birds are collected from May to October every year.

[c] ND indicates no finding of WNV evidence among mosquito pools or corvids tested, or no detection human cases of WNND.

for human cases. NAT screening is started in a week from the detection of the second positive mosquito pool or the positive bird. Following evidence produced by the environmental surveillance plan, in 2013 the NAT test was progressively introduced throughout seven (Bologna, Ferrara, Modena, Parma, Piacenza, Ravenna, Reggio Emilia) of the nine provinces in the Emilia-Romagna Region and stopped on 30 November.

Laboratory analysis

Mosquito and bird samples

Mosquito pools and bird samples were tested by a real-time polymerase chain reaction (RT-PCR), according to the method of Tang et al. [23]. Although this method is designed to detect WNV, other flaviviruses such as Usutu virus (USUV) can produce a positive signal if these are present in a sample at high concentration, therefore a traditional pan-flavivirus PCR, targeted to NS5 gene fragment, according to Scaramozzino et al. [24] and a traditional PCR protocol for WNV with the primers described in Lanciotti et al. [25], were applied on WNV positive samples to confirm results. Amplicons obtained were sequenced by an automated fluorescence-based technique following the manufacturer's instructions (ABI-PRISM 3130 Genetic Analyzer, Applied Biosystems, Foster City, CA). Traditional PCR designed for the detection of USUV, useful in differential diagnosis, was applied to the same samples. All confirmed WNV positive samples were sent to the

Table 2

Surveillance parameters obtained in the years of WNV activity, at the province level, Emilia-Romagna region, Italy, 2009–2013

Year	Province	Date of initial symptoms in first human case of WNND	Collection date of first WNV positive pool	Number of days that mosquitos anticipated WNND	VI max (Culex pipiens)	Collection date of first WNV positive corvid	Lag time (in days) between finding WNV in mosquitoes and birds	WNND cases (n)	Incidence WNND (cases/ 100,000)
2010	Bologna	–	–	NC[a]	0.00	1 Aug	NC[a]	0	0
2010	Modena	–	23 Aug	NC[a]	0.14	4 Aug	-19	0	0
2013	Modena	3 Aug	03 Jul	31	0.94	31 Jul	28	7	1.40
2013	Ferrara	6 Aug	17 Jul	20	0.87	31 Jul	14	5	1.12
2013	Bologna	15 Aug	17 Jul	29	0.63	3 Aug	17	1	0.24
2013	Reggio E.	16 Aug	17 Jul	30	0.81	6 Aug	20	6	1.43
2013	Parma	11 Sep	19 Jul	54	0.55	2 Sep	45	1	0.34
2013	Ravenna	–	24 Jul	NC[a]	0.27	30 Aug	37	0	0
2013	Piacenza	–	13 Aug	NC[a]	0.37	8 Aug	-5	0	0

NC: not calculated; VI: vector index; WNND: West Nile neuroinvasive disease; WNV: West Nile virus.

[a] Not calculated because no WNND cases were detected.

National Reference Centre for Animal Exotic Diseases (CESME, Teramo) for confirmation, sequencing and determination of lineage.

Human samples

Blood donors: WNV screening was performed on single plasma samples by NAT and transcription mediated amplification (TMA) methods on fully automated system Tigris and Panther (Novartis). Repeatedly reactive samples were confirmed by RT-PCR on single sample on fully automated system Cobas 201 (Roche). Screening tests have been centralised to NAT Laboratory of the Blood Donors Biological Qualification Unit, AUSL of Bologna.

Patients with neuroinvasive disease: WNV RNA detection in human plasma, serum and cerebrospinal fluid samples (CSF) was performed by RT-PCR methods [26]. The presence of WNV-specific IgM and IgG antibodies in serum and cerebrospinal fluid (CSF) samples was tested by immunofluorescent antibody assay (IFA, Euroimmun) and further confirmed by microneutralisation assay (MNTA) [27].

Cost evaluation

As referred in the Introduction a cost evaluation analysis was conducted considering the two possible scenarios (A and B) during the five year period. Direct cost of each PCR performed on mosquito pools or birds was calculated at 15.00 EUR (personnel included). The cost of mosquito collection, species determination and pools preparation has been determined as a lump sum for the whole season.

We estimated an overall cost of 50.00 EUR per consignment of wild bird to the laboratory (each consignment consists on average of 3.5 birds; range: 1–22). The laboratory diagnosis of a human case was calculated and found to have a mean cost of 74.00 EUR (personnel not included). The cost for a single NAT-test on a blood-donor's sample was 11.32 EUR in 2013, 12.10 EUR in 2012 while in the previous years it was 12.00 EUR. All costs per unit included value added tax (VAT).

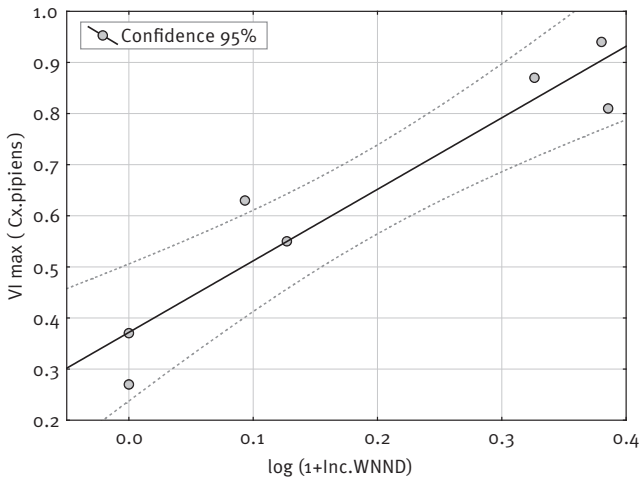
Statistical analysis

Vector Index (VI) was calculated referring to the traps activated in the administrative border of each provinces (nomenclature of units for territorial statistics, NUTS3 level) by means of the formula $VI = \Sigma N_i P_i$ (where N is the average number of *Cx. pipiens* collected per trap/night and P is the Maximal Likelihood Estimation (MLE) of infection, estimated using the PooledInfRate 4.0 software [28]). As traps were activated mainly with fortnightly periodicity a series of VI values were obtained during the season; VImax is the maximum value the VI achieved during the season in a province.

Linear regression analysis was used to perform the correlation between VI and seasonal incidence of WNND human cases at the province level, using the R Stats Package [29]. WNND cases incidence were transformed in log(1+cases incidence) to normalise the data and control the variance.

Figure 2

Correlation between maximum vector index values (at province level in *Culex pipiens*) and the cases of West Nile neuroinvasive disease, Emilia-Romagna, Italy, 2009–2013



Results

Entomological and ornithological integrated surveillance data

During the five year period, WNV activity was registered in the Emilia-Romagna region in 2009, 2010 and 2013, while no virus circulation was detected in 2011 and 2012. In the period between 2009 and 2010 WNV lineage 1 strain [30] was found, while in 2013 WNV lineage 2 was detected [31]. In 2009, mosquitoes were first in signalling the virus activity with five weeks anticipation to the first human case. In 2010 birds were first in signalling the virus circulation but no WNND cases were registered. In 2013 mosquitoes were first in signalling the virus circulation one month before the onset of the first WNND case (Table 1).

The 2010 and 2013 collected data allowed for a more precise analysis at the province level (Table 2). This was neither possible in 2009 nor in 2011 and 2012 because in 2009 the surveillance system was not sufficiently standardised, while in 2011 and 2012 no virus circulation was detected. In 2010, we did not register any WNND cases, but WNV circulation was detected during August in two provinces (Bologna and Modena) (Table 2). In 2013, in all five provinces where WNND cases occurred, both mosquitoes and birds signalled the virus circulation some weeks before the detection of human cases, in the range of 20 to 54 days for mosquitoes and slightly less anticipation for birds (3–12 days) (Table 2).

Table 3

Confirmed West Nile neuroinvasive disease human cases, Emilia-Romagna, Italy, 2009–2013 (n=29)

Year	Surveillance period	Confirmed WNND cases (n)	Men (n)	Age median in years (range)	Deaths (n)
2009	15 Jun–31 Oct	9	8	72 (62–78)	3
2010	15 Jun–15 Nov	0	–	–	–
2011	15 Jun–15 Nov	0	–	–	–
2012	15 Jun–15 Nov	0	–	–	–
2013	15 Jun–30 Nov	20	12	78 (42–86)	5

WNND: West Nile neuroinvasive disease.

The correlation between VI values and WNND cases incidence at the province level was very high with $R^2 = 0.87$ ($F_{1,5} = 32.80$ and $p < 0.01$) for VI mean (mean value during weeks 28–35) and with $R^2 = 0.90$ ($F_{1,5} = 43.53$ and $p < 0.002$) for VI max (peak seasonal value).

A positive correlation was also found between the date of collection of the first observed positive mosquito pool and the VI max (VI max values at province level correlate with the *Julian day* number of the first WNV positive pool with $R^2 = 0.74$, $F_{1,5} = 13.88$ and $p < 0.02$).

WNND cases were observed in provinces with VI max above 0.5, while no human cases were registered in Ravenna and Piacenza provinces where the VI max resulted below 0.5.

Human cases

In the Emilia-Romagna region human WNND cases were identified and reported in 2009 [4,19] and 2013, while in 2010, 2011 and 2012 no cases were reported (Tables 1 and 3). In 2013 with the integrated surveillance system, the blood donor screening system identified 12 positive blood samples, of which four were detected before the first WNND human case in Emilia-Romagna.

Cost analysis

The analysis for cost evaluation was performed comparing two different scenarios: scenario A, implemented in Emilia-Romagna from 2009 to 2012, following the national surveillance guidelines; and scenario B, implemented in 2013, based on the results of the regional integrated surveillance system. In scenario A the only expenses are related to blood screening (Table 4). In scenario B, the cost evaluation analysis shows slight yearly variations in the entomological and ornithological surveillance related costs, with a larger yearly variation in blood screening related costs (Table 5). The economic comparison of the two scenarios shows that in some years scenario B would save the entire expense for blood screening (e.g. year 2011), in other cases, as year 2013, the integrated surveillance system, required higher costs, but enabled the identification of four positive donors, otherwise undetectable in the case of adopting scenario A. By considering the whole five year period the cumulative costs of environmental surveillance and guided blood screening resulted in costs of EUR 2,093,441, while the cumulative costs of blood screening without surveillance guidance resulted in costs of EUR 2,560,200.

Discussion

The WNV surveillance system developed in recent years in the Emilia-Romagna region demonstrated positive evidence in terms of sensitivity (capacity to detect WNV circulation even when at the enzootic level), early detection (capacity to detect the virus circulation well before the appearance of human WNND cases) and area specificity (capacity to indicate the spatial distribution of the risk for WNND human cases). In the 2010 season, WNV activity was detected in two provinces (Modena and Bologna) at very low level and no human cases were registered. A similar situation happened in the 2013 season in the provinces of Piacenza and Ravenna.

The integrated surveillance programme, which enabled to estimate virus circulation in the range of three to four weeks before the appearance of the first human WNND cases at the province level, may support a more

Table 4

Cost evaluations for scenario A – national plan for West Nile virus surveillance, Emilia-Romagna, Italy, 2009–2013

Year	Blood screening surveillance			Overall surveillance cost
	Blood units tested (n)	Positive blood units detected (n)	Blood screening cost (EUR)	
2009[a]	35,552	0	426,624	426,624
2010[a]	66,689	0	800,268	800,268
2011[a]	60,258	0	723,096	723,096
2012[a]	0	0	0	0
2013[b]	53,898	8	610,212	610,212
Total	216,397	8	2,560,200	2,560,200

In the national surveillance plan, screening of blood units is performed in a given year between 1 July and 30 November in all blood units of provinces where a human West Nile neuroinvasive disease (WNND) case or equine case of West Nile virus (WNV) infection has been registered in the previous year. For other provinces that year, blood unit screening is only initiated a week after an equine case of WNV infection or a human WNND case is detected in the current season.

[a] The national surveillance plan scenario was implemented in Emilia-Romagna during the year in question.

[b] In 2013, the Emilia-Romagna region adopted a surveillance system, which differed from the national surveillance plan. Costs that would have been generated by implementing the national surveillance plan have been estimated for the region, based on knowledge that no human WNND or equine WNV infection was detected in the whole region in the previous year, and knowledge of the time of occurrence of human WNND cases in given provinces of the region in 2013.

Table 5

Cost evaluations for scenario B –regional integrated West Nile virus surveillance system, Emilia-Romagna, Italy, 2009–2013

Year	Entomological and ornithological surveillance					Blood screening surveillance			Overall surveillance cost
	Mosquito collection cost (EUR)	Mosquito screening cost[x] (EUR)	Bird collection cost[b] (EUR)	Bird screening cost[a] (EUR)	Total (EUR)	Blood units tested (n)	Positive blood units detected (n)	Blood screening cost (n)	
2009[c,d]	50,000	28,380	16,900	16,065	111,345	44,295	0	531,540	642,885
2010[c,d]	50,000	34,770	11,550	12,180	108,500	11,679	0	140,148	248,648
2011[c]	50,000	23,325	14,650	12,810	100,785	0	0	0	100,785
2012[c]	50,000	28,815	15,500	18,480	112,795	0	0	0	112,795
2013[e]	60,000	39,510	18,400	29,880	147,790	74,242	12	840,538	988,328
Total	**260,000**	**154,800**	**77,000**	**89,415**	**581,215**	**130,216**	**12**	**1,512,226**	**2,093,441**

WNV: West Nile virus.

Entomological and ornithological surveillance has been conducted during the whole study period in the Emilia-Romagna region, however the results of this surveillance were not effectively taken into account for blood screening surveillance until 2013. Before 2013 the national WNV surveillance plan was in place in the region, whereby some particular rules for screening blood units were applied. In 2013, an integrated regional WNV surveillance system was implemented in Emilia-Romagna, which requires that WNV nucleic acid testing screening is applied to all blood donors in a province after reports of at least two positive mosquito pools or one positive bird by the entomological or ornithological surveillance network, within the limits of the province. NAT screening is started a week after the detection of the second positive mosquito pool or positive bird.

[a] Including for each positive sample, the cost of three polymerase chain reactions and the cell culture and sequencing.

[a] Free because voluntary birds consignments.

[c] In this year, blood screening surveillance in Emilia-Romagna does not follow the integrated regional WNV surveillance system, but the national WNV surveillance plan. However, based on entomological surveillance results, it is possible to predict how many blood units would have been screened should the regional surveillance system have been followed, and derive the costs accordingly.

[d] In this year, the blood units that would have been screened by the integrated WNV regional surveillance system happened to have been screened according to the national surveillance plan, so the number of positive blood units that would have been detected via the integrated WNV regional surveillance system is known.

[e] Integrated regional surveillance system implemented in Emilia-Romagna.

evidence-based policy of blood screening, thus avoiding blood units' analyses in case of virus absence, even in areas which were affected in the previous year. As the WNV epidemiology is largely unpredictable by modelling, an integrated surveillance is required to support the risk assessment.

Once validated during a sufficient number of seasons the entomological and ornithological surveillance may be considered as a valid tool to guide the blood and organ donations safety policy, to organise a more evidence-based information to citizens, in order to enhance the adoption of personal protection measures and precautionary behaviour, and eventually to operate adult vector control in sites at high risk (e.g. nocturnal public events in vegetated areas) [32].

The comparative analysis between the two possible scenarios (with and without the environmental surveillance) shows that the integrated surveillance system may have a better performance in terms of costs and public health benefits. In the case of Emilia-Romagna region the cost comparison of the two approaches showed that the surveillance could have allowed the saving of at least EUR 0.5 million in the period from 2009 to 2013 (Table 4 and 5). Differences in costs between the two scenarios may of course be subject to variability depending on the epidemiological circumstances in the long term. Furthermore, during the 2013 season, which came following a year of no WNV detection in the Emilia-Romagna region, the surveillance system assisted the prompt starting of the blood screening before the appearance of any human case, allowing the detection of four WNV positive blood units, which would not have been detected in case of the application of the national plan procedure (as the national plan requires the blood screening in provinces where human cases were reported in the previous year or following the detection of a human case in the current year). So the environmental surveillance may be helpful in to detect WNV circulation in an area in a year which follows an absence of human or equine cases in the previous season, as it may advise about possible risk of infection via transfusion well before the detection of human cases.

The surveillance system, as it has been developed in the Emilia-Romagna region, allowed to detect and monitor the WNV affected areas where preventive sanitary measures may be conveniently adopted. The province level seems the most appropriate administrative NUTS where the implementation of laboratory screening methods, such as NAT screening of blood units, can be conveniently managed.

An extra value of the environmental surveillance is the possibility to detect the circulation of other arboviruses vectored by mosquitoes and other haematophagous

insects readily collected by the CO2 baited traps, such as sand flies, culicoides and black flies, as well. We therefore propose to adopt the WNV environmental surveillance as part of the public health policy in the region.

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Conflict of interest

None declared.

Authors' contributions

Romeo Bellini prepared the first draft of the manuscript; Alessandro Albieri, Paola Angelini, Romeo Bellini, Emanuela Bedeschi, Paolo Bonilauri, Mattia Calzolari, Michele Dottori, Alba Carola Finarelli, Silvano Natalini, Marco Tamba contributed to the environmental surveillance planning and management; Mattia Calzolari and Paolo Bonilauri provided the laboratory results for birds and mosquitoes virological screening; Giada Rossini, Paolo Gaibani, Caterina Vocale, Maria Paola Landini provided the laboratory results for human cases; Claudio Velati, Nadia Pascarelli provided the haemovigilance costs and data on surveillance in the blood donor population; Roberto Cagarelli, Marco Carrieri, Andrea Mattivi, Marco Tamba conducted the data analysis; all authors critically read the manuscript and approved the final submitted version.

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Four-month outbreak of invasive meningococcal disease caused by a rare serogroup B strain, identified through the use of molecular PorA subtyping, England, 2013

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Molecular PorA subtyping provides information that increasingly requires the adaptation of standard public health approaches to outbreak management. We report an outbreak of a rare subtype of meningococcal infection not previously identified in the United Kingdom (UK). The outbreak occurred in the Warwickshire area in England between February and June 2013. Molecular subtyping allowed the identification of additional cases, prompting an enhanced public health response that included efforts to identify potential social networks that might benefit from chemoprophylaxis. It also prompted swabbing to define nasopharyngeal carriage in the focal nursery and helped explain the unusual epidemiological pattern. Without subtyping to identify a link, the additional cases would have been managed as sporadic cases in accordance with current UK guidance.

Introduction

Invasive meningococcal disease (IMD) is a significant cause of mortality and morbidity, with the highest incidence in children under the age of five years. Persistent neurological defects are more common among infants and young children [1,2]. In the United States (US), an estimated 2,600 people are affected and 300 die from the disease each year [3]. In 2012, the incidence of probable and confirmed IMD in the US was 0.18 per 100,000 population [4]. In Europe, 3,808 confirmed cases of IMD were reported across 29 countries in 2011 [5]. Across all age groups in England and Wales, there were 766 cases of IMD in the epidemiological year 2011/12 and the average annual incidence of IMD was 2 per 100,000 [5,6].

In Europe and the Americas, meningococcus serogroup B has been the predominant serogroup, particularly since the introduction of vaccines against serogroup C [1]. Most cases are sporadic [7,8]. When one case occurs in a pre-school group, the absolute risk of becoming a case within the next four weeks is low, about 1 in 1,500 for each child at the same institution [9]. However, a review by the European Centre for Disease Prevention and Control (ECDC) of the available evidence suggested a relative risk of 22.3 (95% confidence interval (CI): 12.1–40.9) [7].

While guidance varies across Europe [7], guidance in the United Kingdom (UK) recommends that prophylactic antibiotics are not given to pre-school groups after a single case [9]. The reasons for this include that any benefit is unclear, that there is the possibility of harm, that antibiotics may cause side effects, provide false reassurance about protection and eradicate naturally immunising strains from the nasopharynx, and that clusters in pre-school groups are rare (about three per year in England and Wales) [9]. Following a second case in an educational setting, the risk of a third case in that setting may be as high as 30–50% [10,11]. In UK guidance, a cluster in a single educational institution is defined as '*two or more cases of meningococcal disease occurring in the same pre-school group [or other educational setting] … within a four week period*' [9]. If the two cases are, or could be, caused by the same serogroup, staff and children would normally be offered prophylaxis [9]. If the notification of invasive meningococcal disease is delayed, close contacts should be offered chemoprophylaxis up to four weeks after onset of illness [9]. ECDC guidance is focused on sporadic cases and does not address responses to clusters [7].

Swabbing is not recommended in the UK during outbreaks because decisions have to be taken before the results are available and because rates of carriage

often bear no relationship to the risk of additional cases [9].

We describe here an outbreak of five cases of meningococcal serogroup B disease that occurred in the Warwickshire area in the UK between February and June 2013. It was unusual in that it did not meet the UK standard definition of an outbreak in an educational setting [9] because it included three cases in a nursery over a four-month period. Others have reported a cluster of cases over a long timescale, e.g. Acheson et al. [12], but this was within a single nuclear family, a setting where there is a higher risk of secondary cases. The authors concluded that it was likely that the strain was reintroduced by a regular non-household contact or unidentified close contact [12]. In addition, Mandal et al. reported an outbreak of 13 cases between January 2008 and November 2010 [13].

In our situation, the cases were only identified as linked through initial serological phenotyping, followed by molecular PorA subtyping undertaken by the national meningococcal reference laboratory. Sequence-based typing is increasingly used as a tool to help outbreak investigation. For instance, whole genome sequencing (WGS) of isolates between 1990 and 2012 showed that WGS has the potential to inform the epidemiological investigation of outbreaks of verocytotoxin-producing *Escherichia coli*. The WGS results prompted further investigation, which in some cases identified previously obscure links between cases [14].

In this report, our aim is to describe how the information provided by the molecular PorA sequence typing informed and changed our standard response. Our focus is the health protection response to the outbreak and in particular, the challenge of interpreting and acting upon additional microbiological information in this setting. All standard public health actions relating to single cases, such as chemoprophylaxis for household contacts, were undertaken at the time of notification.

Outbreak description

During February 2013 a two year-old child (Case 1) attending nursery (Nursery 1), was admitted to hospital with symptoms of lethargy, reduced consciousness and a non-blanching rash. The following day, the health protection team was notified of this probable case [9] of meningococcal disease. Three days after the child was admitted, the reference laboratory reported that meningococcus serogroup B had been isolated from the blood culture (in addition to PCR-positive EDTA blood samples) and 7 days later, the organism was confirmed as *Neisseria meningitidis* phenotypically described as group B, PorB serotype 4, and PorA serosubtype (NT / NT / P1.6). DNA sequencing was used to define the PorA subtype as P1.18–4, 25, 38–1. The isolate had reduced susceptibility (intermediate) to penicillin (minimum inhibitory concentration (MIC) = 0.06 mg/L) but was susceptible to cefotaxime (MIC = 0.002 mg/L), rifampicin (MIC = 0.004 mg/L) and ciprofloxacin (MIC = 0.004 mg/L). Routine typing was performed in accordance with Gray et al. [15].

Nine days after the first case was admitted, a case of probable meningococcal disease in a two year-old child (Case 2) from the same nursery as Case 1 was notified. The child presented with a non-blanching rash, lethargy, vomiting and fever. Given that Case 1 was caused by a strain not preventable by the vaccines available at the time, chemoprophylaxis (and not vaccine) was offered to a defined group of staff and children at Nursery 1. UK guidance recommends that the target group should be 'discrete' and 'make sense to staff and parents' [9]. In this case, we offered chemoprophylaxis to children and staff associated with two of the four classes who were considered to be close contacts. Close contacts were defined as any child or adult at the nursery who spent between four and eight hours in the same room as the two cases. In total, 38 children and nine staff were offered chemoprophylaxis the day after the second case was admitted. All children and staff collected a letter from the nursery to take to their general practitioner, which advised that they had been a contact of cases of meningococcal disease and chemoprophylaxis was recommended. Self-reported uptake of chemoprophylaxis was 100%.

The causative organism for Case 2 was confirmed by both EDTA PCR and culture as meningococcus serogroup B and subsequently as the same PorA subtype

Box

Outcome of the expert discussion following the third case of *Neisseria meningitidis* in a nursery, England, June 2013

Information on the outbreak strain *N. meningitidis* group B, serotype 4, serosubtype P1.NT/NT/6

- Sero-subtyping markers showed that this is an extremely rare strain.
- Since 2007, there have only been two other cases of similar sero-subtypes in the United Kingdom.
- This subtype has not been identified in carriage studies; carriage rate and patterns of carriage are therefore unknown.

Agreed actions

- Offer chemoprophylaxis to all staff and children of Nursery 1.
- Use ciprofloxacin for chemoprophylaxis. Ciprofloxacin had already been used in the initial response to Cases 1 and 2, however:
  - Ciprofloxacin is more effective at eradicating nasopharyngeal carriage than rifampicin;
  - There is no microbiological evidence that the first two cases were resistant to ciprofloxacin.
- Given that this is a rare subtype for which carriage rates, virulence and the effectiveness of chemoprophylaxis are currently unknown, undertake swabbing as part of the operational public health response to inform action:
  - Swabbing should not delay chemoprophylaxis;
  - It is recognised that swabbing does not always identify carriage.

as in Case 1, P1.18–4, 25, 38–1. The isolate from Case 2 had reduced susceptibility (intermediate) to penicillin (MIC = 0.06 mg/L) but was susceptible to cefotaxime (MIC = 0.004 mg/L), rifampicin (MIC = 0.004 mg/L) and ciprofloxacin (MIC = 0.004 mg/L).

In June (over 18 weeks after the first case was admitted to hospital), the health protection team was notified of a probable case of meningococcal septicaemia in a two year-old child (Case 3) who attended the same nursery class as Cases 1 and 2 and who had received chemoprophylaxis in February. While awaiting typing results, given the time between the cases and as per UK guidance, the initial public health response was based on the assumption that the latest case was likely to be unrelated to the previous cases. The case was subsequently confirmed as meningococcus serogroup B by PCR alone and later as subtype PorA P1.18–4, 25, 38–1. All cases made a good recovery with no long-term sequelae.

Public health investigation

Following confirmation 10 days after the third case was admitted, that the third case in Nursery 1 (Case 3) was infected with the same rare subtype by PorA sequencing, the outbreak control team consulted national experts at the Centre for Infectious Disease Surveillance and Control at Colindale and the Meningococcal Reference Unit. The Box summarises information on the subtype and actions agreed at the meeting with the experts, which was held the day after typing results were available for the third case in Nursery 1.

Identifying potentially linked cases

In an attempt to identify others affected by the outbreak strain, molecular PorA subtyping results were sought from all cases of meningococcal disease where typing had been undertaken from the local area (Warwickshire and Coventry) notified between January and July 2013.

Further epidemiological investigation

Telephone interviews were conducted to identify potential links between these cases. Two questionnaires were developed and both were piloted to ensure ease of understanding and use. The first was used to capture information from adult cases and the second was used to obtain information from parents if the case was a child. Questions covered regular activities, events, gatherings and links with local nurseries, schools and mother and toddler groups as well as activities in the week before the case became unwell.

Assessing carriage in Nursery 1

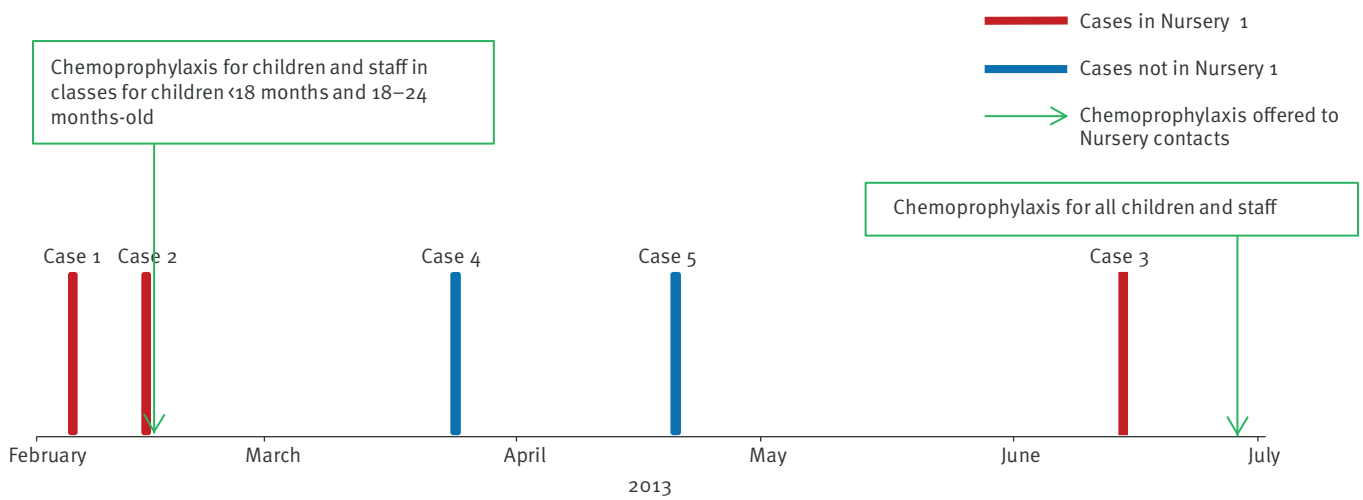
To support the epidemiological investigation, throat swabs were requested from all children and staff of Nursery 1 prior to prophylaxis. Swabbing took place the day after the meeting with the experts. Parental consent was secured for swabbing of children. Parents and staff were informed that individual results would not be disclosed, in line with usual practice. Ethical approval for throat swabbing was not obtained as it was part of the outbreak response.

Additional molecular investigations

The Meningitis Research Foundation Meningococcus Genome Library (MGL; http://www.meningitis.org/genome-library) contains genomic sequence data for all IMD case isolates from the epidemiological years 2010/11 to 2012/13. Genomic sequence data for the three outbreak isolates became available via the MGL in February 2014. Full finetype data (Group: PorA: FetA: multilocus sequence type (ST) and clonal complex (mL)) was queried against the library and the library's

Figure 1

Timeline of meningitis cases, nursery outbreak in England, February to June 2013 (n=5)



Table

Results of throat swabs taken at the nursery in context with the meningitis outbreak in England, February to June 2013 (n=69)

	Number of individuals	Individuals swabbed n (%)	Individuals swabbed who had received prophylaxis in February n (%)	Swabs processed by laboratory[a] n (%)	Carriage n (%)[b]	Carriage of outbreak strain %
Staff	16	15 (94)	7 (47)	14 (88)	0 (0.0)	0
Children	95	54 (57)	12 (22)	53 (56)	1 (1.9)[c]	0
Total	111	69 (62)	19 (28)	67 (60)	1 (1.5)	0

[a] Two swabs were not processed because of unclear labelling.

[b] Carriage was estimated using swab results positive for meningococcus. The denominator used to calculate carriage was the number of swabs taken at the nursery which were processed by the laboratory.

[c] This child was not given antibiotic prophylaxis in February 2013.

BIGSdb genome comparator tool was used to perform partial core-genome comparisons (1,546 loci) between these and related isolates both in the MGL (comprising all English, Welsh and Northern Irish IMD isolates from July 2010 to June 2013) and the wider PubMLST Neisseria database (http://pubmlst.org/neisseria/). NeighborNet phylogenetic networks were produced by applying SplitsTree4 (version 4.12.8) [16] to the distance matrices generated. For two culture negative cases (Cases 3 and 4), non-culture characterisation of sub-variants of the meningococcal factor H binding protein (fHbp) was performed according to Clark et al. [17].

Results

Identifying potentially linked cases

Subsequently, after PorA sequence typing of six geographically and temporally associated group B cases identified by the health protection team (i.e. cases notified within the six months before mid-June 2013), the reference laboratory notified the health protection team of two additional cases with the same PorA subtype. The first was a person in their 60s (Case 4) identified by PCR alone (onset March 2013) whose grandchild attended Nursery 1 and had been offered prophylaxis in February. The second was a two year-old child (Case 5) from an unconnected nursery (Nursery 2) who became unwell with a non-blanching petechial rash in April 2013 and was confirmed by both PCR and culture. The isolate from Case 5 was also of reduced (intermediate) susceptibility to penicillin (MIC = 0.06 mg/L) but susceptible to cefotaxime (MIC = 0.004 mg/L), rifampicin (MIC = 0.004 mg/L) and ciprofloxacin (MIC = 0.004 mg/L). Nursery 2 is situated 12.1 km from Nursery 1. Four further PCR-confirmed group B cases in children investigated in the area in February to June yielded PorA subtypes P1.22, 9, 35–1, P1.7–2, 4, 37 and two cases with P1.19, 15, 36, all quite distinct to the outbreak strain. Figure 1 shows the timeline of the five P1.18–4, 25, 38–1 cases and public health actions taken.

Further epidemiological investigation

Interviewing did not identify social links among cases except Nursery 1 for four of the five cases.

Assessing carriage within Nursery 1

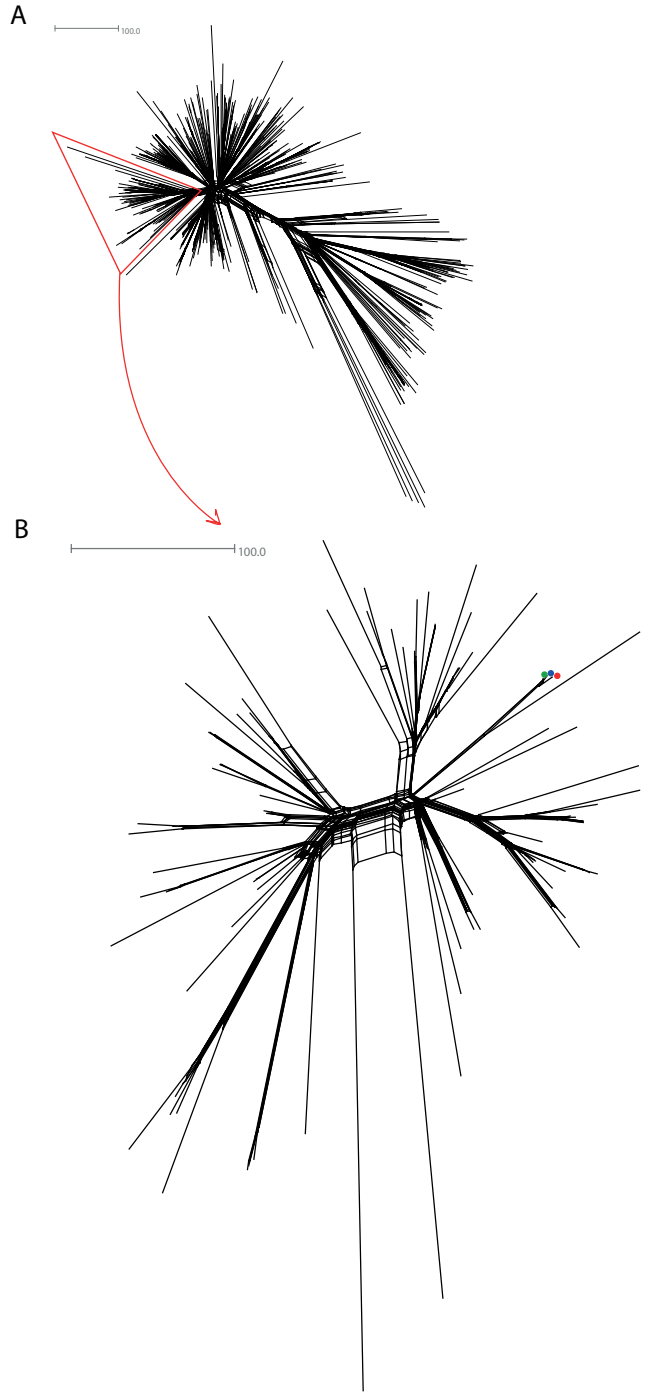
In order to test for meningococcal carriage, throat swabs were collected from 15 of 16 staff and 54 of 95 children (Table). Swabs were taken from all staff and children who consented and were able to attend the nursery on the day of swabbing. Swabbing was not repeated so as not to delay chemoprophylaxis. It was not possible to collect throat swabs from all children if parental consent was not obtained or the children did not tolerate the procedure. One member of staff was not swabbed because they were abroad at the time of swabbing. The swabs were screened for any query *Neisseria* species. *Neisseria lactamica* was not tested for. A meningococcus was isolated from one child who had not been identified as eligible for the initial prophylaxis, and was phenotypically characterised as group NG, serotype 21 and sero-subtype (NT / NT / NT) with PorA P1.18–1, 30–9, 38. The isolate was resistant to penicillin (MIC = 0.50 mg/L) but susceptible to cefotaxime (MIC = 0.008 mg/L), rifampicin (MIC = 0.016 mg/L) and ciprofloxacin (MIC = 0.008 mg/L). The outbreak strain was not identified from any swab taken.

Additional molecular investigations

The three available outbreak isolates (Cases 1, 2 and 5) possessed the finetype B: P1.18–4,25: F1–5: ST-1194 (cc41/44), which was unique within the PubMLST Neisseria database (including the MGL). A partial core genome comparison among all cc41/44 genomes on the PubMLST Neisseria database (n = 484, of which 334 were also in the MGL; accessed 22 May 2014) indicated that the three outbreak isolates formed part of a discrete cluster of 85 isolates, all of which were isolated in the UK or Ireland. In a further partial core genome comparison, Cases 1 and 2 differed from one another at 11 of 1,250 variable loci and from Case 3 at 20 and 22 loci, respectively. When treating putative recombinations (differences among two or more neighbouring loci) as single differences, the corresponding figures

Figure 2

Phylogenetic network analyses of *Neisseria meningitidis* outbreak isolates and closely related isolates in the PubMLST Neisseria database and Meningococcus Genome Library



Network A is based on a partial core genome comparison of all cc41/44 isolates on the PubMLST Neisseria database including those of the Meningococcus Genome Library (MGL) representing all English, Welsh and Northern Irish isolates from July 2010 to June 2013 (inclusive). The red triangle highlights a discrete cluster of 85 isolates including those of outbreak Cases 1, 2 and 5. Network B is based on a separate partial core genome comparison of the cluster of 85 isolates in which isolates from Cases 1, 2 and 5 are highlighted green, blue and red, respectively. Scale bars indicate the number of differences among 1,546 loci compared.

were 6, 10 and 9 loci, respectively. By contrast, the average distance between case one and the remaining 82 closely related isolates (not adjusting for putative recombinations) was 256 differences (standard deviation: 66, range: 142–502) (Figure 2).

In terms of fHbp, the isolates from Cases 1, 2 and 3 all possessed alleles for peptide 9, the isolate from Case 4 possessed an allele for peptide 30, and the isolate from Case 5 an allele for peptide 4.

Discussion

The health protection team identified three plausible explanations for the outbreak. Firstly, the initial prophylaxis at Nursery 1 may not have eradicated carriage within the nursery. It is unlikely that prophylaxis failed to eradicate carriage for the following reasons:

(i) Given the length of time between the cases at Nursery 1 and the negative swabs from nursery staff and children who were not given prophylaxis initially, and given that meningococcal carriage is low in this age group of children [18], it is unlikely that the initial prophylaxis did not include enough people and the outbreak strain was reintroduced to the affected class from within the nursery. In addition, the existence of a case in the wider community without a link to Nursery 1 makes this unlikely. However, it should be noted that not all children and staff at Nursery 1 were swabbed, and therefore a carrier may have been missed.

(ii) Uptake of prophylaxis was assumed to be good since all parents and staff collected the antibiotic prescriptions and reported taking chemoprophylaxis.

(iii) It is unlikely that the organism was resistant to the antibiotic used for initial prophylaxis given that resistance to ciprofloxacin is unusual [19] and swab results were negative for the outbreak strain among those who received prophylaxis, although not all were tested. In addition, the isolates from all three culture-confirmed cases (Cases 1, 2 and 5) were ciprofloxacin-sensitive.

Secondly, the notified cases could have been the beginning of a community outbreak. Subtyping results of recent local cases of meningococcal infection identified no additional cases with the outbreak strain and no subsequent cases (as of 25 October 2013).

Finally, there may be another undefined social network connecting the cases. This explanation is plausible because the initial two cases occurred within nine days, while the final case occurred more than 18 weeks later. Transmission could occur via one common contact or via social networks in the wider community. We tried unsuccessfully to identify social links through interviewing. However, an unidentified network is still the most plausible explanation given the known links in time and place and the person characteristics.

The partial core genome comparisons confirmed that the isolates from Cases 1, 2 and 5 were very closely related to one another but were relatively distant from any other English, Welsh or Northern Irish isolates collected between July 2010 and June 2013. This strongly supports earlier conclusions, based on spatiotemporal evidence and routine typing data, that Cases 1 and 2 constituted a genuine outbreak and that the outbreak was protracted, extending to Case 5 in the wider community. They also confirmed the absence of additional, but previously unidentified, culture-confirmed cases dating back to June 2010.

The rarity of PorA P1.18–4,25 (unique within the MGL to the three outbreak isolates) indicated the inclusion of the non-culture (confirmed by PCR only) Cases 3 and 4 in the outbreak investigation. The now routine typing target *fhbp*, however, differed between Case 4 (peptide 30), Case 5 (peptide 4) and Cases 1 to 3 (encoding peptide 9). The difference between Case 5 and Cases 1 and 2, given the close relationship identified through core genome comparisons, is likely to have arisen through recombination (the alleles were substantially different) which is not uncommon in meningococci [20]. This highlights the potential of a single antigen gene to, somewhat misleadingly, distinguish very closely related isolates. Thus the differing fHbp variant did not preclude the inclusion of Case 4, especially given the spatiotemporal link and the rare PorA variant. Routine real-time whole genome sequence analysis of case isolates and, eventually, non-culture specimens, may help us to confirm or rule out such uncertain, protracted outbreaks in a suitable timescale to influence management. At present, isolates are added to the MGL in batches at the end of the epidemiological year. Nonetheless, the detailed molecular characterisation that became available later did not support a different approach to that taken by the outbreak management team.

cc41/44 is a diverse clonal complex that has been a major cause of IMD throughout Europe and further afield for over a decade [21]. The sequence type ST-1194 first occurred in the PubMLST Neisseria database in 1999 when it was assigned to an Irish isolate. Further ST-1194 isolates have since been added representing ten European countries (Czech Republic (n=6), France (n=2), Germany (n=10), Ireland (n=11), Italy (n=3), the Netherlands (n=1), Slovenia (n=1), Spain (n=1), Sweden (n=1) and the UK (n=38)), as well as the US (n=4) and South Africa (n=1), indicative of widespread distribution of the strain. The corresponding isolates were associated with 14 different PorA subtypes, of which P1.18–4, 25, however, was only found among the outbreak isolates described herein. Similarly, in the phylogenetic analyses, these occupied an isolated sub-lineage in which they diverged from a single Irish isolate (isolated 2011) possessing the relatively common PorA P1.18-1,3. Thus, while PorA may serve to highlight potentially related cases occurring subsequently in the UK or further afield, only genomic analyses can confirm this.

There is now growing evidence that outbreaks of meningococcus group B are different from meningococcus C outbreaks as they may be prolonged [13]. Our outbreak fits this pattern and highlights that guidance that is predominantly based on experience of meningococcus C outbreaks may not always be appropriate for serogroup B outbreaks. However, in response to the recent licensure of a vaccine targeting capsular group B meningococci, new recommendations for preventing secondary cases have been prepared in the UK [22].

The availability of meningococcal typing information led to a substantially different public health response to the situation as cases, other than the initial two, would have been treated as sporadic. The important lesson learnt was therefore that subtyping results should be reviewed early in incidents with a potential epidemiological link involving the same meningococcal serogroup, even when cases occur outside the recognised time limits used to define a cluster. Since the occurrence of this cluster, new guidance in the UK has been published which recommends that for clusters involving two or more confirmed/probable cases who are or could be infected with the same capsular group B strain, vaccination using the recently licensed vaccine should be offered to the same group that would receive antibiotic chemoprophylaxis [22]. In this instance, vaccine would have been offered after the second case in Nursery 1 and may have prevented subsequent cases.

Conclusion

In the investigation of our outbreak, we did not identify any other link among cases than attendance at Nursery 1. However, the length of time between illness onset and interview may have affected the respondents' recollection of details that could have identified potential links. Therefore, to identify potential links among social networks we suggest that a standard questionnaire should be administered to all cases as soon as an outbreak is suspected. Use of research from social science around networks and social interaction could also help develop robust methods for identifying social links between cases.

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Conflict of interest

None declared.

Authors' contributions

CC, RG, KN, MT, ML, SG and TF were members of the outbreak team. CC and TF prepared the initial manuscript. CC, RG, JH, KN, MT, ML, SG, JL, AC, SC and TF contributed to the subsequent editorial revisions. SG, JL, AC and SC performed laboratory investigations and genomic analyses.

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