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Human infection with avian influenza A(H7N9) virus re-emerges in China in winter 2013

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Through a national surveillance system for unexplained pneumonia, a severe case of influenza A(H7N9) in a man in his mid-30s was identified in Zhejiang Province, China on 14 October 2013. Epidemiological and clinical findings were consistent with the patterns reported during the outbreak in spring 2013, and laboratory findings showed that the virus had 99.6% identity with earlier H7N9 viruses identified in humans in the spring except for five mutations in the NA gene.

Introduction

Since 2004, all hospitals from the 11 cities of Zhejiang Province have participated in enhanced surveillance throughout the year of patients with pneumonia without explanation. On 14 October 2013, this system identified a new case of human infection with influenza A(H7N9). Here we describe how the case was discovered, and report the epidemiological, clinical and virological characteristics compared with the previous laboratory-confirmed influenza A(H7N9) cases in Zhejiang Province.

Since the first case was confirmed on 31 March 2013, a total of 135 human infections with avian influenza A(H7N9) have been reported in China, including 45 deaths. They occurred mainly in eastern China during March and June, with the peak incidence in April [1]. After the last case with illness onset on 28 July in Guangdong Province in the south of China, no further influenza A(H7N9) cases were reported in China until 14 October.

In Zhejiang, a province neighbouring Shanghai, the influenza season usually lasts throughout the year, with a peak between November and February. In 2013, it was one of the areas most affected by the spring outbreak of influenza A(H7N9) during April [2]. A total of 46 confirmed cases, including 11 fatal cases, occurred in five of the 11 cities (›3,000,000 inhabitants) in Zhejiang Province. Eleven of the 46 cases were clustered with four live poultry markets [2]. The decrease in the number of new human cases since mid-April may have resulted from containment measures taken by the Zhejiang authorities, such as closing live bird markets, or from a change in seasons, or a combination of both factors. Until 14 October, no confirmed cases had been reported in Zhejiang Province since 18 April. The public health response in the province had ended on 17 May.

Methods

Identification of cases

Suspected cases of H7N9 virus infection are identified through the Chinese surveillance system for pneumonia of unexplained origin [3]. On identification of a suspected case, local centers for disease control and prevention (CDCs), including prefecture and provincial CDCs, conduct the initial field investigations and obtain respiratory specimens, which are shipped to the provincial CDC for H7N9 laboratory testing [3].

Definition of cases and contacts

The definitions of probable, confirmed, severe case and surveillance case were based on Chinese guidance of diagnosis and treatment and Chinese guidance of the surveillance programme (second edition of 2013) for humans infected with H7N9 avian influenza [3].

Based on the Chinese guidance for control and prevention of human infections with H7N9 avian influenza (second edition of 2013), contacts were defined as (i) those who did not wear personal protective equipment

Table 1a

Origin of HA, NA and PB1 genes of human reference strains of avian influenza A(H7N9) included in phylogenetic analysis

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI457805, EPI457804, EPI457803	HA, NA, PB1	China	2013-04-03	A/chicken/Shanghai/S1079/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI440701, EPI440700, EPI440699	HA, NA, PB1	China	2013-04-02	A/pigeon/Shanghai/S1069/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI457797, EPI457796, EPI457795	HA, NA, PB1	China	2013-04-03	A/chicken/Shanghai/S1080/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI457845, EPI457844, EPI457843	HA, NA, PB1	China	2013-05-03	A/chicken/Jiangxi/SD001/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI447618, EPI447716, EPI447720	HA, NA, PB1	China	2013-04-21	A/Shandong/01/2013	Shandong CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al.
EPI447599, EPI447893, EPI447897	HA, NA, PB1	China	2013-03-18	A/Shanghai/07/2013	Shanghai CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al.
KC885956, KC885958, KC885961	HA, NA, PB1	China	2013-04-18	A/Zhejiang/DTID-ZJU01/2013	The First Affiliated Hospital, College of Medicine, Zhejiang University	The First Affiliated Hospital, College of Medicine, Zhejiang University	Chen H, Yuen K
EPI447604, EPI447829 , EPI447836	HA, NA, PB1	China	2013-04-12	A/Beijing/01-A/2013	Beijing CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al.
EPI440685, EPI440684, EPI440683	HA, NA, PB1	China	2013-04-03	A/chicken/Shanghai/S1053/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI440693, EPI440692, EPI440691	HA, NA, PB1	China	2013-04-03	A/environment/Shanghai/S1088/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI439502, EPI439500, EPI439501	HA, NA, PB1	China	2013-03-05	A/Shanghai/2/2013	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al.
EPI457749, EPI457748, EPI457747	HA, NA, PB1	China	2013-04-11	A/chicken/Zhejiang/SD033/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI477402, EPI477404, EPI477400	HA, NA, PB1	China	2013-04-24	A/Zhejiang/20/2013	Zhejiang Provincial Center for Disease Control and Prevention	Zhejiang Provincial Center for Disease Control and Prevention	Sun Y, Zhang Y.
EPI439507, EPI439509, EPI439508	HA, NA, PB1	China	2013-03-20	A/Anhui/1/2013	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al.
EPI447609, EPI447780, EPI447784	HA, NA, PB1	China	2013-04-10	A/shanghai/13/2013	Shanghai CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al
EPI443028, EPI443029, EPI447942	HA, NA, PB1	China	2013-03-25	A/Zhejiang/01/2013	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al
EPI447614, EPI447744, EPI447748	HA, NA, PB1	China	2013-04-03	A/Zhejiang/02/2013	Zhejiang CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al
EPI457733, EPI457732, EPI457731	HA, NA, PB1	China	2013-04-16	A/duck/Zhejiang/SC410/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI457757, EPI457756, EPI457755	HA, NA, PB1	China	2013-04-11	A/chicken/Zhejiang/SD019/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI477410, EPI477412, EPI477408	HA, NA, PB1	China	2013-10-14	A/Zhejiang/22/2013	Zhejiang Provincial Center for Disease Control and Prevention	Zhejiang Provincial Center for Disease Control and Prevention	Sun Y, Zhang Y.

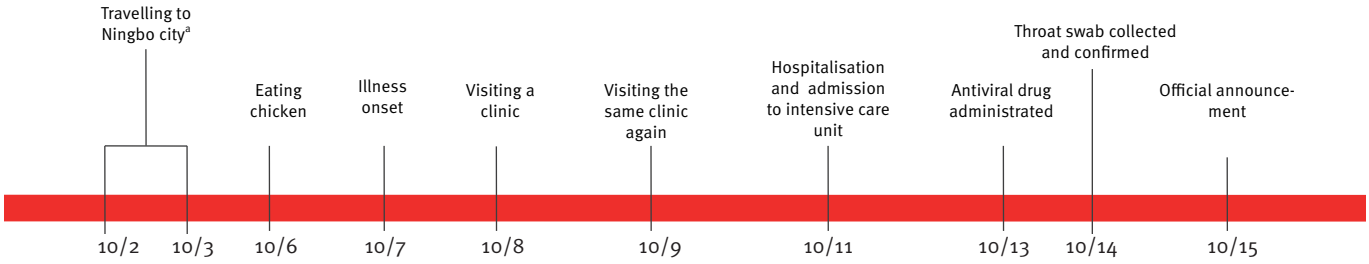
Table 1B

Origin of HA, NA and PB1 genes of human reference strains of avian influenza A(H7N9) included in phylogenetic analysis*

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI457861, EPI457860, EPI457859	HA, NA, PB1	China	2013-04-16	A/chicken/Jiangsu/SC099/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI457789, EPI457788, EPI457787	HA, NA, PB1	China	2013-04-03	A/chicken/Shanghai/S1358/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
KF055468, KF055470, KF055467	HA, NA, PB1	China	2013-04	A/Zhejiang/HZ1/2013	First Affiliated Hospital, School of Medicine, Zhejiang University	First Affiliated Hospital, School of Medicine, Zhejiang University	Wu H, Wu N, Guo C, et al.
EPI457627, EPI457629, EPI457628	HA, NA, PB1	China	2013-04-03	A/pigeon/Shanghai/S1423/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI447612, EPI447758, EPI447762	HA, NA, PB1	China	2013-04-07	A/Shanghai/8/2013	Shanghai CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al.
EPI457659, EPI457661, EPI457660	HA, NA, PB1	China	2013-04-03	A/environment/Shanghai/S1438/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI457675, EPI457677, EPI457676	HA, NA, PB1	China	2013-04-03	A/environment/Shanghai/S1436/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI457667, EPI457669, EPI457668	HA, NA, PB1	China	2013-04-03	A/environment/Shanghai/S1437/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI457651, EPI457653, EPI457652	HA, NA, PB1	China	2013-04-03	A/environment/Shanghai/S1439/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI447596, EPI447916, EPI447920	HA, NA, PB1	China	2013-03-30	A/Jiangsu/01/2013	Jiangsu CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al.
EPI457851, EPI457853, EPI457852	HA, NA, PB1	China	2013-04-16	A/chicken/Jiangsu/SC537/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
KC899667, KC899669, KC899671	HA, NA, PB1	China	2013-04	A/chicken/Zhejiang/DTID-ZJU01/2013	First Affiliated Hospital, School of Medicine, Zhejiang University	First Affiliated Hospital, School of Medicine, Zhejiang University	Wu H, Wu N, Yao H, et al.
EPI457619, EPI457621, EPI457620	HA, NA, PB1	China	2013-04-17	A/wild pigeon/Jiangsu/SD001/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI457763, EPI457765, EPI457764	HA, NA, PB1	China	2013-04-22	A/chicken/Zhejiang/SD007/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang Q, Shi J, Deng G, et al.
EPI447598, EPI447903, EPI447910	HA, NA, PB1	China	2013-04-02	A/Shanghai/05/2013	Shanghai CDC	WHO Chinese National Influenza Center, Virology Institute, Chinese CDC	Wang D, Gao R, Yang L, et al.
EPI439489, EPI439486, EPI439487	HA, NA, PB1	China	2013-02-26	A/Shanghai/1/2013	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Wang D, Gao R, Yang L, et al.
JQ906576	HA	China	2011-06	A/duck/Zhejiang/12/2011(H7N3)	Institute of Bioengineering, Zhejiang Academy of Medical Sciences	Institute of Bioengineering, Zhejiang Academy of Medical Sciences	Hai-Bo W, Ru-Feng L, En-Kang W, et al.
KF259698	NA	China	2011	A/duck/Jiangxi/21714/2011(H11N9)	Centre of Influenza Research, School of Public Health, The University of Hong Kong	Centre of Influenza Research, School of Public Health, The University of Hong Kong	Lam T, Wang J, Shen Y, et al.
FJ581435	PB1	China	2007	A/chicken/Zhejiang/HJ/2007(H9N2)	Animal Husbandry and Veterinary Medicine of Fujian Academy of Agricultural Sciences	Animal Husbandry and Veterinary Medicine of Fujian Academy of Agricultural Sciences	Wan C, Huang Y

Figure 1

Timeline of potential exposures and medical consultation, laboratory-confirmed influenza A(H7N9) case, Shaoxing city, Zhejiang Province, China, October 2013



[a] Ningbo city is a neighbouring city to Shaoxing.

when diagnosing and treating suspected or confirmed cases or otherwise taking care of the patient; (ii) those who lived together or were in close contact with a suspected or confirmed case within 10 days of illness onset; (iii) those the epidemiologist determined as close contacts [4].

Data collection

All available medical records were reviewed by three clinicians, using a standardised data collection tool. Furthermore, epidemiologists and local public health doctors interviewed the patient's relatives, colleges and medical staff using a standard questionnaire. Pharyngeal swabs from the patient and his 25 contacts (n=26 in total), as well as nine environmental specimens from a live bird market nearby the patient's living place, were collected and submitted to Zhejiang CDC at 4 ºC for detection of influenza A(H7N9) RNA.

Laboratory testing

Viral RNA was extracted using Qiagen RNeasy Mini Kit. Real-time RT-PCR was used to detect influenza type A, subtype H7 and N9 with the protocol and specific primer and probe sets provided by China CDC [5]. Seasonal influenza viruses (A1, A3, or B), and H5N1 viruses were also tested by real-time RT-PCR [5]. Complete genomic fragments of the H7N9 virus were amplified directly from the clinical sample, and sequencing was performed with an ABI 3730XL automatic DNA analyser. The nucleotide sequences were determined by dideoxy sequencing, using an ABI Prism BigDye Terminator cycle sequencing kit. Phylogenetic trees were constructed by maximum likelihood method with GTR+I+Γ4 model using MEGA 5.1 to estimate the relationship with selected influenza A virus strains obtained from the Global Initiative on Sharing Avian Influenza Data (GISAID) database. The sequences from reference strains used in the genetic analysis were obtained from the EpiFlu database of the GISAID (Table 1). The full genome sequence was submitted to GISAID (A/Zhejiang/22/2013(H7N9); accession no. EPI477399-414).

Case description

A male case in his mid-30s was diagnosed with laboratory-confirmed influenza A(H7N9) virus in Shaoxing City on 14 Oct 2013. His illness began on 7 October. The patient had no smoking history, and had no occupational exposure to poultry. He has been living in Shaoxing city for seven years, sharing with one colleague a dormitory located 1 km away from his daily workplace. The dormitory is located in a village where free-range chickens are kept. The patient did not report any contact with patients with influenza-like illness or visits to live-bird and animals markets within 10 days of illness onset except that he travelled to a neighbouring city on 2–3 October and ate cooked chicken on 6 October.

Illness began with influenza-like symptoms; including cough and 40.5 ºC fever on 7 October. The patient consulted a township hospital twice, on 8 and 9 October. He was first admitted to the township hospital on the morning of 11 October and then transferred to Shaoxing municipality hospital because of progressive dyspnoea and shortness of breath. At midnight on 11 October, his condition deteriorated and he was transferred to the intensive care unit. The patient was reported to Zhejiang CDC as a suspected influenza A(H7N9) case on 14 October. One throat swab was collected on October 14 and tested positive for influenza A(H7N9) with real-time PCR on the same day. The government announced the case on 15 October (Figure 1).

A chest radiograph on 9 October revealed bilateral interstitial pneumonia, and consolidation was noted in a chest computed tomography scan on 11 October. The results of the clinical biomarkers are listed in

Table 2

Clinical characteristics at admission, laboratory-confirmed influenza A(H7N9) case, Shaoxing City, Zhejiang Province, China, October 2013

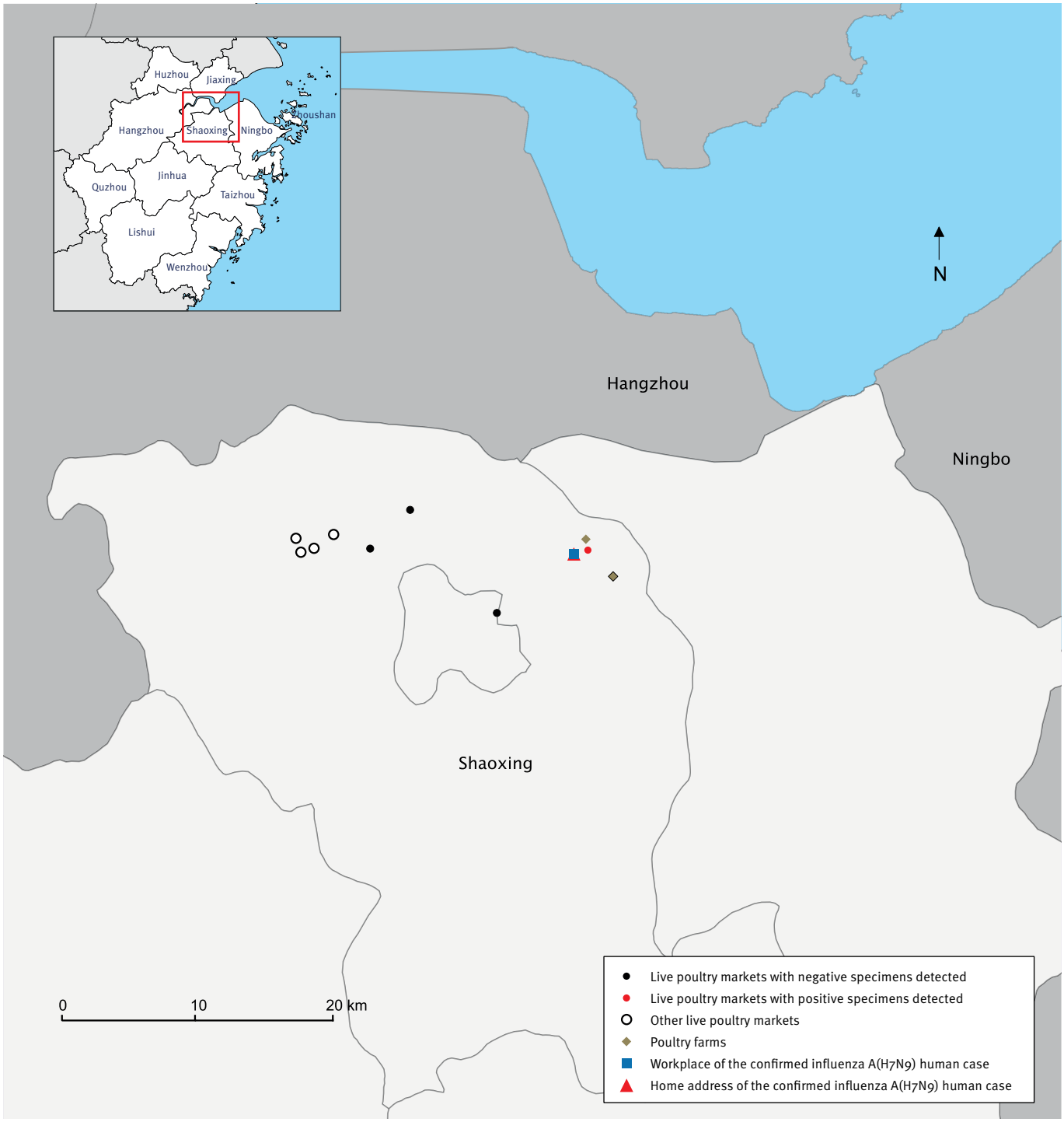
Characteristics	Patient	Normal value
Clinical symptoms and signs		
Fever	40.5 ºC	-
Cough	Yes	-
Cough with blood-tinged sputum	Yes	-
Shortness of breath	Yes	-
Dyspnoea	Yes	-
Chest pain	No	-
Abdominal pain	No	-
Diarrhoea	No	-
Nausea	Yes	-
Vomiting	Yes	-
Skin ecchymosis	No	-
Coma	Yes	-
Blood cell count		
White blood cell	3.51x109 cells/L	3.5–9.5 x109 cells/L
Neutrophils	3.34x109 cells/L	1.8–6.3 x109 cells/L
Lymphocytes	0.12x109 cells/L	1.1–3.2 x109 cells/L
Platelets	172 x109 cells/L	125–320 x109 cells/L
Biomarker		
Alanine aminotransferase	75 U/L	9.0–50 U/L
Aspartate aminotransferase	165.2 U/L	15–40 U/L
Lactate dehydrogenase	1,050.1 U/L	109.0–245 U/L
Creatine kinase	16,737 U/L	38–174 U/L
C-reactive protein	184.07 mg/L	0–8 mg/L
Prothrombin time	12.8 s	11–15 s
Blood gas analysis		
Oxygen tension	56.3 mmHg	83–108 mmHg
Carbon dioxide tension	22.0 mmHg	22.0–29.0 mmHg
Blood oxygen saturation	89.2%	95–98%
Chest findings		
Chest X-ray on 8 October	Bilateral lung markings increased	-
Chest computed tomography on 11 October	Bilateral interstitial pneumonia and consolidation	-
Complications		
Septic shock	No	-
Respiratory failure	Yes	-
Acute respiratory distress syndrome	Yes	-
Acute renal damage	No	-
Encephalopathy	No	-
Multiple organ failure	No	-
Diffuse intravascular coagulation	No	-
Secondary infections	No	-
Treatment		
Oxygen therapy	Yes	-
Extracorporeal membrane oxygenation	No	-
Continuous renal replacement therapy	No	-
Antibiotic therapy	Cefuroxime sodium + levofloxacin lactate + imipenem/ cilastatin	-
Antiviral agent	Yes	-
Glucocorticoid therapy	No	-
Intravenous immunoglobulin therapy	Yes	-
Mechanical ventilation	Positive end expiratory pressure	-

Table 2. The case was diagnosed as a laboratory-confirmed case of influenza A(H7N9) infection with severe pneumonia combined with two complications: acute respiratory distress syndrome (ARDS) and acute respiratory failure (type I). Treatment with oseltamivir was started at a dose of 150 mg twice daily on 13 October. Endotracheal intubation and mechanical ventilator support (positive end expiratory pressure, PEEP) were given on 11 October because of acute respiratory failure. His condition continued to worsen despite further treatments including oxygen, antibiotic therapy (cefuroxime sodium + levofloxacin lactate + imipenem/ cilastatin) and intravenous immunoglobulin therapy (Table 2). As of 24 October, the patient has remained in intensive care and his condition remains serious.

The case had 25 close contacts, including 21 healthcare workers, one sibling, one workplace colleague, one patient exposed in the same ward and one roommate in the dormitory. All contacts without clinical symptoms were negative for influenza A(H7N9) virus RNA by real-time RT-PCR performed on 15 October during the first five days of medical observation.

Figure 2

Distribution of the human influenza A (H7N9) case and the live poultry and wholesale market in Shaoxing city, Zhejiang Province, China, October 2013



Environmental samples

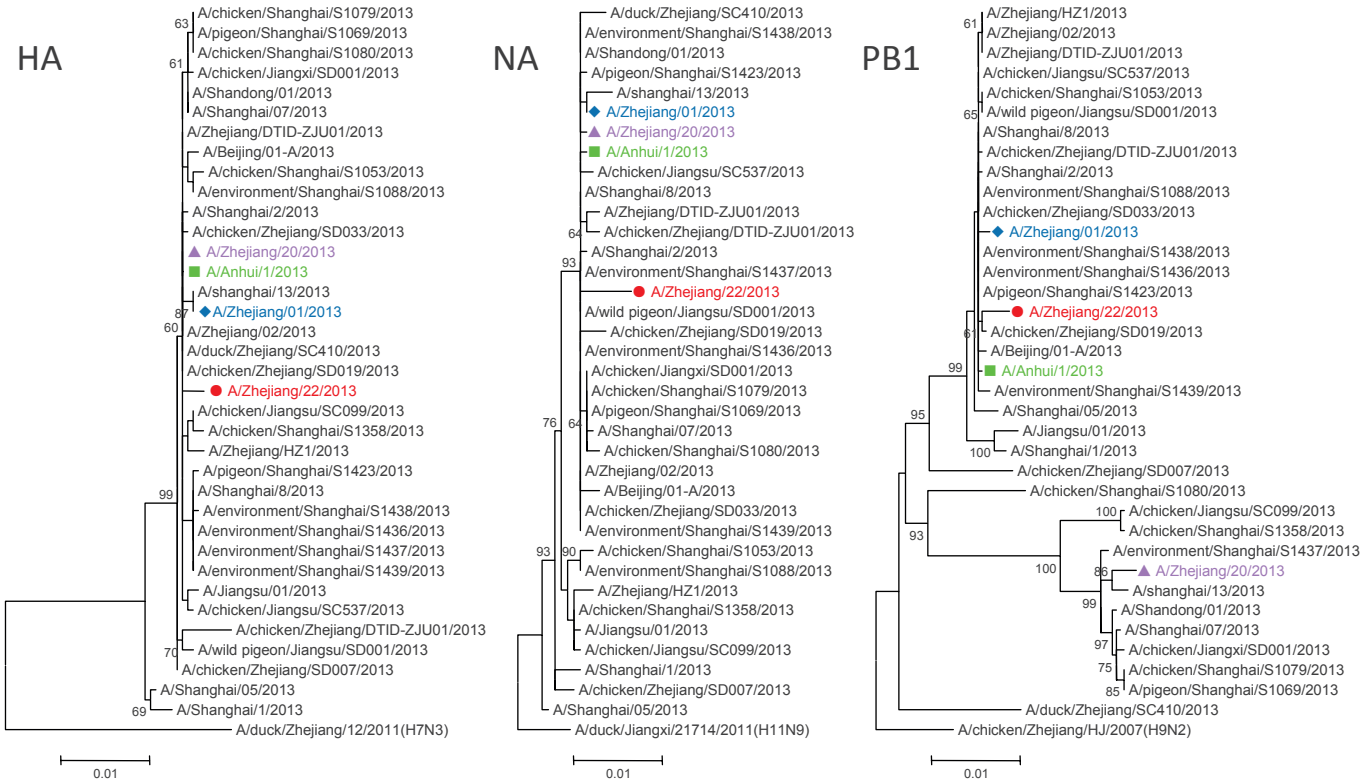
Two of the nine environmental samples collected from the live poultry market and the secondary wholesale market were positive for influenza A, H7 and N9; the other seven environmental samples remained negative for these three targets (Figure 2).

Sequence analysis

Phylogenetic analysis showed the A/Zhejiang/22/2013(H7N9) (ZJ/22) virus was in the same cluster with the World Health Organization (WHO)-recommended A(H7N9) vaccine virus, A/Anhui/1/2013(H7N9) (Figure 3).

Figure 3

Phylogenetic analysis of HA, NA and PB1 genes of the reemerged influenza A (H7N9) isolate, Shaoxing City, Zhejiang Province, China, October 2013



Red circle: A/Zhejiang/22/2013(H7N9), current case in October 2013;
green square: A/Anhui/1/2013(H7N9), 2013 selected influenza vaccine strain;
blue diamond: A/Zhejiang/01/2013(H7N9), the first case in the spring 2013 from Zhejiang;
violet triangle: A/Zhejiang/20/2013(H7N9), the first case in the spring from Shaoxing city of Zhejiang.

The selected characteristic amino acids of the ZJ/22 virus were similar to other H7N9 viruses (Table 3), which included the mammalian-adaptive mutations previously reported [5], such as Q226L in the HA gene. The R294K mutation in the NA gene of H7N9 virus has been reported to confer reduced sensitivity to oseltamivir and to be related to the administration of antivirals [5]. Our sample was collected only a few hours after the patient received the oseltamivir treatment, it remained R in position 294 of the NA gene.* Interestingly, five amino acid mutations, V241I, K266E, N327T, N346D and K465R were detected in the NA gene that had not been identified in previous H7N9 viruses. The ZJ/22 viruses possessed 627E in the PB2 gene, which has been found in few of the human H7N9 isolates and all of the H7N9 isolates from avian or environmental samples [6]. The D701N mutation in PB2 was not detected. However, the PB1 gene of ZJ/22 presented in a different cluster with the ZJ/20 virus that had previously been isolated from the confirmed case in Shaoxing city in April 2013.

Discussion

We report here a severe case of laboratory-confirmed influenza A(H7N9) virus infection this winter in Zhejiang Province, identified through enhanced surveillance of pneumonia of unknown cause. The event shows that influenza A(H7N9) has returned three months after the last case had been confirmed in China in summer, and around five months since the spring outbreak in eastern China. It indicates a possible risk of a larger outbreak of influenza A(H7N9) this winter [7]. The epidemiological, clinical and laboratory findings in this case were similar to the previous cases in Zhejiang Province this spring, with no evidence of sustained person-to-person transmission. However, this case differed from the previous cases in that it was a younger severe case with no obvious underlying diseases and no obvious recent direct contact with live poultry. Most laboratory-confirmed cases in the past had been older patients over the age of 60 years reporting recent exposure to poultry, generally at live bird markets [3,5,7]. Epidemiological and clinical data have been shared with WHO and other international partners.

Table 3

Selected characteristic amino acids of the A/Zhejiang/22/2013(H7N9) virus isolated from a laboratory-confirmed influenza A(H7N9) case, Shaoxing City, Zhejiang Province, China, October 2013

Genes	Function associated	Sites	**A/Zhejiang/ 22/2013**	A/Anhui/ 1/2013	A/Zhejiang/ 1/2013	A/Zhejiang/ 20/2013
HA (H3-Numbering)	Without N-glycosylation and increased virus binding to human-type receptors	160	**A**	A	A	A
	Specific mutation[a]	222	**P**	Q	Q	Q
	Receptor binding site	186	**V**	V	V	V
		226	**L**	L	I	L
		228	**G**	G	G	G
NA	Drug resistance	119	**R**	R	R	R
		120	**E**	E	E	E
		152	**D**	D	D	D
		153	**R**	R	R	R
		224	**I**	I	I	I
		226	**R**	R	R	R
		276	**H**	H	H	H
		278	**E**	E	E	E
		294	**R**	R	R	R
		296	**N**	N	N	N
		372	**R**	R	R	R
	Specific mutation[a]	241	**I**	V	V	V
		266	**E**	K	K	K
		327	**T**	N	N	N
		346	**D**	N	N	N
		465	**R**	K	K	K
PB2	Enhanced transmission	627	**E**	K	K	E
		701	**D**	D	D	D
PB1-F2	Increased pathogenicity		**90 aa**	90 aa	90 aa	25 aa
M2	Amantidine resistance	31	**N**	N	N	N

[a] These mutations have not been found in previously reported H7N9 viruses.

The surveillance system for unexplained pneumonia covering in all hospitals of Zhejiang Province was set up since 2004 ran routinely for 10 years. In 2013, following the confirmation of the first influenza A(H7N9) case on 1 April, the surveillance system was expanded and strengthened, and one confirmed influenza A(H7N9) case was discovered through this system during the spring outbreak, but no further cases were discovered until 14 October. This observation is consistent with seasonality of influenza A(H7N9) cases, much like human cases of influenza A(H5N1) in China that have mainly been occurring in the winter months [8].

The clinical features in this new case were similar to the cases reported in the spring in Zhejiang Province and other areas of China [9]. They shared the acute onset and rapid development to severe and sustained hypoxia, accompanied by respiratory failure, ARDS, etc. Although the patient received broad-spectrum antibiotics, supportive management and mechanical ventilation, his condition deteriorated. Without underlying comorbid conditions or other risk factors, the case did not receive oral oseltamivir until seven days after disease onset, which may have contributed to disease progression as early treatment with neuraminidase inhibitors can be effective [7,9].

Although no other influenza A(H7N9) outbreaks had been identified in animals in Shaoxing city before onset of the present case's illness and although he had no history of direct exposure to birds, the isolated virus had a high degree of similarity to H7N9 viruses

previously reported in humans, with 98.5%–99.6% (see Figure 3). Five mutations in the NA gene have never been reported in those strains isolated in spring 2013. The origin of these mutations is not clear, and any potential change in pathogenicity or transmissibility remains to be determined. In addition, positive PCR results were obtained in two of the nine samples from live bird markets, which is consistent with continuing circulation of this virus in poultry and epidemic spillover leading to human cases [10,11]. Within 10 days before his illness onset, the case had spent two days in a rural area, and wild animals cannot be excluded as a possible source, although the incubation period would then have been very long. If influenza A(H7N9) transmission continues in poultry, human infections may become more frequent when temperatures decrease in China this winter.

This study had some limitations: Firstly, we were unable to comprehensively investigate the exposure and travel history because the case has been unconscious in the intensive care unit since 11 October. Secondly, medical observation of contacts is still ongoing without any positive findings to date. Thirdly, the H7N9-positive isolates from birds from the markets have not been sequenced because the viral load in the environment was too low. Finally, we have just begun a serological survey to identify subclinical infections among local residents associated with exposures in the affected live poultry markets and poultry farms.

Conclusion

Human infection with influenza A(H7N9) virus has re-emerged in winter 2013, without substantial genetic change in the virus, signalling the potential for a new epidemic wave this winter. It is important to monitor the pandemic potential of this re-emerging virus which has apparently continued to circulate in an animal reservoir during the summer. Based on experiences in the spring, enhanced and expanded surveillance in the human and animal populations help to ensure early discovery and diagnosis of suspected cases, while hygiene campaigns and closure of live poultry markets can reduce the risk of severe cases and deaths. In particular, enhanced surveillance in poultry would be helpful if it can identify the H7N9 virus and inform early control measures before human infections occur. In the longer term, reformation of the poultry farming, distribution and purchasing system may be required to reduce human risk of infection with avian influenza viruses.

Note added in proof

A second severe laboratory-confirmed influenza A(H7N9) case was identified on 23 October in Jiaxing City, Zhejiang Province, while this manuscript was in production. The case is a man in his late 60s with no underlying disease who lived in a rural area and whose occupation included transporting and selling poultry.

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Conflict of interests

None declared.

Ethics Statement

For all sampling activities official permits were approved by the Chinese medical ethical committee, and the National Health and Family Planning Commission (NHFPC) approved the study. We got the written consent of collecting samples by the relatives of this patient.

Authors' contributions

Conceived and designed the experiments: Enfu Chen, Yin Chen, Lijun Fu, Zhiping Chen, Benjamin J. Cowling. Epidemiological investigation: Haiyan Mao, Dayan Wang, Michael Y. Ni, Peng Wu, Zhenyu Gong, Zhao Yu. Performed the experiments of virology: Tingting He, Zhen Li, Jian Gao. Contributed reagents/materials/analysis: Yuelong Shu. Drafted the manuscript: Shelan Liu. Conceived and coordinated the study: Shichang Xia, Hongjie Yu.

* Authors' correction:

In the first column of Table 1B, the segment IDs in the last three rows were amended to include one segment ID per row.

In addition, the sentence, 'Our sample was collected before the patient received the oseltamivir treatment, it remained R in position 294 of the NA gene' was corrected to read, 'Our sample was collected only a few hours after the patient received the oseltamivir treatment, it remained R in position 294 of the NA gene'.

These corrections were made on 25 October 2013 at the request of the authors.

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Study of a measles outbreak in Granada with preventive measures applied by the courts, Spain, 2010 to 2011

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Measles had practically been eliminated in Granada since the systematic vaccination of children with two doses introduced in 1984. However, in 2009 the disease returned in the form of small outbreaks. This study describes the measles outbreak that occurred in Granada from October 2010 to August 2011 and the measures imposed to control it. Information was sourced from the records of the Andalusian epidemiological surveillance system. A total of 308 cases were recorded, representing an incidence rate of 33.6 cases per 100,000 inhabitants. The first wave of the epidemic took place in Granada city, with the majority of cases occurring among families who lived in the Albaycín neighbourhood and were opposed to vaccination for ideological and/or religious reasons. The initial cases were in unvaccinated children aged 1 to13 years. The outbreak later spread throughout the province. To control the outbreak, the vaccination schedule for the exposed children was brought up to date. The Regional Ministry of Health decided to take legal action in order to ensure vaccination of those in the initial nucleus of the outbreak.

Introduction

Between 2003 and 2009, substantial progress was made toward the previous World Health Organization (WHO) goal of measles elimination in the European Region by 2010. However, since late 2009, measles virus transmission has increased, and outbreaks have become widespread. In 2011, measles outbreaks were reported in 36 of 53 WHO European Region member states. Overall, the primary reason for the increased transmission and outbreaks of measles in the WHO European Region is failure to vaccinate susceptible populations [1]. The European Surveillance System (TESSy) detected a total of 30,567 cases of measles throughout the European Union (EU) and European Economic Area (EEA) [2].

The mean coverage of vaccination against measles increased from 1990 to 2008 in the WHO European Region, reaching an estimated average measles vaccine coverage of 95% or above in 30 of the Region's 53 Member States [3]. The appearance of outbreaks, particularly in Western Europe and more recently in Spain, has been related to the existence of pockets of susceptible people. These can often be divided into two main groups: a growing number of susceptible children of parents who distrust vaccinations, and those belonging to disadvantaged groups [3-6]. In Spain, 45 measles outbreaks were recorded across 17 autonomous regions in 2011, reaching a total of 3,647 cases [7]. The majority of these occurred in Andalusia (n=2,113 cases), Madrid (n=601) and Catalonia (n=315) [7,8].

In Andalusia, childhood vaccination against measles began in 1984. The measles-mumps-rubella (MMR) vaccine is administered at the age of 15 months. Since 1990 a second dose has been administered at the age of 11 years. In 2004, the age for the second dose has been changed to three years. Before 1984, between 8,000 and 98,000 cases of measles had been recorded annually, representing an incidence rate of 130 to 1,500 cases per 100,000 inhabitants per year. After the introduction of vaccines, the incidence rate fell dramatically, with just three cases per 100,000 inhabitants per year recorded in 2002 and 2004. Two small outbreaks were recorded in 2003 (Almeria, 185 cases) and 2008 (Algeciras, 247 cases) [9]. In 2010 an epidemiological alert protocol for measles was introduced in Andalusia, which set out the steps to take in the event of an outbreak or sporadic measles cases recorded [10].

In Granada Province, the incidence rate for measles had continued to fall since the two major epidemics in 1983 and 1985, with no cases at all recorded in 2001 and 2002. This trend was interrupted in 2009 with an outbreak of 22 cases among children in the south of the province, most of whom had not been vaccinated for ideological reasons [11,12]. In October 2010, a measles outbreak began in the Albaycín neighbourhood of the city of Granada and later spread across the rest of the city and throughout the province. Initial data for this outbreak have been published on previous occasions [5,6].

Table

Measles outbreak cases with prior contact to other confirmed measles cases, Granada Province, 2010–11 (n=130)

Prior contact	Residents	Professionals	Total
Attendance of family event	6	0	6
School	16	0	16
Household contact	71	0	71
Health centre	20	11	31
Workplace	0	1	1
Case in another country	5	0	5
Total	**118**	**12**	**130**

The objective of this study was to describe and analyse the characteristics of the outbreak recorded in Granada Province, and the measures taken to control it.

Methods

A descriptive analysis was done of the measles cases recorded in Granada during the epidemic period, on the basis of the variables of patient, location and time. Cases were considered confirmed i) when there was a laboratory diagnosis of infection or ii) when the rash had first appeared seven to 18 days earlier and they had an epidemiological link to a laboratory confirmed case. Laboratory confirmation was carried out by serology, (detection of specific IgM) and/or PCR or by isolating the virus in a throat culture or in urine. In accordance with the existing protocol, cases were considered clinically compatible when they met the clinical criteria but there was no laboratory confirmation or link with a confirmed case [10].

Outbreak data were obtained through the Andalusian Epidemiological Surveillance System's (Sistema de Vigilancia Epidemiológica de Andalucía, SVEA) alert network and processed using Microsoft Excel and Epi Info version 3.4.3. The univariate analysis included the calculation of means and medians for quantitative variables, and frequencies and percentages for qualitative variables. Incidence rates per 100,000 and by age group were also calculated.

Results

Outbreak description

On 25 October 2010, two cases of measles were recorded in unvaccinated children from the Albaycín neighbourhood of Granada. From that date until 27 August 2011 (week 34), considered to be the epidemic period, 372 suspected cases of measles were recorded throughout Granada Province: 287 of these were confirmed and 21 were compatible cases, and 64 were rejected. These 308 recorded measles cases represented an incidence rate of 33.6 cases per 100,000 inhabitants over the period of the outbreak.

In the first wave of the outbreak between 10 and 12 October 2010, there were six confirmed cases, all involving children of between one and 13 years-old who belonged to a religious community in Albaycín and had attended a family event that took place between 15 and 16 days before their onset of symptoms. A second and third wave of new cases were identified, with 10 and 16 cases, respectively, who had known epidemiological links with earlier cases within the family, neighbourhood, at school or at the hospital (Table).

With the progression of the epidemic, 148 cases occurred in the city of Granada and municipalities in the surrounding metropolitan area for whom there was no known contact with earlier cases. Finally, in late spring and early summer 2011, the outbreak reached the south of the province, where the last cases were recorded on 27 August 2011.

In 31 cases (10% of the total), measles infection was acquired at a healthcare centre. Eleven of the registered cases were healthcare workers; nine of them were between 25 and 36 years-old and two were older than 45 years. Six were doctors in training. There is no evidence of secondary cases as a result of transmission via these healthcare workers.

The average age of cases was 15 years (range: 25 days to 48 years). The measles incidence was highest in children under the age of 16 months (679.5 cases per 100,000 inhabitants), followed by children aged between 16 months and four years (106.6 cases per 100,000 inhabitants). Among the total number of cases, 80 (26%) were younger than 16 months and 134 (44%) were between 15 and 34 years-old. There were no cases older than 48 years (Figure 1).

The age distribution among the initial cases, which occurred in the Albaycín neighbourhood, was different from the later cases in the outbreak, with a predominance of cases (21 of 26 cases) in children between 16 months and 14 years-old (Figure 1).

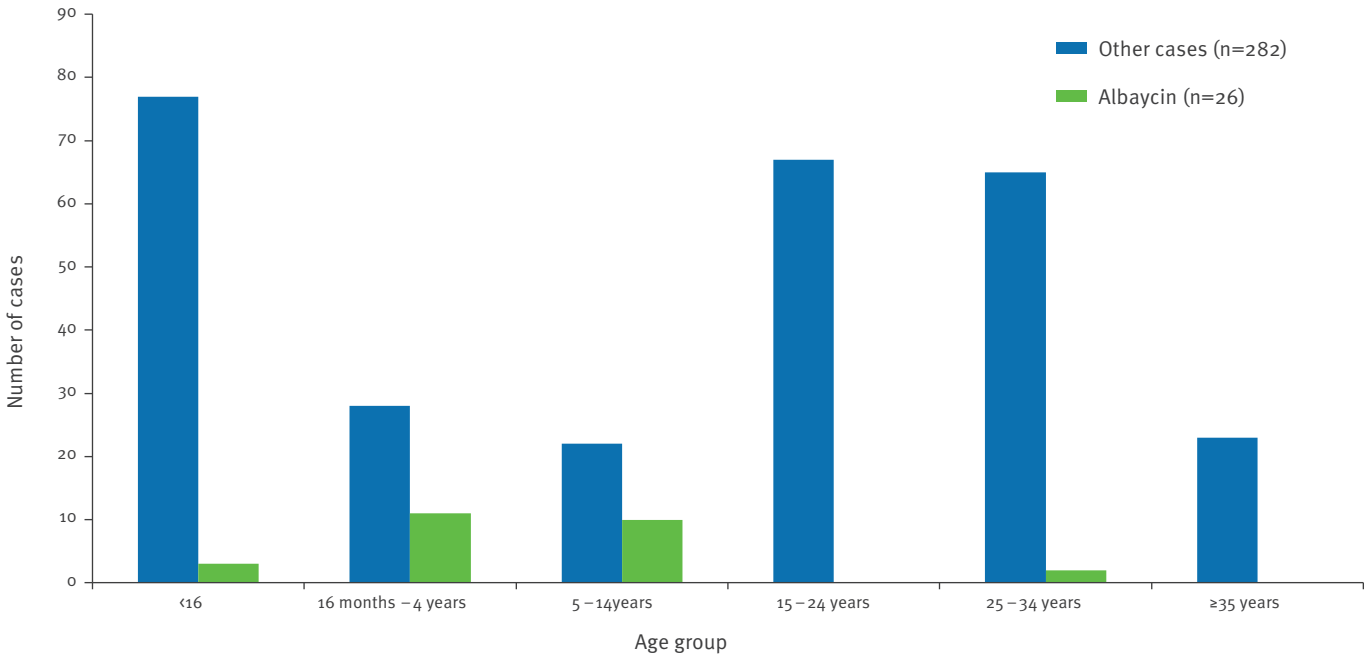
The sex ratio (male/female) was 1.18 for the entire outbreak. The hospitalisation rate was 23% (71 cases), lowest for the age group from five to 14 years (6.2%) and highest for the age group 34 years and above (47.8%). No deaths were recorded during the outbreak period.

Laboratory confirmation

Of the 287 confirmed cases, 254 (89%) were confirmed in the laboratory: 56 cases (22%) by PCR alone, 80 (32%) by serology (positive IgM) alone, and 118 cases (46%) with both methods. The genetic sequence of 22 samples that were sent to the National Microbiology Centre at the Carlos III Healthcare Research Institute, WHO reference laboratory for measles in Madrid, Spain, corresponded to the B3 genotype. These 22 samples had been taken at different times and places during the outbreak.

Figure 1

Measles outbreak cases, Granada province, 2010–11 (n=308)



Vaccination coverage

In 89 % of the 287 confirmed cases, the patient had not received any dose of MMR vaccine. Moreover, 81% of the 138 patients aged between 16 months and 24 years (the age range in which a high level of vaccination coverage is to be expected) had not received any dose of the vaccine. One dose of MMR had been given to 31 cases, for 10 of them documented. No case had received the second MMR dose.

Control measures

From the moment the first case was declared, the measures given in the alert protocol for measles – namely, isolating the cases and monitoring contact were adopted in order to prevent new cases from developing (Figure2).

The initial actions were aimed at the affected five institutions: two childcare centres (children aged 0–3 years) with one and two cases, two primary schools (children aged 3–12 years) in the neighbourhood with one and 10 cases, and one secondary school (children aged 12-16 years) in the city centre with one case. In four of the five schools, the parents were cooperative in terms of checking and keeping up to date with the vaccination schedule for the children. However, in the primary school in which the first cases had appeared, the vaccination coverage was low (63% of the ca. 200 pupils). Meetings with the parents were organised and informative letters were sent to the homes of all children in schools and childcare centres in Albaycín, requesting that the parents present the children's vaccination records and/or give written consent for their children to be vaccinated. Some parents refused the proposed vaccination on religious or ideological grounds (anti-vaccination beliefs based on naturopathy or alternative medicine).

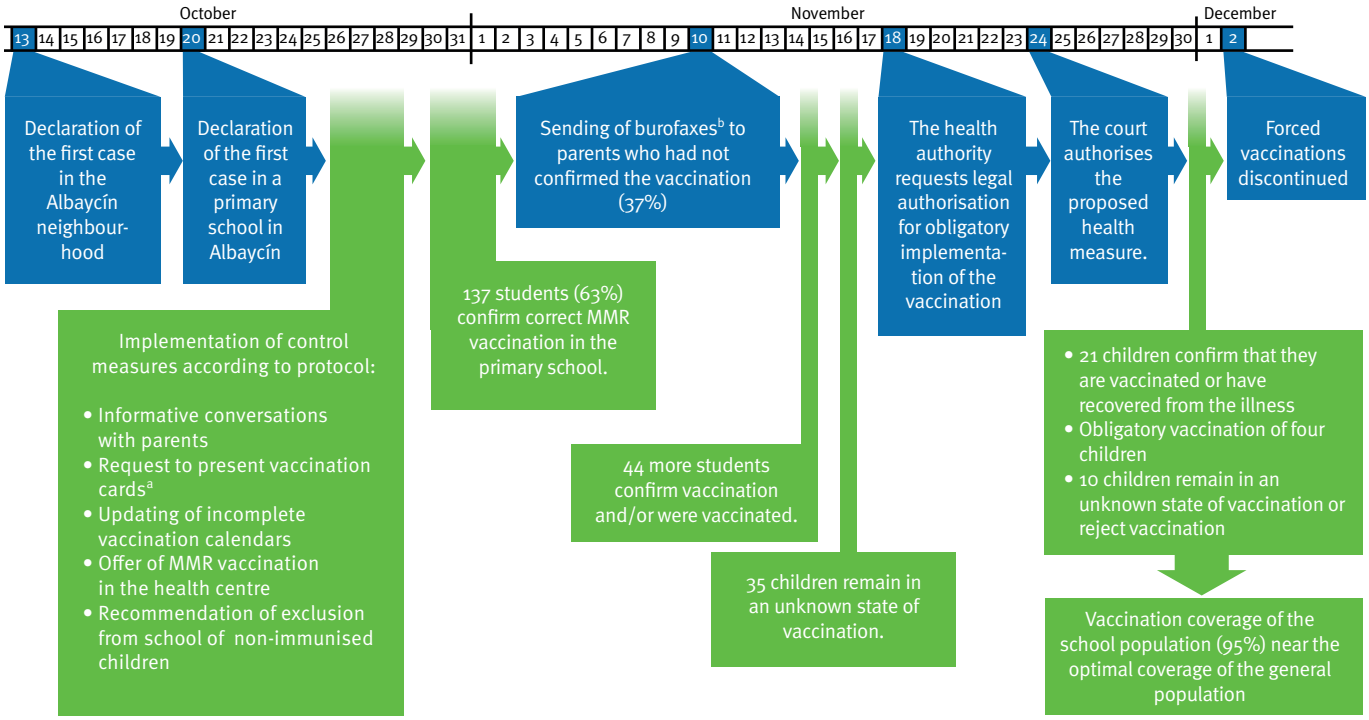
On 10 November 2010, burofaxes (a reliable means of communication in Spain) were sent to the parents of 79 students demanding the vaccination of the child or the presentation of the vaccination card in the school. In response, 44 children were either vaccinated or presented their card (Figure 2).

As the parents of 35 pupils at the primary school either failed to respond or rejected the vaccination, the Regional Ministry of Health requested authorisation from Granada Contentious-Administrative Court on 18 November 2010 to adopt the measures it considered urgent and necessary for public health, in accordance with the Special Measures for Matters of Public Health Act (Organic Law 3/1986 of 14 April 1986). The Ministry requested the forced vaccination of those children whose parents had expressly refused it or had failed to submit the child's vaccination record. Six days later, the presiding judge agreed to "*authorise the forced vaccination of the 35 children named in the list provided by the Provincial Office. The vaccination may be performed at the primary school, at the hospital or in the children's homes, and is to be performed by specialist healthcare professionals*".

The parents of said children were informed of this decision immediately. As a result of the decision, four children were vaccinated and a further 21 provided

Figure 2

Control measures adopted during the measles outbreak in Granada Province, 2010–11



MMR: measles-mumps-rubella.

[a] The request for vaccination cards was put out in different meetings and by letter.

[b] Burofax: a means of notification that confirms the content of the communication and serves as evidence in court and public administrations.

evidence that they had already been vaccinated or had already had measles (four of them had contracted measles during the ongoing epidemic). Some families rejected the vaccination.

On 2 December 2010, the provincial office of the Regional Ministry of Health submitted a written report to the court stating that it believed the situation of special risk detected at the primary school to have passed, as the proportion of pupils with immunity, whether through vaccination or contracted disease, exceeded 95%, which was considered sufficient to control the outbreak and render the forced vaccination of the remaining ten children unnecessary. The presiding judge responded that it was up to the Ministry to decide whether or not to carry out the authorised measures.

On 22 July 2013, the High Court of Justice of Andalusia refused the appeal lodged by two parents against the forced vaccination of their children.

Another measure was adopted during November 2010, when new cases of measles occurred, many of them in infants outside the Albaycín neighbourhood: the Regional Ministry lowered the age for the first dose of the MMR vaccine and authorised its administration to all children aged 12 months in the city of Granada and to those in other municipalities where one or more cases had been reported, for the period while the risk remained. This measure was modified two weeks later after measles cases were recorded in children under the age of 12 months: the Ministry decided to lower the vaccination age further to include all infants aged six months and older in the city of Granada and in those municipalities in which one or more cases had been recorded.

At the beginning of the outbreak, two cases were recorded who had contracted the infection at the paediatric accident and emergency department at a hospital in the city of Granada. Consequently, and in accordance with the alert protocol, the decision was made to isolate suspected cases at the accident and emergency department and to distribute information concerning the outbreak to the medical services that could potentially be affected. Serological tests and vaccination of healthcare workers under the age of 40 years was

recommended. A few tests were negative and there is only a record of four of those workers being vaccinated.

Discussion

Measles incidence rates as high as those reached in Granada Province during this outbreak have not been recorded for more than 20 years. The median age of the cases diagnosed at the start of the outbreak in 2010 was two years, which was much younger than the median age (19 years) of the cases diagnosed in 2011. The first waves of the epidemic involved many unvaccinated children living in the Albaycín neighbourhood. We think the main reason was that their parents were opposed to vaccination [6,12,13]. The higher age of cases in 2011 is suggestive of a historical pocket of susceptible adults (born before the start of the vaccination regime in 1984 but who did not contract the disease because vaccination rapidly interrupted the circulation of the virus). This phenomenon was also observed in other outbreaks occurring in 2011 in Spain and other European countries such as France which reported the largest number of cases (approximately 14,000). The median age of cases in those outbreaks were, respectively, 18 years (Spain) and 16 years (France) [2].

Due to the effectiveness of the vaccine, the majority of cases in the Granada outbreak occurred in an unvaccinated population. These were not only children under the age of 15 months not eligible for the first dose of the vaccine, but also a larger than anticipated unvaccinated population of susceptible adults born before 1984 and people who had not been vaccinated on ideological and/or religious grounds or as a result of belonging to an underserved population [12].

Coercive measures can be necessary to control outbreaks in certain situations

[12-14]. At the start of this outbreak, there was a risk of the disease spreading to the rest of the city because a significant proportion of parents in the Albaycín neighbourhood were not willing to support the catch-up vaccinations. This led the Regional Ministry of Health to adopt a highly unusual measure: it requested the Contentious-Administrative Court to authorise the forced vaccination of 35 children. The court's decision in a situation of conflict between the right to choose not to be vaccinated and the protection of the health of the collective was a significant boost to the control activities: it changed the attitudes of some of the parents, prompting them to have their children vaccinated, and made it possible to determine that many of the other children were not susceptible to measles.

A precedent of a coercive measure adopted by the health services and supported by the legal authorities had occurred in Berlin in 2010, during an outbreak of measles at a private school (a 'Waldorfschule') where many pupils had not been vaccinated as a result of their parents' anti-vaccination stance. The public health authorities decided to adopt the legal measures foreseen in the legislation, such as withdrawing from school those pupils who were unvaccinated and who had not already contracted (and recovered from) measles. The parents lodged an appeal against this decision with Berlin Administrative Court; however, the judge found in favour of the utility and legality of the measure [15].

Exclusion from school of children that were unvaccinated and/or not immune was recommended in the Albaycín school, but never imposed by the education or legal authorities. However, faced with the inefficiency of this recommendation, the health authority later decided to appeal to the court to apply forced vaccination as a measure of control. According to the literature consulted, this measure had not previously been taken in Spain or in other countries in Europe.

A significant proportion of the exposed healthcare workers, such as training medical specialists and other young professionals, were part of the group considered susceptible on account of their age (25 to 35 years-old). Given that on this occasion, the efforts made to vaccinate health workers were not sufficient to prevent the disease, it would be important to ensure the vaccination schedule for these professionals is up to date when they start work [11,16,17]. Contact with other patients in the accident and emergency departments of the hospitals involved led to the infection of at least 20 people who had visited the hospital for other reasons. This highlights the importance of reinforcing the early diagnostic isolation and treatment measures for suspected measles cases during an outbreak.

The most frequently recorded genotype in the latest outbreaks in Spain (Madrid, Seville and Valencia) and across Europe was the D4 genotype [18]. The B3 genotype isolated in the Granada outbreak is less common in Spain and the rest of Europe and could have been imported from Africa [19].

In conclusion, the measles outbreak recorded in Granada during 2010-11 was notable for the low vaccination coverage among certain sections of the population, the role played by nosocomial transmission of the disease, and the use of court-approved coercive measures to gain control over the outbreak.

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Conflict of interest

None declared.

Authors' contributions

All authors contributed to the writing of the manuscript and agree with the results and conclusions.

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Outbreak of West Nile virus infection among humans in Serbia, August to October 2012

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We describe the first reported outbreak of West Nile virus (WNV) infection in humans in Serbia in August to October 2012 and examine the association of various variables with encephalitis and fatal outcome. Enzyme-linked immunosorbent assay (ELISA) was used for detection of WNV-specific IgM and IgG antibodies in sera and cerebrospinal fluid. A total of 58 patients (mean age: 61 years; standard deviation: 15) were analysed: 44 were from Belgrade and its suburbs; 52 had neuroinvasive disease, of whom 8 had meningitis, while 44 had encephalitis. Acute flaccid paralysis developed in 13 of the patients with encephalitis. Age over 60 years and immunosuppression (including diabetes) were independently associated with the development of encephalitis in a multivariate analysis: odds ratio (OR): 44.8 (95% confidence interval (CI): 4.93–408.59); p=0.001 (age over 60 years); OR: 10.76 (95% CI: 1.06–109.65); p=0.045 (immunosuppression including diabetes). Respiratory failure requiring mechanical ventilation developed in 13 patients with encephalitis. A total of 35 patients had completely recovered by the time they were discharged; nine patients died. The presence of acute flaccid paralysis, consciousness impairment, respiratory failure and immunosuppression (without diabetes) were found to be associated with death in hospital in a univariate analysis (p<0.001, p=0.007, p<0.001 and p=0.010, respectively).

Introduction

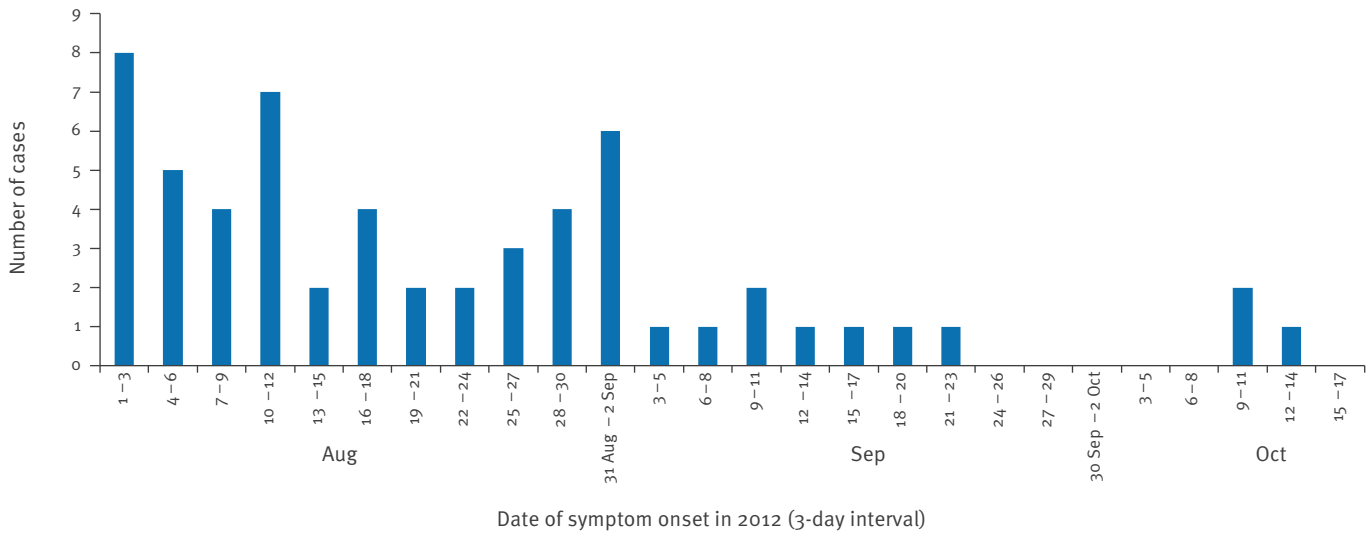
West Nile Virus (WNV) is a single-stranded RNA, mosquito-borne virus that belongs to the *Flaviviridae* family. It was first identified in the West Nile district of Uganda in 1937 in a woman who presented with a mild febrile illness [1]. During the next decades, the virus spread through Africa and Asia: by the mid-20th century, it had appeared in Europe [2]. The first cases were reported in Israel (in the World Health Organization European Region) in the 1950s, followed by France in 1962–63 [2]. The first large outbreak in humans in Europe was in Romania, in 1996–97, and subsequent outbreaks were also reported in Russia in 1999 and Spain in 2004 [3-5]. Recently, ecological conditions were favourable for the spread of WNV in the Mediterranean, central and south-east Europe, causing outbreaks in countries neighbouring Serbia: Italy in 2008–10, Hungary in 2008, Albania and the former Yugoslav Republic of Macedonia in 2011 [6-9]. In Greece in 2010, 262 cases of WNV human infection were reported, including 197 patients with neuroinvasive disease [10]. In the following year (2011), there was another outbreak in Greece [11]. At the same time, epidemics were recorded in Turkey in 2010 and 2011, and Russia in 2010 [12,13]. Taken together, these data indicated that WNV was circulating in nearby countries, suggesting that human cases of WNV infection could be expected in Serbia as well. In 2011, Lupulovic et al. reported the first serological evidence of WNV infection in Serbia as a zoonosis: they showed that 42 (12%) of 349 horses analysed had WNV-specific neutralising antibodies [14].

It was first thought that WNV caused a mild, influenza-like disease, but the epidemics in Romania in 1996–97 and New York in 1999 demonstrated that this virus was also a neuropathogen, causing severe neurological disease [3,15]. Today, it is known that around 20% of people who become infected have symptomatic disease, and one of 150 infected has neuroinvasive disease, which can present as meningitis, encephalitis and acute flaccid paralysis (AFP) [16,17]. The clinical presentation of AFP usually resembles that of poliomyelitis and AFP has been recognised as a separate entity of neuroinvasive disease due to WNV infection [16]. Possible, but extremely rare, clinical presentations of WNV infection are chorioretinitis, pancreatitis, fulminant hepatitis and myocarditis [18-20].

We describe here the clinical and epidemiological characteristics and outcome of patients in the first reported outbreak of WNV infection in humans in Serbia in 2012. We also sought to determine the association of various variables with encephalitis due to WNV infection and fatal outcome.

Figure 1

Human cases of West Nile virus infection by date of symptom onset, Serbia, August–October 2012 (n= 58)



Methods

Inclusion criteria

At the Clinic for Infectious and Tropical Diseases, Clinical Centre Serbia in Belgrade, we carry out specific screening for WNV infection (we have no data about specific screening in other medical centres in Serbia). In the study presented here, we enrolled patients with acute WNV infection treated at our clinic from 1 August to 31 October 2012.

Case definition

Diagnostic criteria for probable and confirmed cases were established according to the European Union case definition for West Nile fever [21]. A person with fever (≥37.5 °C), meningitis or encephalitis met the clinical criteria.

Patients presenting with meningitis, encephalitis and/or AFP were considered as having neuroinvasive disease. Meningitis was defined as the presence of fever, clinical signs of meningeal inflammation, including headache, nuchal rigidity, Kernig's sign or Brudzinski signs, photophobia or phonophobia, and the presence of cerebrospinal fluid (CSF) pleocytosis (›5 leucocytes/mm^3; norm: 0–5 leucocytes/mm^3), elevated protein levels (›0.45 g/L; norm: 0.15–0.45 g/L) and normal (2.6–3.1 mmol/L, 50–60% of serum glucose levels) or mildly decreased (2.4–2.6mmol/L) CSF glucose level [22,23].

Encephalitis was defined as the presence of fever, encephalopathy (decreased or altered level of consciousness, lethargy or personality change) and/or focal neurological signs (weakness, cranial nerve palsies), seizures or movement disorders (tremor, parkinsonism, ataxia) and the presence of CSF pleocytosis (›5 leucocytes/mm^3), elevated protein levels (›0.45 g/L), and normal (2.6–3.1 mmol/L, 50–60% of serum glucose levels) or mildly decreased (2.4–2.6mmol/L) CSF glucose level [22,23].

AFP was defined as acute onset of limb weakness or paralysis with progression over 48 hours. Limb weakness was characterised by at least two of the following: asymmetry; areflexia/hyporeflexia of affected limbs; absence of pain; electrodiagnostic studies consistent with an anterior horn cell lesion; CSF pleocytosis (›5 leucocytes/mm^3) and elevated protein levels (›0.45 g/L) [22].

Laboratory criteria for case confirmation was the presence of at least one of the following: (i) isolation of WNV from blood or CSF; (ii) detection of WNV nucleic acid in blood or CSF; (iii) WNV-specific antibody response (IgM) in CSF; (iv) WNV IgM high titre and detection of WNV IgG, and confirmation by neutralisation [21].

In our study, all patients who were diagnosed as confirmed cases had a WNV-specific IgM antibody response in CSF. Isolation of the virus and reverse transcription-polymerase chain reaction (RT-PCR) for detection of WNV nucleic acid were not performed.

Laboratory criteria for a probable case were the presence of WNV-specific IgM and IgG antibody response in serum in the absence of WNV-specific IgM antibodies in CSF.

None of the patients had a history of flavivirus vaccination. All of the patients with neuroinvasive disease had sterile CSF bacterial cultures and negative serological tests for other common causes of viral encephalitis, especially herpes viruses.

Serology tests

Acute phase CSF and acute and convalescence phase serum samples (on the first day of hospitalisation and within 10–14 days after symptom onset) were collected to be tested for the presence of IgM and IgG WNV-specific antibodies. The samples were transported at 4 °C and stored at –25 °C until testing. Samples were analysed by enzyme-linked immunosorbent assay (ELISA) (Anti-West Nile Virus ELISA (IgM) and Anti-West Nile Virus ELISA (IgG), EUROIMMUN, Medizinische Labordiagnostika AG), which was performed by the National Reference Laboratory for Arboviruses of the Institute of Virology, Vaccines and Sera 'Torlak' in Belgrade.

Patient data

Patients' demographic characteristics (age, sex, region of residence within the country), comorbidity (hypertension, cardiovascular disease including cardiomyopathy, coronary disease, previous stroke and myocardial infarction, diabetes, immunosuppression), presenting symptoms and signs, clinical findings, biochemical analysis of blood and CSF along with the results of serological tests were recorded, as well as the functional outcome at hospital discharge. Immunosuppression was defined as a state of reduced immune response due to, for example, immunosuppressive therapy, malignancy and radiotherapy. Consciousness impairment was classified as mild if the Glasgow Coma Score (GCS) was >8, and serious if the GCS was ≤8.

Statistical analyses

All data were analysed using descriptive and analytical statistics. For the univariate analysis, chi-square test and Fisher's exact test were applied for categorical variables and Student's t-test for continuous variables. Binary logistic regression analysis was performed to estimate the independent association of several variables with the development of encephalitis. Covariates were selected for entry into the model based on the results of univariate analysis ($p<0.2$) and judgement of clinical importance (i.e. if a known risk factor for encephalitis). Odds ratios (ORs) and 95% confidence intervals (CIs) were obtained.

The association of several variables with fatal outcome was analysed using univariate analysis: multivariate analysis could not be performed due to the small number of deaths. The Statistical Package for the Social Sciences (SPSS) software for Windows (version 17.0) was used for statistical analysis. Statistical significance was set at 0.05.

Results

A total of 58 patients who met the diagnostic criteria were included in our study. Of these, 45 were diagnosed as confirmed cases and 13 as probable cases, according to the case definition. The first cases had symptom onset in the beginning of August 2012 and the last in the first half of October (Figure 1).

Figure 2

Distribution of human cases of West Nile virus infection, Serbia, August–October 2012 (n=58)

* This designation is without prejudice to positions on status, and is in line with UNSCR 1244 and the ICJ Opinion on the Kosovo Declaration of Independence.

Of the 58 patients, 32 reported spending prolonged periods of time outdoors (every day during the summer, for more than two hours per day) or close (500 metres) to a river, or living next to a river and all had had multiple mosquito bites in the three weeks before symptom onset.

The majority of patients (n=33) were from Belgrade; 11 others were from Belgrade suburbs. A further seven were from the district of Srem, six from South Banat and one from North Banat (Figure 2). The first diagnosed case of WNV infection was in Pancevo, in South Banat. None of the patients had travelled abroad in the four weeks before symptom onset, except for one patient who had travelled to Montenegro (17 days before symptom onset), where no human cases of WNV infection had been reported.

Most patients (n=52) had neuroinvasive disease, while six presented with mild acute febrile illness. Among the six, three presented with fever and maculopapular

rash, one patient had pneumonia and two had a prolonged febrile illness with intensive myalgia.

Of the 52 patients with neuroinvasive disease, 44 had encephalitis and 8 meningitis. Of those with encephalitis, 10 presented with ataxia and dysmetria, suggesting rhombencephalitis, while 13 had AFP affecting one or more limbs. There were no patients with AFP without encephalitis.

The mean age of all 58 patients was 61 years (standard deviation: 15), while 33/44 patients with encephalitis were over 60 years old (Table 1). Patients with encephalitis were statistically significantly older than those with mild febrile illness or meningitis (p<0.0001). Age over 60 years and immunosuppresion including diabetes were found to be associated with the development of encephalitis by univariate analysis (p<0.0001 and p=0.024, respectively; Table 1), and were confirmed to be independently associated with encephalitis by multivariate logistic regression analysis after adjustment for confounders (sex, cardiovascular disease, hypertension) (OR: 44.8; 95% CI: 4.93–408.59; p =0.001 (age over 60 years) and OR: 10.76; 95% CI: 1.06–109.65; p=0.045 (immunosuppression including diabetes)).

Cytological and biochemical analysis of CSF from the 52 patients with neuroinvasive disease revealed pleocytosis (mean: 145 leucocytes/mm^3 (SD: 124), with lymphocyte predominance in 38 patients. In all 52 patients with neuroinvasive disease, the protein level was mildly elevated, at a mean of 1.09 g/L (SD: 0.52) and the mean glucose level was normal.

The frequency of self-reported symptoms and clinical signs in the 52 patients with neuroinvasive disease are shown in Table 2.
Among patients with encephalitis, neurological manifestations, including focal neurological deficit and/or consciousness impairment, were recorded a mean of 4 days (SD: 3) after symptom onset. Consciousness impairment appeared from the first to the 10th day of illness (median: 4th day). Among 33 patients who developed an altered state of consciousness, 13 had qualitative consciousness impairment (disorientation, confusion, agitation), while the others also had quantitative consciousness impairment (somnolence, sopor, coma). Six of the 33 had severe consciousness impairment (GCS≤8).

Respiratory failure requiring mechanical ventilation developed in 13 patients with encephalitis a mean of 12 days (SD: 6) after symptom onset (median: 11 days; range: 3–21). Five of these patients had only mild or no quantitative consciousness impairment at the time respiratory failure occurred. The mean duration of mechanical ventilation was 11 days (SD: 17) (median: 4 days; range: 2–50 days).

Of the 58 patients, 35 had completely recovered by the time they were discharged (Figure 3). Nine patients died.

All patients who died had encephalitis: eight of the nine were over 60 years-old. The presence of acute flaccid paralysis, consciousness impairment, respiratory failure and immunosuppresion (without diabetes)

Table 1

Demographic characteristics of and comorbidites in patients with different forms of West Nile virus infection, Serbia, August–October 2012 (n=58)

Characteristic	Total n=58	Mild febrile illness n=6	Meningitis n=8	Non-encephalitis n=14	Encephalitis n=44	Univariate analysis		Multivariate analysis	
						P value[a]	OR[a] (95% CI)	P value[a]	OR[b] (95% CI)
Mean age (SD)	61 (15)	40 (13)	54 (10)	48 (13)	65 (13)	<0.001	–	–	–
Aged >60 years	34	0	1	1	33	<0.001	39.0 (4.6–333.3)	0.001	44.8 (4.9–408.6
Male	40	3	5	8	32	0.210	2 (0.6–7.0)	–	–
Immunosuppression including diabetes	18	0	1	1	17	0.024	8.2 (1.0–68.4)	0.045	10.8 (1.1–109.7)
Immunosuppression excluding diabetes	5	0	0	0	5	0.237	–	–	–
Hypertension	33	0	6	6	27	0.182	2.1 (0.7–7.2)	–	–
Cardiovascular disease	11	0	1	1	10	0.186	3.8 (0.4–32.9)	–	–

CI: confidence interval; OR: odds ratio; SD: standard deviation.

[a] Univariate analysis was performed to evaluate the difference between patients with encephalitis and those with other forms of the infection (meningitis, mild febrile illness).

[b] Multivariate logistic regression analysis was performed after adjustment for confounders (sex, cardiovascular disease, hypertension).

Table 2

Frequency of self-reported symptoms and clinical signs in patients with neuroinvasive disease due to West Nile virus infection, Serbia, August-October 2012 (n=52)

Signs and symptoms	Number of patients
Fever (≥37.5 °C)	52
Neurological manifestations	44
Fatigue	40
Consciousness impairment	33
Headache	32
Vomiting	29
Myalgia	17
Respiratory failure	13
Acute flaccid paralysis	13
Diarrhoea	9
Rash	5
Conjunctivitis	3

were found to be associated with a fatal outcome in a univariate analysis ($p<0.001$, $p=0.007$, $p<0.001$ and $p=0.010$, respectively) (Table 3).

Discussion

In Serbia, the summer of 2012 was among the warmest summers in recent years, with a dense mosquito population leading to the appearance of the first cases of human WNV infection in the country [24]. The majority of the patients were from urban regions, mainly from Belgrade and its suburbs, possibly due to specific screening in our clinic. It could also be related to the fact that Belgrade is situated at the confluence of two rivers (Danube and Sava). People enjoy swimming and other leisure activities (10–50 metres from the rivers) during the summer months and there is also a large number of houses on stilts and boat restaurants where many people gather in the summer evenings: there are dense mosquito populations by the rivers, especially in the evening [25]. Although epidemics of WNV infection were first reported in rural areas, the epidemics in Romania in 1996 and New York, United States, in 1999 demonstrated the spread of this infection in urban areas as well [3,15]. In our study, the majority of diagnosed patients had neuroinvasive disease, but we assume that there were more patients with WNV infection who had influenza-like febrile disease, but who did not seek medical help due to the symptoms being mild or were treated in primary care.

During the outbreaks in Israel, New York and Romania, most of those diagnosed with WNV infection had neuroinvasive disease [2,3,15,26]. In our study population, encephalitis was the most frequent form (in 44 of 58 patients). Similar results were reported in Romania (1996) and the United States (1999), with meningoencephalitis being present in 60–65% of patients with WNV infection with neuroinvasive disease [17]. In our study, encephalitis was seen mainly in older people and those with any kind of immunosuppression including diabetes, which was also reported by Nash et al. and Murray et al. [15,27].

A notable proportion of our patients with neuroinvasive disease developed AFP. In the outbreaks and Romania and Russia, paresis or paralysis was recorded in 15–20% of patients, while in the New York epidemics, the proportion of patients with paresis was notably higher, reaching 50%, while about 10% had paralysis [3,4,15,26].

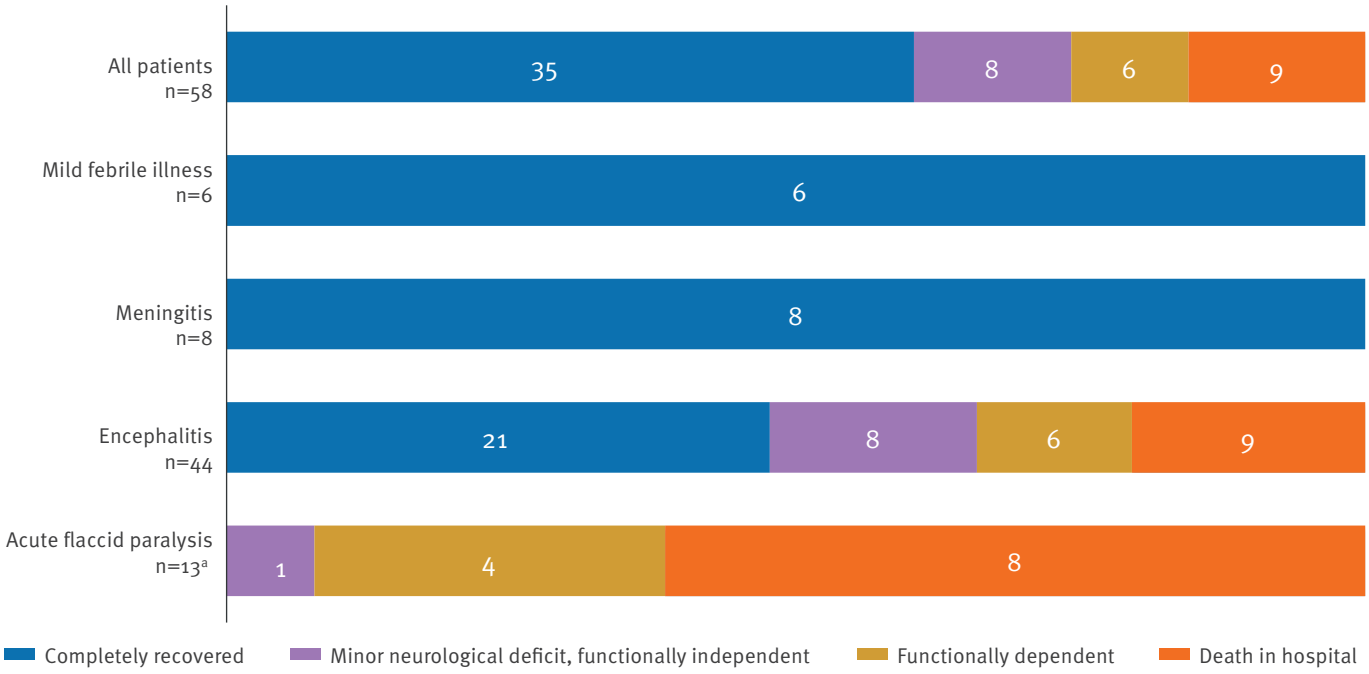
Another symptom that was observed more often in patients with encephalitis due to WNV infection compared with patients with encephalitis caused by other pathogens was respiratory failure and the need for ventilation support. In our patients, respiratory failure mostly developed during the later stages the illness, usually around the 12th day after symptom onset. Respiratory failure during encephalitis due to WNV infection has also been described by others [28,29]: it was usually not accompanied by consciousness impairment profound enough to cause central respiratory failure [29]. It has been assumed that respiratory failure in neuroinvasive disease due to WNV infection is caused by respiratory muscle weakness including both diaphragm and intercostal muscles, similar to that in poliomyelitis [30,31]. Pathological inspection of the spinal cord of patients who had died from respiratory failure in WNV infection revealed gliosis, neuronal loss and inflammation in the anterior horns of the cervical spinal cord and brainstem [32].

The case fatality rate (CFR) of 16% (9/58) in our study is similar to CFRs reported in the literature for WNV infection. In recent epidemics, the overall CFR was lower, ranging from 4% to 14%, but higher rates were found in older patients and those with encephalitis [4,15,20,26,33,34]. Among patients with encephalitis, the CFR reached 18% in the United States and 24% in Israel, while CFR among patients of age over 70 years was 29% [15,26]. High rates of patients with encephalitis, AFP and fatal outcome were usually observed in the first outbreaks, when the virus was introduced to a non-immune population [15,26]. In our study, the CFR reached 20% (9/44) in patients with encephalitis.

Veterinary and entomological investigations related to WNV have also been performed in our country. Djuricic et al. analysed 3,618 sera collected from several animal species and humans from different areas of Serbia in 2008–12, revealing that WNV was circulating in 9 of 18 tested locations in Serbia [35]. The percentage of seropositive results varied from 0.42% in Pozarevac (horses and humans) to 6.45% in Novi Pazar (dogs). Among the species tested, the highest seropositivity was registered in horses (3.97%) [35]. Petrovic et al. examined 92 sera from 30 migratory and resident

Figure 3

Functional outcome of patients at hospital discharge according to different forms of West Nile virus infection, Serbia, August–October 2012 (n=58)



The numbers represent the number of patients with the specified outcome.

[a] All had encephalitis.

wild birds species collected during 2012 in Vojvodina: WNV antibodies were detected in seven samples [36]. WNV RNA was also detected in birds, mostly among raptors: genetic analysis of isolated virus strains demonstrated that they belonged to lineage II, similar to those detected in Greece and Hungary [36]. During August 2012, a total of 3,000 *Culex pipiens* mosquitoes were collected from three locations in Belgrade: testing of 150 pools of females confirmed the presence of WNV nucleic acid in 10 of the pools [37]. Tests in various parts of Serbia suggest that WNV is circulating not only in the area of Belgrade and Vojvodina [35]. Screening for human cases should therefore be further strengthened in summer and autumn in regions of the country not previously affected by WNV. In 2013, there were even more reported cases (n=260) [38], of which 180 were treated in our clinic.

This study has some limitations. It was a single-centre study, and although it was performed at the main and largest clinic for infectious diseases in Serbia, there are other hospitals in which patients with WNV infection might have been treated. For this reason, no incidence rates could be calculated at a national or regional level. Another limitation is the small number of patients. While some of the results were statistically significant and concur with previous publications, the CIs of the ORs were wide. The study included only six patients with mild febrile illness, making it difficult to determine correlates of encephalitis. A larger sample size would be necessary for confirmation of the results. A case–control study comparing clinical characteristics of patients with encephalitis due to WNV infection with those of encephalitis patients with a different aetiology would also provide more information and might reveal specific characteristics of encephalitis due to WNV infection.

Given the number of patients with WNV infection in 2012 in Serbia, a number of public health measures were undertaken. These included intensifying activities to reduce the number of mosquitoes in indoor and outdoor environments (e.g. using insecticides, destroying mosquito habitats), systematic extermination of larvae and adult forms of mosquitoes, education of the population on how to avoid or decrease the risk of being bitten by potentially infected mosquitoes (e.g. through poster, leaflets, television and newspapers) [37]. The Ministry of Health has set up a project to detect WNV in mosquitoes in Serbia. A team from the Institute for biocides and medical ecology – comprising physicians, veterinarians and laboratory staff – will set traps to collect adult forms of mosquitoes, which will be tested for the presence of WNV (by RT-PCR) in urban regions and near large rivers in various parts of Serbia. Surveillance of human cases has also been improved as WNV infection became mandatorily notifiable, in August 2012.

Table 3

Characteristics of patients with West Nile virus infection who died (n=9) and those who survived (n=49), Serbia, August–October 2012

Characteristic	Total number of patients with the characteristic	Number of patients with the characteristic who died	Number of patients with the characteristic who survived	P value	OR (95% CI)
Age >60 years	34	8	26	0.067	7.1 (0.8–61.0)
Male	40	4	36	0.119	0.3 (0.1–1.2)
Immunouppression (including diabetes)	18	4	14	0.438	2.0 (0.5–8.6)
Immunosuppression (excluding diabetes)	5	4	1	0.010	38.4 (3.6–413.7)
Encephalitis	44	9	35	0.096	–
Acute flaccid paralysis	13	8	5	<0.001	70.4 (7.2–685.1)
Consciousness impairment	33	9	24	0.007	–
Respiratory failure	13	9	4	<0.001	–

CI: confidence interval; OR: odds ratio.

These actions should help avoid future outbreaks of human WNV infection and raise the awareness of healthcare providers about the emergence of the virus in Serbia. In patients with mild influenza-like disease of unknown origin and those with neuroinvasive disease during late summer and early autumn, WNV should be considered a possible causative pathogen.

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Conflict of interest

None declared.

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Retrospective identification of human cases of West Nile virus infection in Austria (2009 to 2010) by serological differentiation from Usutu and other flavivirus infections

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There is increasing evidence for the spread of West Nile virus (WNV) in southern, eastern and central Europe. In parallel, another flavivirus, the antigenically closely related Usutu virus, was introduced from Africa and first detected in Austria (2001), followed by Spain (2003), Hungary (2005), Italy (2006), Switzerland (2006) and Germany (2007). In Austria, human WNV infections have not previously been documented, although the virus was isolated from birds and detected in mosquitoes in 2008 and 2009. We therefore conducted a retrospective search for human cases of WNV infection using serum and cerebrospinal fluid samples collected from patients with central nervous system (CNS) disease in the summers of 2009, 2010 and 2011. Although all samples were negative for WNV by polymerase chain reaction, quantitative evaluation of standardised antibody assays with purified flavivirus antigens (including Usutu virus, which cross-reacts with WNV even in neutralisation assays) provided serological evidence for three autochthonous WNV infections in Austria: two in 2009 and one in 2010. Our data highlight the importance of raising awareness of WNV infections in Austria and neighbouring countries and suggest including testing for this infection in routine diagnostic practice of CNS diseases.

Introduction

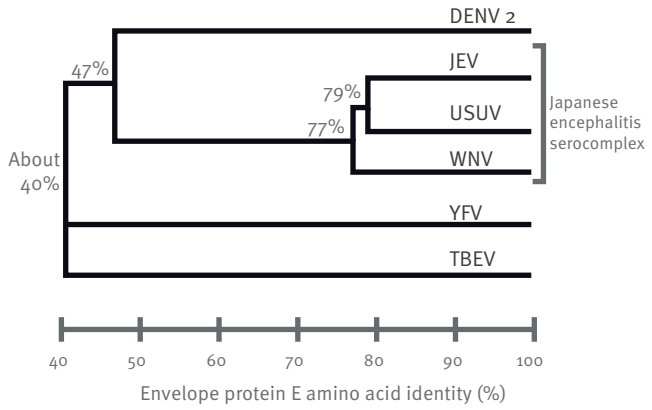
West Nile virus (WNV) is a mosquito-borne member of the genus *Flavivirus* (Family *Flaviviridae*) [1], which has a wide geographical distribution and is found in regions of Africa, Asia, Australia, Europe and the Americas [2]. Although most human infections are subclinical or result in mild febrile disease, about 1 in 150 infected individuals develop severe neurological symptoms with a potentially lethal outcome [3]. There is evidence for an increase and spread of WNV infections in Europe, especially in south-eastern countries [4,5]. This was most noticeable in the Central Macedonian Region of Greece in 2010, where human WNV infections (n=262) resulted in 197 neurological cases and 35 deaths [6]. This was the third-largest human WNV outbreak in the World Health Organization (WHO) European region, after those in Romania in 1996 (Bucharest region; 393 cases and 17 deaths) and Russia in 1999 (Volgograd region; 826 cases and 40 deaths) [2]. In addition to such locally restricted epidemics, intensified surveillance programmes have documented the presence of the virus in animals and substantial numbers of human infections have been documented since the beginning of the 2013 transmission season in Greece (n=86), Hungary (n=29), Italy (n=66), Romania (n=22), Croatia (n=16), Serbia (n=260) and Russia (n=177) as well as sporadic human infections in Bosnia and Herzegovina (n=3), former Yugoslav Republic of Macedonia (n=1) and Montenegro (n=2), as of 10 October 2013 [5]. Both genetic lineages of WNV (I and II) have been associated with human disease and new introductions (most likely through migratory birds), as well as overwintering of the virus in mosquitoes, have been observed [4].

Similar to the situation with WNV, there is increasing documentation of human and animal Usutu virus (USUV) infections in Europe since the first reports of its introduction in Austria in birds in 2001 [7], with virus isolation and/or serologically confirmed infections in animals or humans in Hungary, Italy, Spain, Switzerland and Germany [8]. Although human USUV infections are believed to take a mild or asymptomatic course, impairment of neurological function has also been described in two persons with underlying diseases [8].

Since WNV viraemia is short-lived, the amount of virus in serum and cerebrospinal fluid (CSF) samples has frequently dropped below levels detectable by polymerase chain reaction (PCR) when central nervous system (CNS) symptoms start and patients are hospitalised [9]. Therefore laboratory diagnosis usually has to rely

Figure 1

Dendrogram by percentage amino acid identity of the envelope protein E from various flaviviruses



DENV 2: dengue virus type 2 (strain 16681; GenBank accession number NC_001474)

JEV: Japanese encephalitis virus (strain Nakayama; GenBank accession number U44966)

USUV: Usutu virus (strain SAAR; GenBank accession number AY453412)

WNV: West Nile virus (strain NY99; GenBank accession number DQ211652)

YFV: yellow fever virus (strain 17D; GenBank accession number X03700)

TBEV: tick-borne encephalitis virus (strain Neudoerfl; GenBank accession number U27495)

on the detection of specific antibodies [10]. However, the results of these assays may be biased by cross-reactive antibodies induced by other autochthonous or imported flavivirus infections and/or vaccinations. These include tick-borne encephalitis virus (TBEV), which is endemic in large parts of Europe, with high vaccination rates in certain countries (85% in Austria [11]), dengue viruses (DENVs), yellow fever virus (YFV) (travel vaccination), Japanese encephalitis virus (JEV) (travel vaccination) and USUV. The problem of cross-reactivity is most pronounced with viruses belonging to the same flavivirus serocomplex. WNV – like USUV – is a member of the Japanese encephalitis (JE) serocompex [1,12]: these viruses have approximately 80% identical amino acids in the envelope protein E (Figure 1), the major target of neutralising antibodies. With such closely related viruses, cross-reactivities are not only observed in enzyme immunoassays but also in virus neutralisation assays [12] that are proposed for discriminating between different flavivirus infections [10]. Viruses of different serocomplexes (e.g. TBEV, DENV, YFV) have only about 40% identical amino acids in E (Figure 1). Cross-neutralisation is usually not observed in these cases [12], but cross-reactivity is still detectable in enzyme-linked immunoassay (ELISA) and haemagglutination inhibition assays [13].

Austria is adjacent to countries that have reported human cases of WNV infection (Italy and Balkan countries in the south as well as Hungary in the east [4,5]) and the virus was isolated in the eastern part of Austria from birds of prey in 2008 and 2009 [14] and overwintering mosquitoes (2008–09; Norbert Nowotny, personal communication, September 2012). Human cases, however, have not been documented in Austria to date. We therefore aimed to conduct a retrospective search for human cases of WNV infection by analysing serum and CSF samples from patients who had been sent to our department in the summer of 2009, 2010 and 2011 with suspected TBE or other CNS infections, but whose samples did not yield laboratory evidence of a recent viral infection. For this purpose, we designed quantitative serological methods that – even in the presence of cross-reactive antibodies – allowed us to discriminate between infections with closely related flaviviruses of the JE serocomplex.

Methods

Human sera

We analysed patients' serum and CSF samples that had been submitted to the Department of Virology, Medical University of Vienna during 2009 to 2011 for virological laboratory diagnosis of CNS infections (most of them being suspected cases of TBE). We used the following criteria for the selection of patient samples: (i) time window of August and September for sample collection date, because most of the WNV infections in other European countries have been reported in these months [15]; (ii) age more than 70 years, because elderly people are more likely to develop CNS symptoms than younger people [15]; and (iii) availability of serum and, if possible CSF, taken upon hospitalisation.

The controls used in the quantitative IgM and IgG ELISAs were sera from WNV-, USUV- and JEV-infected people obtained from the following: (i) an external assay quality control study performed by the European Network for Diagnostics of Imported Viral Diseases, ENIVD (WNV, USUV); (ii) a commercially available JE Detect IgM Capture ELISA from InBios, Seattle, WA, United States; and (iii) TBE cases confirmed at the Department.

All samples were tested with the approval of the local ethics committee.

West Nile virus IgM screening

For the screening of WNV-specific IgM antibodies in patient sera, the commercial WN Detect IgM Capture ELISA (InBios; Seattle, WA, United States) was used.

Table

Patient information of cases of West Nile virus infection, Austria, 2009–10 (n=3)

Patient information	Case 1 (2009)	Case 2 (2010)	Case 3 (2009)
Age group in years at symptom onset	80–85	75–80	10–15
Place of residence	South of Vienna	Vienna	Vienna
Stay outside place of residence during incubation period	No	No	No
Clinical picture	Fever (38.5 °C); encephalitis	Fever (39.5 °C); meningitis	Fever (39.4 °C); exanthema; no neurological symptoms
TBE vaccination	No record	Irregular vaccination schedule Last vaccination >10 years before hospitalisation	Regular vaccination schedule Last vaccination about 3 years before hosptitalisation
Samples[a]	Day 0: serum and CSF Day 879: serum	Day 0: serum and CSF	Day 0: serum Day 83: serum Day 111: serum

TBE: tick-borne encephalitis.

[a] Day 0: sample obtained upon hospitalisation of the patient.

Quantitative IgM and IgG determination for West Nile virus and closely related flaviviruses

IgM and IgG antibody titres were determined by ELISA using purified formalin-inactivated preparations [16,17] of WNV (strain NY99; GenBank accession number DQ211652), USUV (strain SAAR; GenBank accession number AY453412), JEV (strain Beijing; GenBank accession number L48961) and TBEV (strain Neudoerfl; GenBank accession number U27495) as antigens (25 ng/well) directly coated to the solid phase of non-treated microtitre plates (Nunc, Thermo Fisher Scientific, Waltham, MA, United States). After blocking with phosphate-buffered saline containing 2% lamb serum and 2% Tween 20 for 30 minutes at 37 °C, 10-fold serial dilutions of sera (starting dilution 1:100) in blocking buffer were added and incubated for 1 hour at 37 °C. In the case of the IgM ELISA, sera were pre-incubated with rheumatoid-factor-IgG-absorbent (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany). Biotin-labelled goat anti-human IgM or IgG (Pierce Protein Biology Products, Thermo Fisher Scientific, Waltham, MA, United States) together with Streptavidin–Peroxidase (Sigma-Aldrich Inc., St. Louis, MO, United States) was used for detection.

Titration curves were established using the absorbance values at 490 nm and fitted by a four-parameter logistic regression (GraphPad Prism 5.0). Titres were then determined by calculating the intersection of the fitted curve with the cut-off, which was defined as the threefold of the mean absorbance value obtained with eight flavivirus antibody-negative sera.

Determing IgG avidity

As an additional marker for recent infection, we determined the relative avidities of WNV-specific IgG antibodies. For this purpose, we used the same IgG ELISA as described above, except for a wash step after serum incubation in which either 6M urea or phosphate-buffered saline pH 7.4 was added for 5 minutes at room temperature [17,18]. The dilution curves were fitted using a four-parameter logistic regression (GraphPad Prism 5.0) and the titres were determined at an absorbance value of 0.5 (490 nm). The avidity of each serum was calculated with the following formula: Avidity (%) = (titre with urea/titre without urea) x 100.

Neutralisation test

Serial twofold dilutions (starting dilution 1:10) of heat-inactivated sera (duplicates) were mixed with an equal volume of virus dilution (containing 20–40 $TCID_{50}$ (50% tissue culture infective dose) of WNV strain NY99) and incubated for one hour at 37 °C. Vero cells were added and incubation was continued for four to six days. Presence of virus in the supernatant was assessed by the occurrence of cytopathic effects. Neutralisation test (NT) titres ≥20 were considered positive.

Detection of West Nile virus RNA

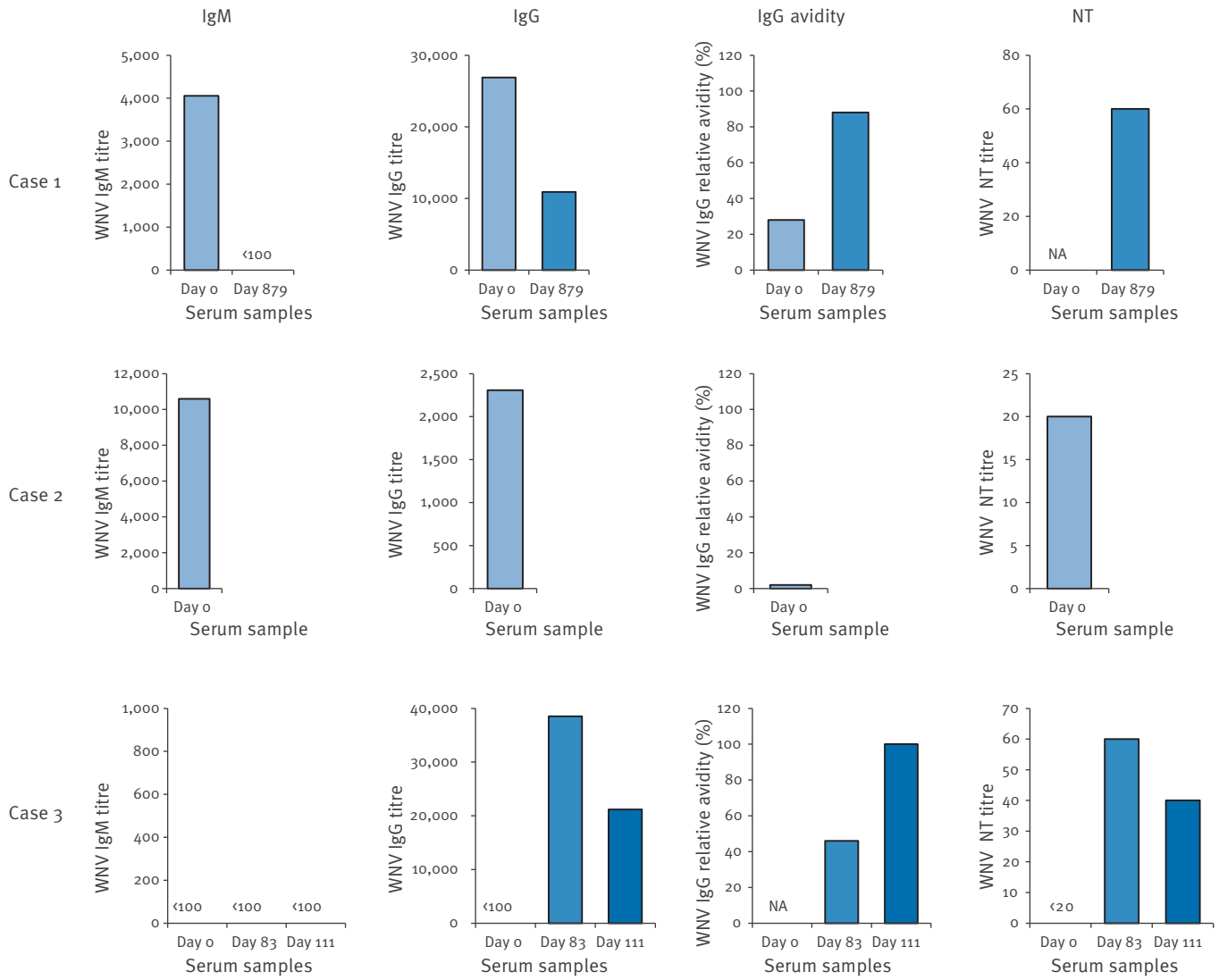
WNV RNA was extracted from 200 µl serum or CSF and eluted in 50 µl using the automated NucliSENS easyMAG extractor (bioMérieux, Marcy l'Etoile, France). A real-time TaqMan PCR for the detection of WN virus lineages 1 and 2 with primers and probe located within the conserved WN virus 3'-noncoding region was used, as described elsewhere [19]. The PCR was controlled using sera from an external proficiency panel as standards [20].

Results

Samples of 110 patients with neurological symptoms and unknown aetiology were selected for this

Figure 2

West Nile virus serology of sera obtained upon hospitalisation (day 0) and follow-up samples from human cases of West Nile virus infection, Austria, 2009–10 (n=3)



NA: not analysed; NT: neutralisation test; WNV: West Nile virus.
‹100 (IgM, IgG): no antibodies detectable at starting serum dilution of 1:100.
‹20 (NT): no antibodies detectable at starting serum dilution of 1:20.

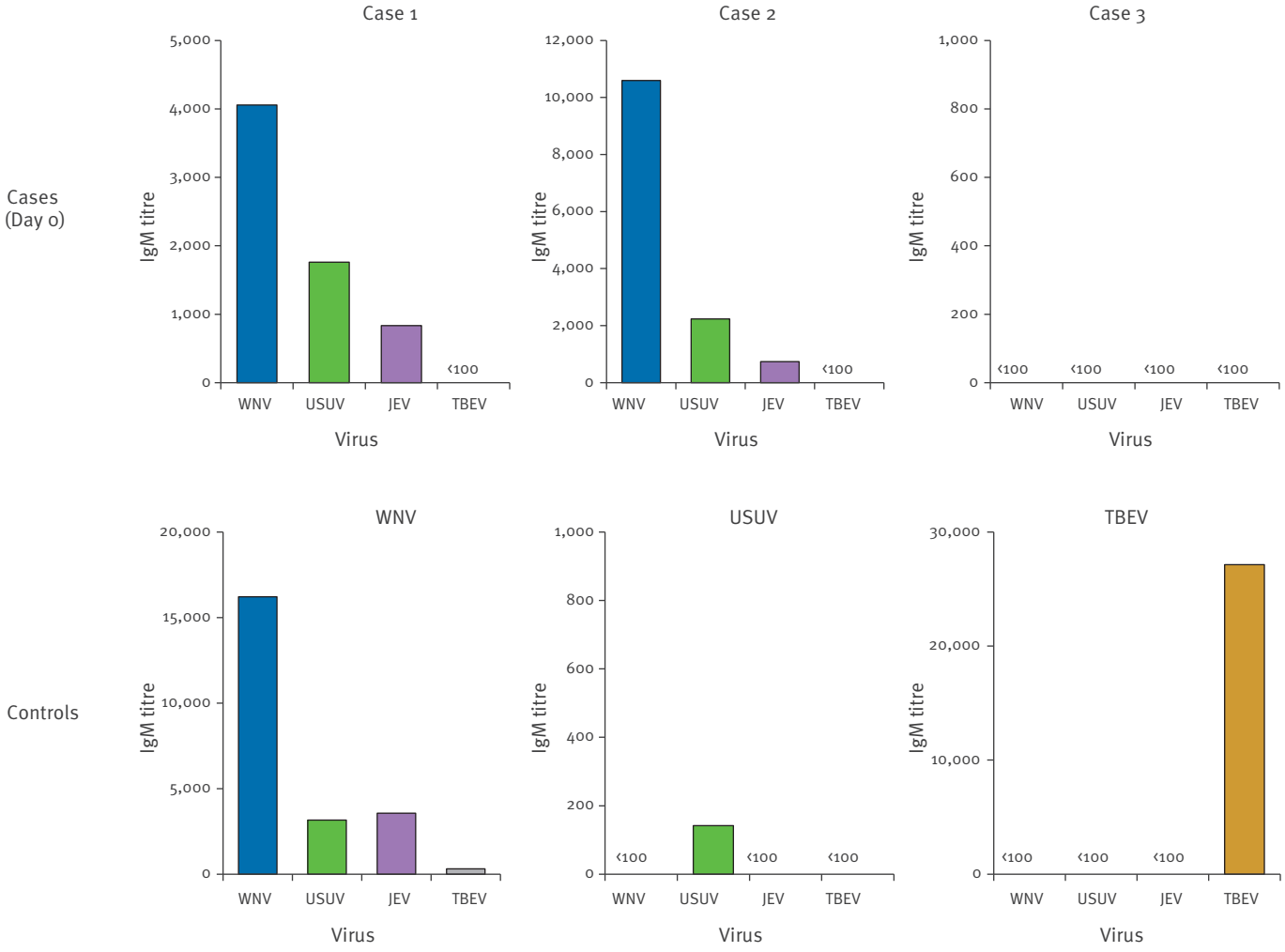
retrospective analysis of human WNV infections in Austria. Single serum samples from each patient were tested using WNV IgM ELISA as a first qualitative screen. Of these, two were IgM-positive, suggesting possible recent WNV infections. For 82 of the 110 patients, there was sufficient serum available for PCR testing (and for 35 of the 82, CSF was also available): all 85 sera and 35 CSF samples were PCR-negative. This included serum and CSF of the two WNV IgM-positive patients (Cases 1 and 2).

We also tested an additional sample (Case 3), even though it did not meet our selection criteria due to the young age of the patient. During initial tests in 2009, this sample (from an adolescent hospitalised with febrile infection) had shown titre rises in haemagglutination-inhibition assays with various flavivirus antigens, including WNV; however, the result was not verified at that time, because both PCR and WNV IgM ELISA were negative.

Information on the clinical picture, follow-up samples and other relevant available data of these three patients are summarised in the Table.

Figure 3

Quantitative flavivirus IgM ELISA with sera obtained upon hospitalisation (day 0) of human cases of West Nile virus infection, Austria, 2009–10 (n=3) and control sera[a]



ELISA: enzyme-linked immunosorbent assay; JEV: Japanese encephalitis virus; TBEV: tick-borne encephalitis virus; USUV: Usutu virus; WNV: West Nile virus.

‹100: no antibodies detectable at starting serum dilution of 1:100.

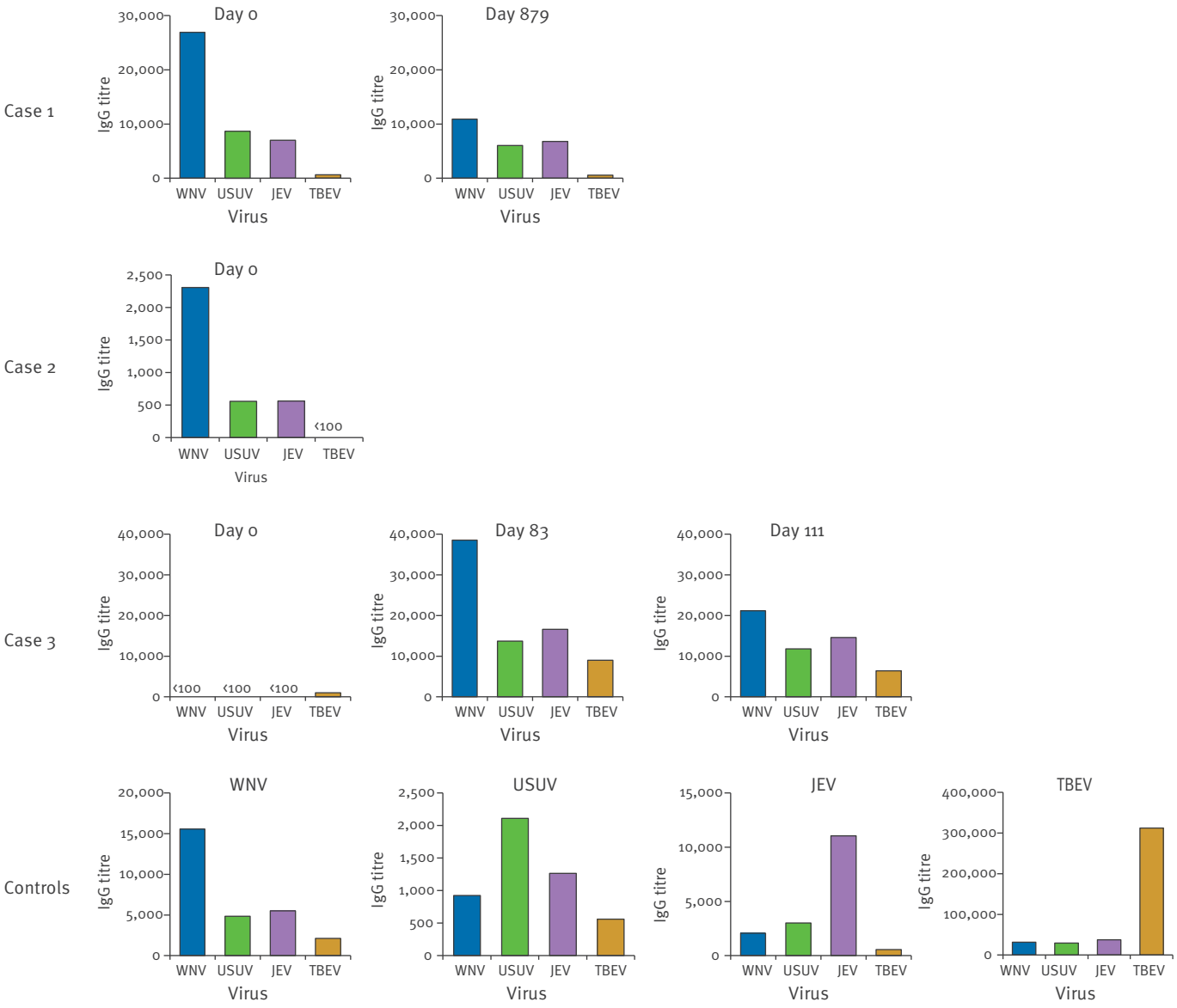
[a] Sera from WNV-, USUV- and TBEV-infected people.

To confirm these three putative WNV infections, we conducted further WNV-specific analyses of initial and follow-up samples, including quantitative IgM and IgG assays, IgG avidity assays and virus NTs (Figure 2). Case 1 had not only IgM on hospitalisation, but also high levels of IgG antibodies, which had decreased in a follow-up sample taken about 2.5 years later. The initial IgG avidity was low but was much higher in the second sample, which was also positive in the WNV NT. For Case 2, only the initial sample was available, containing both IgM as well as low-avidity IgG antibodies and displaying WNV neutralising activity. Both of these cases thus met the laboratory criteria of the European Union (EU) definition for West Nile fever [21]. Case 3 was IgM-negative both in the initial sample as well as in follow-up samples taken 83 and 111 days later. In contrast, IgG antibodies (not detectable in the first sample) were found in the two follow-up samples. The titres of these antibodies decreased from day 83 to day 111, whereas their avidity increased in this period, and both samples were NT-positive. Taken together these data are consistent with a recent infection caused by WNV or a closely related virus in all three cases.

To rule out possible misinterpretations due to cross-reactivity, we performed ELISAs with WNV, USUV, JEV and TBEV under standardised conditions that allowed quantitative comparisons of antibody reactivities. The data obtained with serum samples of the three cases and control sera are summarised in Figure 3 (IgM of initial sera) and Figure 4 (IgG of initial and follow-up samples). Except for Case 3 (with no detectable IgM

Figure 4

Quantitative flavivirus IgG ELISA with sera obtained upon hospitalisation (day 0) and follow-up samples of human cases of West Nile virus infection, Austria, 2009–10 (n=3) and control sera[a]



ELISA: enzyme-linked immunosorbent assay; JEV: Japanese encephalitis virus; TBEV: tick-borne encephalitis virus; USUV: Usutu virus; WNV: West Nile virus.

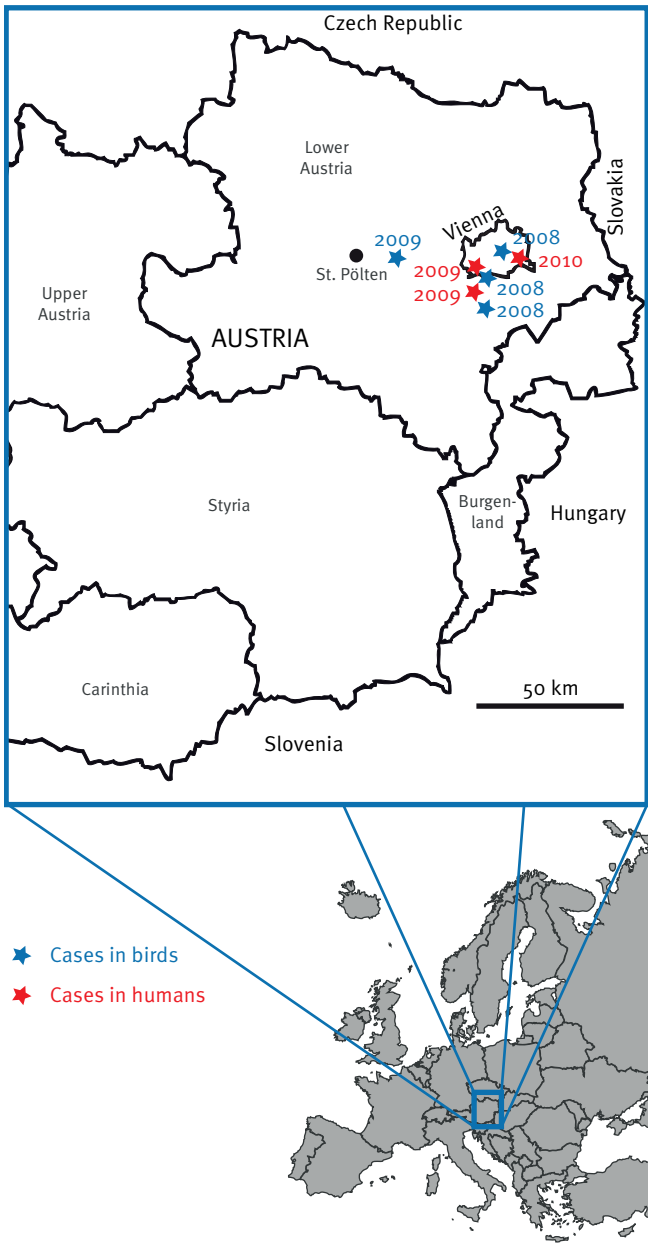
<100: No antibodies detectable at starting serum dilution of 1:100.

[a] Sera from WNV-, USUV- and TBEV-infected people.

antibodies), the cross-reactive IgM patterns of Cases 1 and 2 were similar to that of the WNV control serum and different from those of the USUV and TBEV control sera, i.e. they displayed much higher titres against WNV than against USUV and TBEV. TBEV-cross-reactive antibodies were not detectable in these samples and the TBEV IgM-positive serum did not cross-react in the WNV assay (Figure 3). Similar quantitative differences were also observed in the IgG assays (Figure 4), which in all three cases – like the WNV control serum – always showed the highest reactivity with WNV and thus differed substantially from the patterns obtained with the USUV, JEV and TBEV control sera. It is important to note that the specificity of the IgG ELISA results diminished over time (Cases 1 and 3; Figure 4) and was most prominent in early serum samples. Taken together, these analyses allowed the identification of recent WNV infections in all three cases. This is especially noteworthy with respect to Case 3: the patient's initial sample had neither WNV IgM nor IgG antibodies but only TBEV IgG antibodies due to vaccination. Since none of the patients had a travel history within

Figure 5

Place of residence of human cases and location of birds with West Nile virus infection, Austria, 2009–10 (n=3)



The place of residence of the three patients are indicated in red and the geographical locations of virus isolation from birds with lethal West Nile virus infections in blue [14], together with the year of infection.

the WNV incubation period (Table), these cases were apparently caused by autochthonous WNV infections in Vienna or its surroundings. The place of residence of the three cases matches the locations of previously reported WNV isolations from birds [14] (Figure 5) and/ or mosquitoes (Norbert Nowotny, personal communication, September 2012).

Discussion and conclusions

Our study was prompted by increasing evidence for WNV infections in Europe and led to the retrospective identification of three human cases of WNV infection in Austria that were probably autochthonous. The putative sites of infection were in Vienna and its surroundings and matched the locations of previously reported WNV isolation from birds and/ or mosquitoes. Consistent with the short viraemia in most of the documented human cases [10] and data from other studies [9], we were unable to detect the virus in serum and/ or CSF samples taken at the time of hospitalisation, and therefore we cannot make any statements on the genetic lineage of WNV that caused these infections. Lineage 2 viruses, however, are the more likely candidates, because these were isolated from birds in the same geographical area in 2008 and 2009, whereas lineage 1 viruses have not yet been detected in Austria.

In addition to WNV, the closely related USUV also emerged in central and southern Europe, following its first detection outside Africa in Austria in 2001 [7,8]. This virus cross-reacts with WNV in serological assays, including virus NTs [12]. Since the initial samples of all three patients with suspected WNV infection were PCR-negative, we established serological assays (based on inactivated purified virions) that allowed the differentiation between infections with WNV and USUV as well as other closely related flaviviruses. As expected, these assays displayed strong cross-reactivities between WNV and USUV (and JEV, another member of the same flavivirus serocomplex), not only in IgG but also in IgM assays. Only the comparison of the patterns obtained in quantitative and standardised immunoassays allowed a clear distinction between these infections. Our study thus points to the important fact that the serological differentiation between WNV, USUV and other viruses of the same flavivirus serocomplex requires comparative quantitative in addition to qualitative serological analyses, even when the NT (usually considered the most discriminating assay [22]) is positive.

Of all European countries, Austria has the highest seroprevalence of antibodies to TBEV because about 85% of its population has been vaccinated against it at least once [11]. Since TBEV and WNV infections both display very similar clinical pictures, distinguishing between WNV and TBEV antibodies will be frequently required in routine diagnostic practice. The present and previous studies of our group [23] as well as others [24], however, indicate that the extent of cross-reactivity between the two viruses – especially in IgM assays – is so low that distinguishing between recent infections is usually unproblematic.

Somewhat unexpectedly, one of our three cases (Case 3) had no measurable WNV-specific IgM antibodies, neither in the sample taken at symptom onset nor 83 and 111 days later, and would therefore not meet the EU case definition for West Nile fever [21]. All other parameters, however, were consistent with a recent

WNV infection, including IgG seroconversion, increase of IgG avidity, WNV-specific NT and the reactivity patterns with USUV, JEV and TBEV. One possible interpretation would be that the first serum sample was taken before the onset of the antibody response and that IgM antibodies produced later on had already declined to non-detectable levels after 83 days. Consistent with the vaccination history, this patient had TBEV-reactive IgG antibodies at the time of symptom onset, which increased in the course of the WNV infection, presumably due to the boosting and/or new induction of cross-reactive antibodies (Figure 4). To what extent the immunity against TBEV might have contributed to a low and/or delayed WNV-specific IgM response remains unresolved and a matter of speculation.

Considering that our search criteria were restricted to people over 70 years of age (about 13% of the total population in Austria) [25] and that only less than 1% of infections (1/150) progress to severe CNS symptoms [10,22], it is justified to assume that the real number of infections in the east of Austria was at least in the range of several hundred in the study years. Our data are consistent with recent findings of increasing WNV antibody titres in human plasma collected in Austria, Germany and the Czech Republic from 2006 to 2010 [26] and support activities to increase vigilance for this infection as a potential public health problem in central Europe [27]. In this context, it is also important to strengthen the awareness of clinicians for WNV infections, especially during the summer months, and to include tests for WNV into routine diagnostic practice of CNS diseases.

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Conflict of interest

None declared.

Authors' contributions

KS, SWA and FXH conceived and designed the experiments. KS and SWA performed the experiments. KS, FXH and SWA analysed the data. FXH, KS and SWA wrote the paper.

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Letter to the editor: Seafarers: a new risk group for meticillin-resistant *Staphylococcus aureus* (MRSA)

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To the editor: We applaud the successful implementation of meticillin-resistant *Staphylococcus aureus* (MRSA) screening programme on the German side of the Dutch–German border region (EUREGIO) [1]. The described strategy is based on the risk-based admission screening approach [1].

From our experience with 'search and destroy' (S and D) in the Netherlands, we learned that it is essential to evaluate and timely enlarge risk categories when epidemiology changes to prevent MRSA of unknown origin (MUO), i.e. not fitting any defined risk factors [2]. Herewith, we report our recent finding of a previously unrecognised risk group for MRSA: seafarers.

In 2010, we noticed that clinical cultures with unexpected MRSA in the Harbour Hospital (Port of Rotterdam, the Netherlands) were mainly from seafarers. Although the Harbour Hospital is especially equipped for seafarers, this patient population only accounts for 1.2% of all admissions [3]. Since seafarers are not considered an MRSA risk group, all seafarer patients were screened (nose and throat) at the Emergency Department of the Harbour Hospital in a six month prospective surveillance (2011). Perineum and wounds were screened additionally, if active infection or skin lesions were present. Detection of MRSA was performed as previously described [4], and *spa* typing was done at the reference laboratory of the National Institute for Public Health and the Environment (RIVM) [5]. Furthermore, since the hospital is visited by a large number of seafarers, a case–control study was designed to identify risk factors for MRSA carriage among seafarers, in order to identify specific risk factors within this putative new risk group.

Cases were defined as seafarers with a positive MRSA culture of any site, whereas controls had a negative MRSA culture. Data on demographics, medical history, laboratory and naval parameters were collected retrospectively. Data were then analysed by univariate (chi-squared, Fisher's exact test) and multivariate analysis (logistic regression model).

In the study period 124 seafarers (men, 22–51 years of age) were included. Four seafarer patients had an unknown MRSA status and were excluded. MRSA prevalence among seafarers was 5.8% (95% confidence interval (CI): 4.6–7.1) and the incidence rate was 24.8/1,000 seafarer population. Seven MRSA-positive seafarers were identified as cases, leaving 113 MRSA-negative seafarers as controls. Of seven MRSA carriers, four had wounds, of which three were cultured MRSA positive as well. Furthermore, of seven MRSA strains, five had similar *spa* repeat successions: t019 (twice), t122, t975 and t4557. The remaining two were t4845 and t9231. Nationality was only known for 32 seafarers.

Table

Risk factors for meticillin-resistant *Staphylococcus aureus* (MRSA) in seafarers, Rotterdam, the Netherlands

Risk factor	MRSA positive (N=7) n(%)	MRSA negative (N=113) n(%)	P-value	Odds ratio (95% CI)
Male sex	7 (100)	112 (99)	–	–
Specialist involved with patient				
Internal medicine	2 (29)	21 (19)	0.61	–
Surgery	5 (71)	59 (52)	0.45	–
Physical examination				
Presence of wounds or abscesses	4 (57)	5 (4)	<0.01	40.8 (5.9–278.3)
Pus detected	2 (28)	2 (2)	0.01	26.7 (2.9–241.3)

CI: confidence interval; OR: odds ratio.

Presence of wounds or abscesses was the only risk factor (p<0.01) in univariate analysis and in the multiple regression model with an OR of 40.8 (95%CI: 5.9–278.3). The multiple regression model was based on forward selection with 'presence of wounds or abscesses', 'detection of pus' and 'C-reactive protein (CRP)' (area under the receiver operator characteristic criterion (AUC): 0.75, R2max: 0.4603).

Twenty-five of them were of Asian origin, with 18 seafarers from the Philippines. Of MRSA carriers, nationality was known in two cases: the Philippines and India.

Severe missing data for many proposed variables forced us to exclude many variables, as registration of seafarers was basic due to swift departure and communication difficulties. The remaining risk factors are listed in the Table. Presence of wounds or abscesses was the only significant risk factor ($p<0.01$) in both univariate analysis and multivariate regression analysis (odds ratio (OR): 40.8 (95% CI: 5.9–278.3)). The multivariate regression model was based on forward selection with 'presence of wounds or abscesses', 'detection of pus' and 'C-reactive protein (CRP)' (area under the receiver operator characteristic criterion (AUC): 0.75, R2max: 0.4603). The positive predictive value for finding an MRSA carrier when a seafarer had a wound was 44% whereas the negative predictive value (MRSA carrier when a seafarer has no wound) was 2%. The MRSA carriage did not influence the duration of hospitalisation of the seafarers ($p=0.36$).

In our limited sample of 124 seafarers, the 5.8% MRSA prevalence detected is 52-times higher than the normal prevalence in the Netherlands (0.11% at hospital admission) [4], and the presence of wounds or abscesses gave a 40-times higher risk of being MRSA positive. Some bias might play a role due to our screening procedure. However, this choice was made due to communication difficulties and cultural differences.

Given the high prevalence rate of carriage among seafarers, we recommend that all seafarers should be screened for MRSA in the Netherlands, regardless of wounds or underlying disease, and to apply pre-emptive isolation while awaiting test results, and should be taken into consideration as risk group by other European nations. Further studies are necessary to understand the impact of global transmission of MRSA clones by seafarers.

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Conflict of interest

None declared.

Authors' contributions

Jeroen Peper and Eline Storm collected data. Wouter Lekkerkerk analyzed data and wrote the manuscript. Juliëtte Severin supervised the prospective study and reviewed the manuscript. Perry van Genderen, Margreet Vos supervised overall and reviewed the manuscript.

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Molecular epidemiology of meticillin-resistant *Staphylococcus aureus* (MRSA): think regionally but use globally uniform typing languages

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To the editor: We appreciate the report by Lekkerkerk et al. 'Seafarers: a new risk group for meticillin-resistant *Staphylococcus aureus* (MRSA)' on MRSA of unknown origin, detected in seafarers, which indeed seem to play an important role in explaining the diversity of MRSA *spa* types in an country with low MRSA prevalence as the Netherlands [1].

The report makes clear that the population being at risk can vary from region to region.

As the report describes, it is essential that aside from the commonly known risk factors (e.g. acute care hospital stay in endemic countries), the MRSA-risk population needs to be evaluated on a regional scale and screening protocols need to be adapted according to the result of these evaluations on a regular basis. The goal is to find as many carriers as possible by screening as few patients as necessary. National screening protocols should encourage regional healthcare clusters [2] to assess whether their own risk population is congruent to the one established for the national level.

While seafarers seem to be a major population at risk in harbour cities, farmers are a far more relevant risk group for the Dutch–German border region as demonstrated by the fact that 17% to 29% of all MRSA patients detected at hospital admission on the German side of the border in the period from 2006 to 2012 carried livestock-associated (LA) MRSA *spa* types (predominantly *spa* CC011, MLST CC398) [3]. In comparison, from 2010 to 2011 only 5.4% of MRSA patients included in a nationwide German study could be attributed to LA-MRSA (predominantly *spa* CC011, MLST CC398) [4]. The particular increasing admission prevalence of LA-MRSA in the Dutch–German border region can be explained by the intensive pig farming in this area. Livestock density is known to be associated with occurrence of LA-MRSA in humans [5]. Nearly all studies use common molecular typing methods to distinguish livestock-related isolates from other MRSA. As epidemiology of MRSA seems different from region to region and is changing rapidly, the understanding of the fluctuating epidemiology would not be possible without proper typing.

A retrospective case–control study in the German part of the Dutch–German EUREGIO showed that patients carrying LA-MRSA are different from patients carrying classical hospital-acquired (HA) MRSA with respect to underlying disease, invasive treatment and length of stay in intensive care unit [6]. The key findings of the study were only achievable by molecular *spa* typing data allowing retrospective stratification of patients into carriers of HA-MRSA versus LA-MRSA. Furthermore, a significant proportion (>30%) of patients colonised with LA-MRSA at admission were not associated with contact to farm animals indicating other ways of transmission.

While dissemination of LA-MRSA is discussed here, the report on seafarers makes also clear that without proper molecular typing methods using a globally understandable nomenclature, the unusual MRSA *spa* types found in seafarers would not have been identified. It also shows that publications on microorganisms that are important to clinical microbiology, infection prevention and public health (as e.g. MRSA and carbapenemase-producing *Enterobacteriaceae*) benefit from common typing languages based on publicly available reference databases that ensure excellent quality and a unique nomenclature. This is of particular interest in a time when modern technology such as whole genome-based typing is being implemented for clinical microbiology, infection prevention and public health.

Such common typing languages allow the performance of studies that permit to adapt infection control

measures to the regional need, but from which the results can be also shared and understood worldwide. Moreover, these typing languages own the capacity to become professional tools for daily use in infectious diseases prevention and management.

Conflict of interest

None declared.

Authors' contributions

Conceived and writing the paper: A Jurke, AW Friedrich; Contributed to writing and discussion: R Köck, K Becker; Contributed to discussion: S Thole, R Hendrix, J Rossen, I Daniels-Haardt.

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WHO publishes Global tuberculosis report 2013

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The World Health Organization (WHO) published yesterday the 18th global report on tuberculosis (TB) [1] in a series that started in 1997. It provides a comprehensive and up-to-date assessment of the TB epidemic and of the progress made in TB prevention, care and control at global, regional and country level, using data reported by 197 countries and territories that account collectively for over 99% of the TB cases in the world.

TB remains a major global health problem although TB treatment has saved the lives of more than 22 million people, according to the report, which also reveals that the number of people who fell ill with TB in 2012 was 8.6 million, with 1.3 million TB deaths globally (including 320,000 deaths among HIV-positive people).

Nearly 20 years after the WHO declaration of TB as a global public health emergency, major progress has been made towards 2015 global targets set within the context of the Millennium Development Goals (MDGs). Two years ahead of the deadline, a special 'Countdown to 2015' supplement to this year's report provides full information on the progress to the international TB targets. It assesses the progress towards the 2015 targets and the top priority actions needed to achieve and/or move beyond them.

The report underlines two major challenges: the drug-resistant TB crisis and the fact that around 3 million people (one in three people falling ill with TB) are currently being 'missed' by health systems. A further challenge identified relates to the TB and HIV co-epidemic: while there has been significant progress in the last decade in scaling-up antiretroviral treatment for TB patients living with HIV, less than 60% of the patients were receiving antiretroviral drugs in 2012.

References

1. World Health Organization (WHO). Global tuberculosis report 2013. Geneva: WHO; 23 Oct 2013. Available from: http://apps.who.int/iris/bitstream/10665/91355/1/9789241564656_eng.pdf