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Outbreak of Shiga toxin-producing *E. coli* O157 associated with consumption of watercress, United Kingdom, August to September 2013

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An increase in the number of cases of Shiga toxin-producing *Escherichia coli* O157 PT 2 *stx2* infection was reported in the United Kingdom on 9 September 2013. Of the 19 cases, 13 were interviewed, of which 10 reported consuming watercress purchased from one retailer. The retailer recalled pre-packed bagged salads containing watercress on 12 September. The descriptive epidemiology was supported by a case–case study performed after control measures were implemented.

On 9 September 2013, the Public Health England (PHE) automated outbreak detection system [1] highlighted an increase in the number of cases of Shiga toxin-producing *Escherichia coli* (STEC) serotype O157, phage type (PT) 2, Shiga toxin type 2 (*stx2*), which had been reported through the PHE Gastrointestinal Bacteria Reference Unit, London. During the week commencing 2 September, 12 cases were reported, compared with around one to two cases per week in the preceding months. Routine analyses of multiple-locus variable-number of tandem repeats analysis (MLVA) profiles identified that the STEC isolates in 10 cases shared an identical or single-locus variant (SLV) MLVA profile (the outbreak profile), all reported in England since 30 August. The outbreak strain was intimin (*eae*) positive and haemolysin (*hylA*) positive.

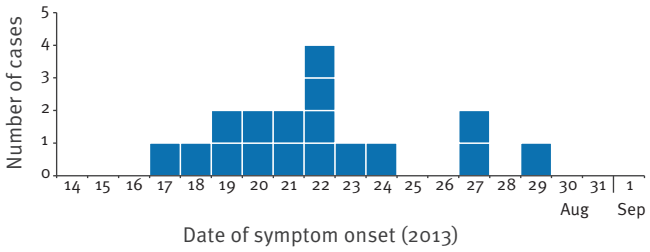
Background and descriptive epidemiology

Routine enhanced surveillance of STEC has been in place in England since 1 January 2009. The STEC Enhanced Surveillance system (SESSy) combines detailed clinical and epidemiological data collected on enhanced surveillance questionnaires with microbiological characterisation of strains. Faecal samples from patients suspected to have STEC or haemolytic uraemic syndrome (HUS) are sent to local hospital laboratories where they are cultured for the presence of *E. coli* O157, then sent to the Gastrointestinal Bacteria Reference Unit for further characterisation. The local laboratories report presumptive isolates of STEC directly to PHE centres, who then arrange for the STEC Enhanced Surveillance Questionnaire (ESQ) to be administered. Contacts of cases that are deemed to pose a risk of onward transmission are screened, as are symptomatic contacts. The PHE Gastrointestinal Bacteria Reference Unit undertakes routine characterisation of isolates in England and Wales, while the Scottish *E. coli* O157/VTEC Reference Laboratory, Glasgow, does so for cases in Scotland. Characterisation includes serogroup, phage and *stx* typing and MLVA for all STEC O157 isolates.

STEC O157 PT 2 is the fourth most common STEC phage type reported in England, with an average of 44 cases reported per year between 2009 and 2012, with a peak of cases in the summer months (unpublished data). Between 30 August and 19 September 2013, 18 cases of STEC O157 PT 2 *stx2* of the outbreak MLVA profile were reported in England (n=14) and Wales (n=4). Health Protection Scotland and the Scottish *E. coli* O157/VTEC Reference Laboratory, Glasgow, were notified of the increase seen in England and Wales and identified one case in Scotland with the outbreak profile. Of the 19 reported cases, 17 were symptomatic primary cases; one was a symptomatic secondary case in the same household as a primary case, and one was an asymptomatic household contact of a primary case identified through contact screening. Symptom onset dates of the primary cases ranged from 17 to 29 August (Figure 1). Primary cases had an unusual demography for cases of STEC infection: they were predominantly female (11/17) (Figure 2), with a median age of 65 years (range: 4–87), whereas the highest incidence of cases in the UK are in children under the age of four [2,3] Cases were geographically dispersed across the United Kingdom (UK). One case reported foreign travel in the

Figure 1

Confirmed primary cases infected with STEC O157 PT 2 *stx*2 of the outbreak MLVA profile, by date of symptom onset, United Kingdom, 17–29 August 2013 (n=17)



MLVA: multiple-locus variable-number of tandem repeats analysis; PT: phage type; STEC: Shiga toxin-producing *Escherichia coli*; STX: Shiga toxin.

seven days prior to symptom onset but UK acquisition could not be excluded. Seven cases were hospitalised, and 14 of the 18 symptomatic cases reported bloody diarrhoea, although no deaths or cases of HUS were reported.

Hypothesis generation

On 9 September, data collected through SESSy for England were reviewed for the 10 outbreak cases. The cases did not report any common travel destinations, or animal or environmental exposures. Scrutiny of the cases' food consumption histories revealed no plausible commonalities with the exception of pre-packed salad, which was reported by 9 of the 10 cases. Three cases specified watercress consumption.

On 10 September, a trawling questionnaire focusing on salad consumption was designed and four cases interviewed. Three of these cases reported consuming watercress bought from a major British retailer, Retailer A. This lead to the null hypothesis that infection with the outbreak strain was not associated with the consumption of watercress.

Case–case study

On 11 September, a case–case study was designed, comparing outbreak cases with cases of other enteric disease, to test the null hypothesis. Outbreak cases for the study were primary symptomatic cases infected with the outbreak strain confirmed by the Gastrointestinal Bacteria Reference Unit, over the age of one year and resident in the UK, with onset of symptoms on or after 17 August 2013. Reference-cases were primary indigenous symptomatic cases of *Salmonella* infection confirmed by the Gastrointestinal Bacteria Reference Unit, over the age of one year and resident in the UK, with onset of symptoms on or after 17 August 2013. Reference-cases were excluded if they were part of recognised outbreaks. Outbreak cases and reference-cases were matched by age group. One reference-case was allocated per outbreak case. Outbreak cases were contacted prior to contacting reference-cases, to ascertain food history and aid trace-back investigations.

Descriptive evidence

Nine outbreak cases were interviewed by telephone using the case–case study questionnaire on 11 September. In total, 10 of the 13 cases interviewed either through trawling (3/4) or the case–case study (7/9) reported consumption of watercress bought from Retailer A, compared with an estimated background watercress consumption of approximately 4% for adults [4-8]. The seven cases interviewed using the case–case study questionnaire who consumed watercress reported that it was pre-packaged, washed and ready to eat.

Control measures

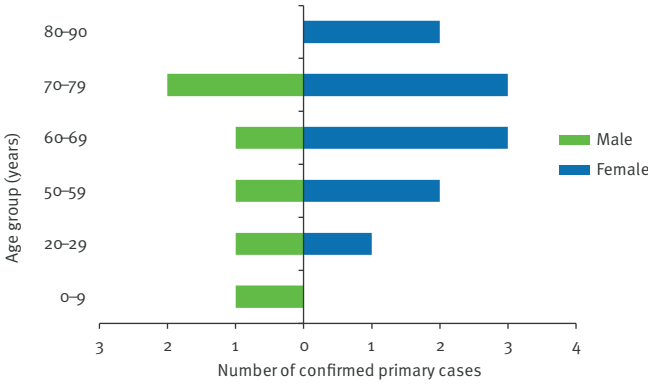
On 12 September, the Outbreak Control Team agreed that the descriptive epidemiological evidence was highly suggestive that watercress from Retailer A was the vehicle of infection and the Foods Standards Agency advised Retailer A to initiate a recall of watercress products. As a precautionary measure, Retailer A recalled six pre-packed bagged salads containing watercress on the afternoon of 12 September.

Food chain and environmental investigation

The Food Standards Agency's food chain investigations identified the supplier and watercress farms that provided all the watercress to Retailer A during August 2013. During that time, Supplier A sourced watercress

Figure 2

Age and sex distribution of confirmed primary cases infected with STEC O157 PT 2 *stx*2 of the outbreak MLVA profile, United Kingdom, August–September 2013 (n=17)



MLVA: multiple-locus variable-number of tandem repeats analysis; PT: phage type; STEC: Shiga toxin-producing *Escherichia coli*; STX: Shiga toxin.

Table

Single variable and multivariable analysis of odds of infection with STEC O157 PT 2 *stx*2 of the outbreak MLVA profile, United Kingdom, September 2013 (n=22)[a]

Variable	Outbreak cases		Reference-cases		Single variable analysis			Multivariable analysis		
	Number exposed	Number unexposed	Number exposed	Number unexposed	OR	95% CI	P value	OR	95% CI	P value
Consumption of:										
watercress	10	1	1	10	100	6.74–∞	0.000	22.7	1.38–1,414.94	0.025
tomatoes	10	1	5	6	12	1.36–∞	0.022	–	–	–
yoghurt	8	3	4	7	4.67	0.81–26.69	0.087	–	–	–
Shopping at:										
Retailer A	9	2	2	9	20.25	2.55–161.09	0.003	4.5	0.06–363.24	0.66

CI: confidence interval; MLVA: multiple-locus variable-number of tandem repeats analysis; OR: odds ratio; PT: phage type; STEC: Shiga toxin-producing *Escherichia coli*; STX: Shiga toxin.

[a] Comprised 11 outbreak cases and 11 reference-cases.

for Retailer A from 10 farms in southern England. The 10 farms and Supplier A have detailed hazard analysis and critical control points (HACCP) plans. While some of the farms have livestock nearby, the watercress is protected from the ingress of livestock and surface water. The water flowing through the watercress beds is deep groundwater of a good microbial quality. Regular microbiological testing (coliform counts) is carried out on the water and pre- and post-packed watercress and all results for 2013 have been satisfactory to date. Samples of watercress from the field and following processing, as well as environmental samples, have been taken by local enforcement authorities at Supplier A's premises to help pinpoint the cause of the contamination. Further investigations into the supply chain of peat and the watercress seeds used by Supplier A are under way.

Further epidemiological investigation

As of 16 September, food histories were obtained for all 17 primary symptomatic cases: from enhanced surveillance questionnaires (n=2), trawling questionnaires (n=4) and case–case study questionnaires (n=11). Of the 17 cases, 15 reported watercress consumption, of whom 13 had purchased the watercress from Retailer A.

Case–case study results

Reference-cases for the case–case study were contacted between 19 and 25 September. Data were imported into STATA for analysis. Variables that had a significant association with infection with the outbreak strain of STEC (odds ratio (OR)›1 and p‹0.1) in single variable analysis were included in multivariable analysis. Unmatched multivariable analysis was performed using a binomial generalised linear model and exact logistic regression.

A total of 11 cases and 11 reference-cases were interviewed by telephone. The mean age of cases was 57 years (standard deviation (SD): 24.08) compared with 55 years (SD: 19.67) in reference-cases. Age and sex had no significant association with being an outbreak case in either single variable or multivariable analysis.

In single variable analysis, outbreak cases were significantly more likely to have consumed watercress, tomatoes and yoghurt, and have shopped at Retailer A than reference cases (Table). In multivariable analysis, consumption of tomatoes was excluded from the final model as it showed a protective effect that was not significant. Yoghurt consumption was excluded as while a significant association was shown, investigations showed multiple types of yoghurt, with no common ingredients, were consumed and it was therefore not a biologically plausible vehicle of infection.

In the final multivariable model, outbreak cases were significantly more likely to have consumed watercress than reference cases (OR: 22.7; 95% confidence interval (CI): 1.38–1,414.94; p=0.025), but there was no significant difference between outbreak cases and reference-cases regarding shopping at retailer A (OR: 4.5; 95% CI: 0.06–363.24; p=0.66) (Table).

Discussion

This outbreak investigation provided strong descriptive epidemiology suggesting the likely vehicle of infection was bagged washed watercress from a specific retailer. Watercress is a well-recognised vehicle of transmission for fascioliasis in many countries [9-13] and a study of microbial contamination of pre-harvest watercress in New Zealand found high levels of *E. coli* and *Campylobacter* in watercress and growing water [14]. A case–control study of over 350 cases of STEC infection reported in England from 1996 to 1997 identified watercress as a risk factor for STEC infection [15]; however, as far as we are aware, this is the first known outbreak of STEC infection associated with watercress.

Control measures were put in place before the case–case study was completed, illustrating that control action may be warranted on strong descriptive epidemiology alone, when supported by microbiological typing. The case–case study supported the descriptive findings, rejecting the null hypothesis that watercress was not associated with infection with the outbreak strain. Cases of the outbreak profile of STEC had increased odds of consuming watercress, though the confidence intervals were wide.

The analytical study had several limitations: the small sample size and the low power of the study, since only one reference-case was recruited per outbreak case. No association was found between infection with the outbreak strain of STEC and Retailer A, probably due to the market share of this retailer, and aforementioned limitations. While no cases of HUS were reported, seven cases were hospitalised and 14/18 reported bloody diarrhoea, suggesting that the outbreak strain did not cause only mild illness. The absence of HUS cases is probably due to the age of the outbreak cases. HUS following STEC infection is predominantly seen in young children [3]: only one case under the age of 10 years was reported in this outbreak.

Outbreaks of STEC infection have previously been associated with salad vegetables, such as spinach [16,17]. Ready-to-eat salad vegetables are vulnerable to contamination with pathogens at the pre-harvest level [18] and have been associated with many outbreaks of food-borne infections [10,16,17,19-23]. While salad vegetables labelled as 'washed' may instil confidence in the consumer, current methods for washing and decontaminating produce cannot guarantee that pathogens, if present, will be removed. It has been demonstrated that STEC can adhere to leaves and become internalised within leafy vegetables [24,25]. The application of controls to minimise the risk of faecal contamination during growing, handling and processing is therefore of fundamental importance in ensuring the safety of fresh produce [26].The STEC Enhanced Surveillance system for England provided invaluable information on potential vehicles of infection in this outbreak, and allowed for rapid production of a hypothesis as to the cause of the outbreak. This was aided by the nature of watercress consumption in the UK: a low proportion of the UK population are thought to consume watercress, but interviewed cases had good recall of eating the product. Interdisciplinary collaboration and cooperation from a major food retailer meant the implicated product was removed from the shelves within 72 hours of the outbreak being notified.

The recall of watercress from Retailer A was well publicised and received media attention, but did not result in the reporting of further cases. The latest date of onset in this outbreak was 29 August 2013, suggesting that the outbreak is over. However, investigations on the identified watercress farms are still ongoing and the source contamination is currently unclear. Possible routes of contamination of the watercress include a failure in control measures protecting the watercress from agricultural run-off, contamination of water or growing materials used in watercress production or contaminated watercress seeds. While the implicated watercress is a UK product and no cases are known outside the UK, until the source of the contamination is identified, the international implications are unclear. The international community should be aware of this novel vehicle of infection for STEC and also be vigilant for cases linked to this outbreak. It is known that watercress seeds are traded internationally, and so if contaminated, there is the potential for cases to occur outside the UK.

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Conflict of interest

None declared.

Authors' contributions

Naomi Launders: Outbreak management, hypothesis generation, study design, data analysis, writing of manuscript. Lisa Byrne: Case identification, hypothesis generation, data analysis, liaison with laboratory staff. Natalie Adams: Data collection and study protocol review. Kirsten Glen: Data collection. Claire Jenkins: Interpretation of typing and Multi-Locus Variable-Number Tandem Repeat Analysis (MLVA). Drazenka Tubin-Delic: Trace-back investigation and provision of food consumption data, communication with retailer, coordination of environmental investigations. Mary Locking: Coordination of Scottish cases, data and response. Chris Williams: Coordination of Welsh cases, data and response. Dilys Morgan: Outbreak lead and chair of the outbreak control team.

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Monitoring West Nile virus (WNV) infection in wild birds in Serbia during 2012: first isolation and characterisation of WNV strains from Serbia

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West Nile virus (WNV), a neurovirulent mosquito-transmissible zoonotic virus, has caused recent outbreaks in Europe, including Serbia from August until October 2012. Although humans can be infected, birds are the main natural WNV reservoir. To assess WNV circulation in northern Serbia, 133 wild birds were investigated. These comprised resident and migratory birds, collected between January and September 2012 in the Vojvodina province. The birds belonged to 45 species within 27 families. Blood sera (n=92) and pooled tissues from respective birds (n=81) were tested by enzyme-linked immunosorbent assay (ELISA), plaque reduction neutralisation test (PRNT) and real-time reverse transcription-polymerase chain reaction (RT-qPCR). WNV antibodies were detected in seven (8%) sera: four from Mute Swans (*Cygnus olor*), two from White-tailed Eagles (*Haliaeetus albicillas*), and one from a Common Pheasant (*Phasianus colchicus*). Five sera neutralised WNV but not Usutu virus. For the first time in Serbia, WNV RNA was detected by RT-qPCR in pooled tissue samples of eight respective birds. WNV RNA was also derived from an additional bird, after a serum sample resulted infective in cell culture. The total nine WNV RNA positive birds included three Northern Goshawks (*Accipiter gentilis*), two White-tailed Eagles, one Legged Gull (*Larus michahelis*), one Hooded Crow (*Corvus cornix*), one Bearded Parrot-bill (*Panarus biramicus*), and one Common Pheasant. Phylogenetic analysis of partial E region sequences showed the presence of, at least, two lineage 2 Serbian clusters closely related to those responsible for recent human and animal outbreaks in Greece, Hungary and Italy. Full genomic sequence from a goshawk isolate corroborated this data. These results confirm WNV circulation in Serbia and highlight the risk of infection for humans and horses, pointing to the need for implementing WNV surveillance programmes.

Introduction

West Nile virus (WNV) a neurovirulent mosquito-transmissible *Flavivirus* is maintained in nature in an enzootic transmission cycle between birds and mosquitoes. Although WNV infections have been described in a wide variety of vertebrates, birds are the main natural reservoir. Hundreds of wild and domestic avian species have been described as susceptible to WNV infection, but many of these showed only subclinical infection [1]. In Europe, the reported seroprevalence in birds has been generally low, 1 to 10%, being usually higher among migratory than resident birds [2-8]. This leads to suggest that migratory birds may play a pivotal role in spreading WNV infection. Nevertheless, some studies pointed to resident birds as important in maintaining WNV circulation in nature [9,10].

Aside from birds, humans and horses are occasionally infected by WNV and sporadic disease outbreaks can occur that may result in fatalities [11]. In Europe WNV has been sporadically detected for decades but, since the 1990s, the number and frequency of outbreaks associated with severe disease including neurological manifestation have increased dramatically, and the virus is spreading throughout the Mediterranean basin and some European surrounding countries, constituting a serious veterinary and public health problem [11].

The genome of WNV is a single stranded RNA molecule of positive polarity with about 11,000 nucleotides that render three structural and seven non-structural proteins [11]. Of five WNV lineages, lineage 1 and 2 are the most widespread in the world [11]. Until 2004, only lineage 1 strains were circulating in Europe, but in 2004, a lineage 2 strain was isolated for the first time in Hungary from a goshawk [12]. Since, lineage 2 strains in several wild birds, mosquitoes, sentinel chickens, and humans have been consecutively isolated in Hungary, Austria, Italy, and also in Greece [12-17], where WNV

accounted for more than 250 human clinical cases and more than 30 deaths.

Few data are available on WNV activity in Serbia. Early reports during the 1970s described the prevalence of anti-WNV antibodies in human populations of the former Yugoslavia that ranged from 1% to 8% depending of the studied region of the country [18-20]. Recently, anti-WNV IgG were detected in 4% (18/451) of human sera collected from 2005 to 2010 in the Vojvodina province, the place of sampling of the present report, and WNV RNA was detected in six of 841 mosquito pools, mainly from 2010 [21]. Twelve per cent of the horses investigated during 2009 and 2010 in the same province tested seropositive [22]. From August to October 2012, an outbreak of WNV clinical infection in humans was reported for the first time ever in the central and northern part of Serbia, including the Vojvodina province [23]. Of 58 reported cases, 45 were confirmed and 13 were probable. Nine were fatal. In light of all previously conducted studies, the objective of the present study was the serological and molecular assessment of WNV activity in wild resident and migratory birds, as virus natural hosts, in the Vojvodina province. The surveillance period which extended from January to September 2012 covered a time before and during the first human WNV outbreak in Serbia.

Methods

Samples

Samples (n=133) from living-captured wild birds, wild birds that died in a rehabilitation centre, or from birds found dead were collected between January and September 2012 in the Serbian province of Vojvodina. Birds collected in the context of bird-ringing activities were captured by traps and mist nets, bled, ringed, and released. From dead birds, blood exudates were collected from heart or from pleural or abdominal cavity. A pool of selected tissues (brain, kidney, liver, lung and spleen) was created from each dead bird. In total 92 blood serum samples from 30 wild bird species were examined for the presence of anti-WNV antibodies. Additionally, 81 pools of tissue samples from birds belonging to 35 species were homogenised as described [24,25] and examined for the presence WNV RNA. Detection of infective virus was assayed on Vero cell culture by standard procedures [26].

Enzyme-linked immunosorbent assay (ELISA)

Anti-WNV IgG was detected by a validated ELISA based on WNV recombinant envelope E (rE) protein [27]. The positive cut-off value was assigned using a positive/negative (P/N) ratio of ≥ 2.

Plaque reduction neutralisation test (PRNT)

Neutralising antibodies were detected by PRNT on Vero cells [28] using a fixed amount (100 plaque-forming unit (PFU)) of cultured passage WNV-NY99 strain [24,26] and two-fold dilutions of serum (starting from 1:20). Neutralising antibody titration was established as the highest serum dilution that inhibits plaque formation by 90% ($PRNT_{90}$). In those samples in which enough serum volume was available, and as control of flaviviral specific reactivity, PRNT was similarly performed with Usutu virus (USUV) strain SAAR 1776, the only other flavivirus of the Japanese encephalitis serocomplex circulating in Europe [29].

Detection of West Nile virus RNA

RNA was extracted from each of 81 homogenised tissues/organs pools [25,27], or from sera that resulted infective in cell culture, using a commercial kit (Speedtools RNA virus extraction kit, Biotools B and M Labs S.A, Madrid, Spain) following manufacturer's instruction. RNA extracts were amplified by real-time reverse transcription-polymerase chain reaction (RT-qPCR) using primers and probes that target the capsid and 5´untranslated genomic regions of both WNV lineage 1 and 2 [2]. Positive samples were further tested employing a primer pair (forward: 5'-CCAAACAATCTGTTGTGGCTCTAG-3' and reverse: 5'-CAGCGAATTTAAACGCTTTTGAAC-3'), designed for the specific detection of WNV lineage 2 and targeting a 194 bp fragment of the E genomic region (nucleotides: 1,709 to 1,903 according to Uganda B956 lineage 2 strain, GenBank accession number: AY532665), and SuperScript One-Step RT-PCR with Platinum Taq (Molecular Probes, Eugene, O), as described [26].

Sequencing and phylogenetic analysis

A fragment targeting the first 1,903 nucleotides of WNV-RNA positive samples was amplified by reverse transcription of viral RNA as above using primers 5'-CAGCGAATTTAAACGCTTTTGAAC-3´and 5'-AGTAGTTCGCCTGTGTGAGC -3', and bi-directionally sequenced (Macrogen Inc. Seoul, Korea). In addition, the complete viral genome sequence of a positive goshawk, here termed SRB-Novi Sad/12 (GenBank accession number: KC407673), was amplified with the appropriate oligonucleotide primers (available upon request), purified, and sequenced.

Nucleotide sequence comparisons and phylogenetic analysis were conducted with the Serbian sequences and representative strains of lineage 1 and 2 retrieved from GenBank. Trees were built by the neighbour-joining method from a multiple alignment using ClustalW and Phylogeny.fr [30] after 100 replications for bootstrapping and visualised with TreeView.

Results

Of 92 wild bird sera tested, seven (8%) were IgG ELISA positive (Table). They belonged to three species: four Mute Swans (species: *Cygnus olor*; order: Anseriformes); two White-tailed Eagles (*Haliaeetus albicilla*; Accipitriformes); and one Common Pheasant (*Phasianus colchicus*; Galliformes). Of the seven ELISA positive samples, five samples neutralised WNV in cell culture with relatively low $PRNT_{90}$ values while one sample from a swan and one from an eagle did not neutralise WNV (Table). Only the White-tailed Eagle serum

Table

West Nile virus surveillance results on wild migratory and resident birds sampled in the province of Vojvodina, northern Serbia, January–September 2012

Order	Family	Resident/ Migratory status	Species	WNV ELISA[a]	WNV PRNT[b]	WNV RT-qPCR[c]
Anseriformes	Anatidae	r/m	*Cygnus olor* - Mute Swan	4/17	3/17	NA
Gruiformes	Rallidae	r/m	*Rallus aquaticus* - Water Rail	NA	NA	0/1
Charadriformes	Laridae	r/m	*Chroicocephalus ridibundus* - Black-headed Gull	0/5	0/5	0/6
		r/m	*Larus michahellis* - Yellow-legged Gull	NA	NA	1/1
		r/m	*Ichthyaetus melanocephalus* - Mediterranean Gull	NA	NA	0/2
Galliformes	Phasianidae	r	*Alectoris graeca* - Rock Patridge	NA	NA	0/1
		r	*Phasianus colchicus* - Common Pheasant	1/1	1/1	1/1
Podicipediformes	Podicipedidae	r/m	*Podiceps cristatus* - Great Crested Grebe	0/1	0/1	0/1
Pelecaniformes	Ardeidae	r/m	*Ardea cinerea* - Grey Heron	0/1	0/1	0/1
		m	*Ixobrychus minutus* - Little Bittern	0/1	0/1	0/3
Ciconiiformes	Ciconiidae	m	*Ciconia ciconia* - White Stork	0/2	0/2	0/3
Accipitriformes	Pandionidae	m	*Pandion haliaetus* - Osprey	0/1	0/1	0/1
	Falconidae	r/m	*Falco tinnunculus* - Common Kestrel	0/2	0/2	0/4
	Accipitridae	r/m	*Circus aeruginosus* - Western Marsh Harrier	NA	NA	0/1
		m	*Buteo buteo* - Common Buzzard	0/4	0/4	0/8
		r	*Accipiter gentilis* - Northern Goshawk	0/3	0/3	3/3
		r	*Accipiter nisus* - Eurasian Sparrowhawk	NA	NA	0/2
		r	*Haliaeetus albicilla* - White-tailed Eagle	2/6	1[d]/6	1/8
Strigiformes	Strigidae	r	*Athene noctua* - Little Owl	NA	NA	0/1
		r	*Tyto alba* - Barn Owl	0/1	0/1	0/3
		m	*Asio otus* - Long-eared Owl	0/4	0/4	0/6
Apodiformes	Apodidae	m	*Apus apus* - Common Swift	NA	NA	0/1
Cuculiformes	Cuculidae	m	*Cuculus canorus* - Common Cuckoo	NA	NA	0/1
Coraciiformes	Meropidae	m	*Merops apiaster* - European Bee-eater	0/1	0/1	0/1
	Coraciidae	m	*Coracias garrulus* - European Roller	0/2	0/2	NA
Columbiformes	Columbidae	r	*Columba Livia domestica* - Domestic Pigeon	0/1	0/1	NA
		r	*Streptopelia decaocto* - Eurasian Collared-dove	0/1	0/1	0/1
Passeriformes	Hirundinidae	m	*Hirundo rustica* - Barn Swallow	0/1	0/1	0/1
	Muscicapidae	r/m	*Erithacus rubecula* - European Robin	NA	NA	0/1
	Turdidae	r/m	*Turdus merula* - Eurasian Blackbird	0/2	0/2	0/3
	Acrocephalidae	m	*Acrocephalus schoenobaenus* - Sedge Warbler	0/4	0/4	NA
		m	*Acrocephalus scirpaceus* - Eurasian Reed-warbler	0/4	0/4	NA
		m	*Acrocephalus arundinaceus* - Great Reed-warbler	0/12	0/12	NA
	Sylviidae	m	*Sylvia atricapilla* - Blackcap	0/2	0/2	NA
	Phylloscopidae	m	*Phylloscopus sibilatrix* - Wood Warbler	NA	NA	0/1
	Corvidae	r	*Pica pica* - Black-billed Magpie	0/1	0/1	0/2
		r	*Corvus cornix* - Hooded Crow	0/1	0/1	1/1
	Passeridae	r/m	*Passer montanus* - Eurasian Tree Sparrow	0/4	0/4	NA
		r/m	*Passer domesticu* - House Sparrow	0/3	0/3	NA
	Fringillidae	r/m	*Carduelis cannabina* - Eurasian Linnet	NA	NA	0/2
		r/m	*Carduelis carduelis* - European Goldfinch	NA	NA	0/6
		r/m	*Carduelis spinus* - Eurasian Siskin	NA	NA	0/1
		r/m	*Chloris chloris* - European Greenfinch	NA	NA	0/1
	Emberizidae	r/m	*Emberiza schoeniclus* - Reed Bunting	0/2	0/2	NA
	Timaliidae	r	*Panurus biarmicus* - Bearded Parrotbill	0/1	0/1	1/1
	Total(27)		**45 species**	**7/92 (8%)**	**5/92 (5%)**	**8/81 (10%)**

ELISA: enzyme-linked immunosorbent assay; m: strictly migratory birds; NA: not applicable; PRNT: plaque reduction neutralisation test; r: strictly resident birds; r/m: resident and migratory birds at the same time, mostly resident but could migrate depending of the weather; RT-qPCR: real-time reverse transcription-polymerase chain reaction; WNV: West Nile virus.

[a] Testing of birds blood sera samples for the presence of anti-WNV IgG antibodies by ELISA (positive/tested). Positive/negative (P/N) values of positive samples: Mute Swans: 2.3, 2.6, 2.3 and 2.7; White-tailed Eagles: 3.9 and 11.2; Common Pheasant: 6.8.

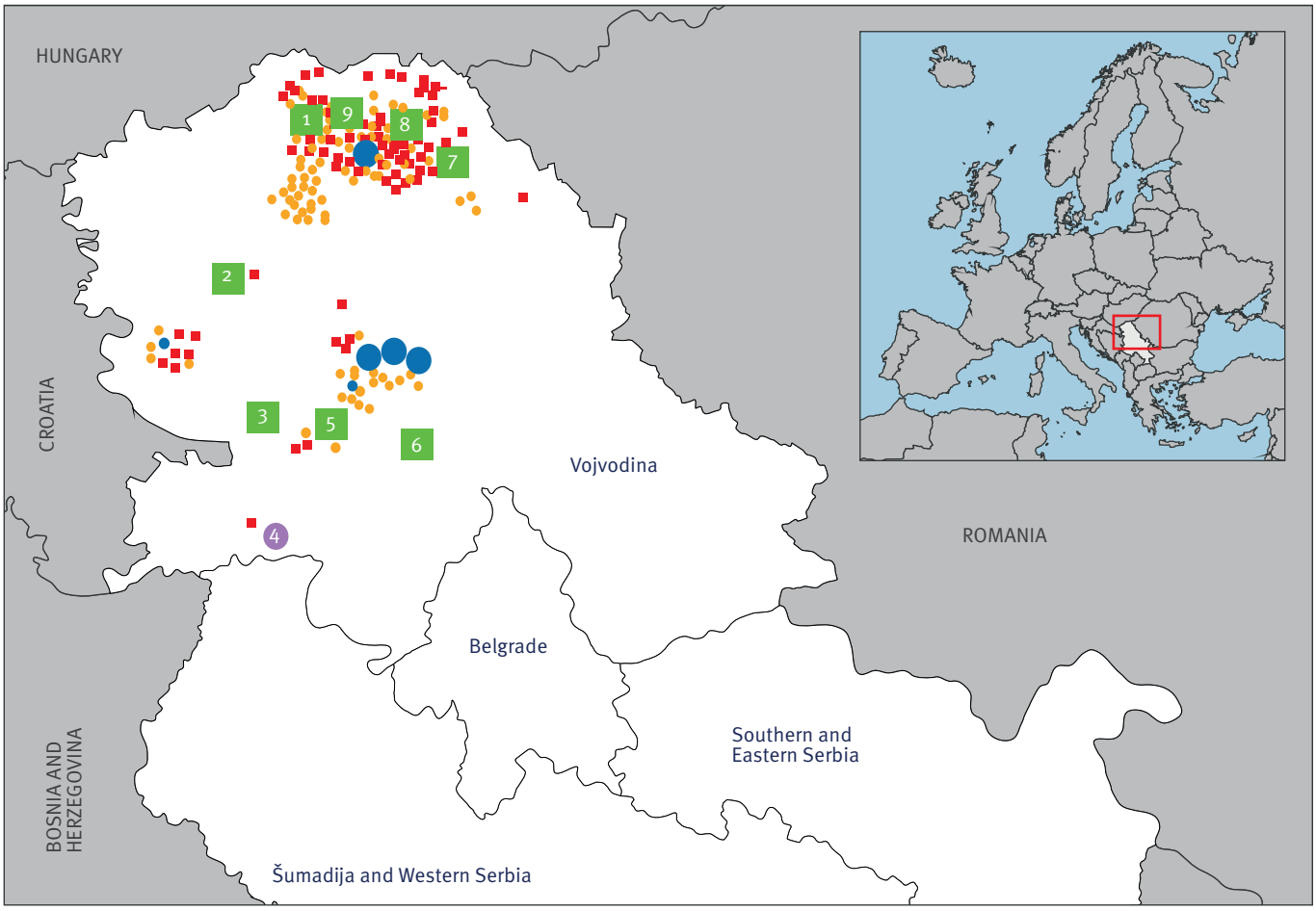
[b] Testing of birds blood sera samples for the presence of anti-WNV IgG antibodies by PRNT (positive/tested). $PRNT_{90}$ titres of positive samples: Mute swans: 56, 63, and 30; White-tailed Eagle: › 160; Common Pheasant: 44.

[c] Testing of bird tissue samples pools for the presence of WNV RNA by RT-qPCR (positive/tested). The complete genome from one Northern Goshawk isolate, SRB-Novi Sad/12 (GenBank accession number: KC407673), was sequenced. Isolates: SRB-7193-13/12 (GenBank accession number: KC407671), SRB-7193-14/12 (GenBank accession number: KC407672), SRB-6989/12 (GenBank accession number: KC407668), SRB-7193-10/12 (GenBank accession number: KC407670) and SRB-7193-3/12 (GenBank accession number: KC407669) from a Common Pheasant, a White-tailed Eagle, a Bearded Parrot-bill, a Hooded Crow and another Northern Goshawk, respectively, were partially sequenced (genome fragment of the E region).

[d] Blood serum sample positive for infectious WNV on Vero E6 cell culture.

Figure 1

Location of birds sampled for West Nile virus testing in Vojvodina province and test results, northern Serbia, January–September 2012



Orange circles: anti-WNV antibodies negative blood sera samples (ELISA and PRNT).
Small red squares: WNV RT-qPCR negative tissue sample pools.
Small blue circles: anti-WNV antibodies positive sera samples (ELISA).
Big blue circles: anti-WNV antibodies positive sera samples (ELISA and PRNT).
Big violet circle: serum sample positive for infectious WNV and for anti-WNV antibodies (ELISA and PRNT).
Big green squares: WNV RT-qPCR positive tissue samples.

ELISA: enzyme-linked immunosorbent assay; PRNT: plaque reduction neutralisation test; RT-qPCR: real-time reverse transcription-polymerase chain reaction; WNV: West Nile virus.

West Nile virus isolates: 1) SRB-7193-13/12 (Common Pheasant, *Phasianus colchicus*); 2) SRB-7193-14/12 (White-tailed Eagle, *Haliaeetus albicilla*); 3) SRB-6989/12 (Bearded Parrot-bill, *Panarus biramicus*); 4) SRB-7193-11/12 (White-tailed Eagle, *Haliaeetus albicilla*); 5) SRB-Novi Sad/12 (full genome sequenced isolate from Northern Goshawk, *Accipiter gentilis*); 6) SRB-7193-10/12 (Hooded Crow, *Corvus cornix*); 7) SRB-7193-3/12 (Northern Goshawk, *Accipiter gentilis*); 8) SRB-7193-4/12 (Yellow-legged Gull, *Larus michahelis*); 9) SRB-7193-7/12 (Northern Goshawk, *Accipiter gentilis*).

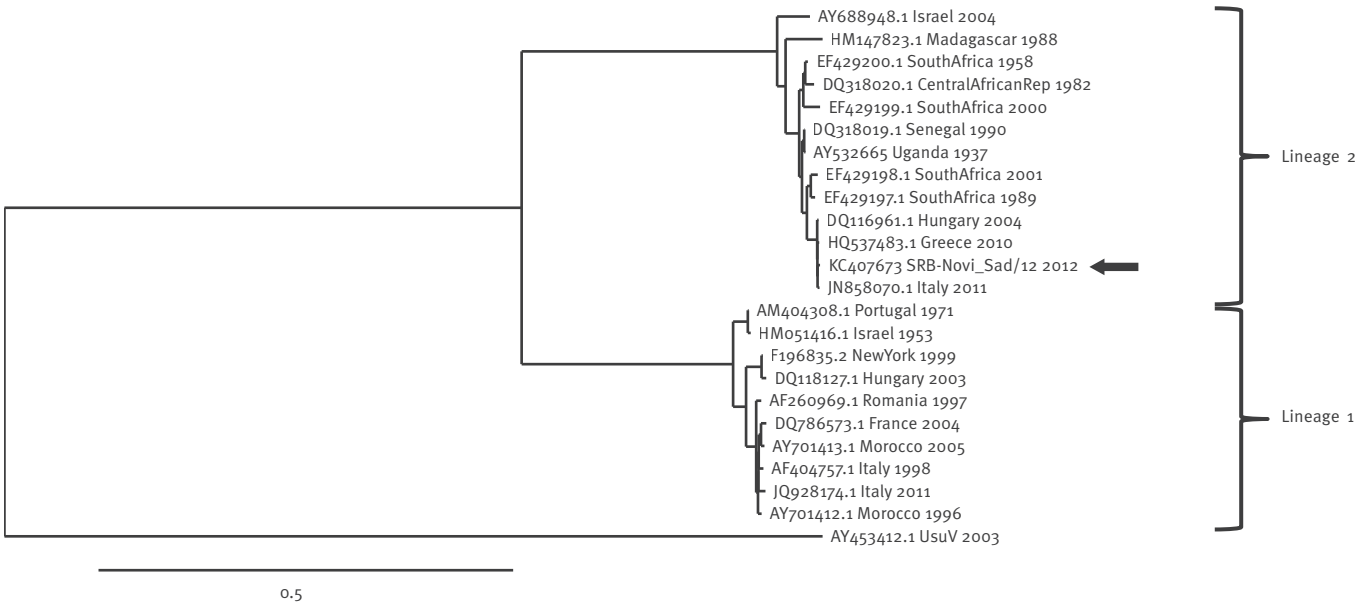
with the highest P/N value (11.2) had a $PRNT_{90}$ >160. Of the 12 sera tested by PRNT for USUV (6 WNV-ELISA positives and 6 negatives), only one (a WNV-ELISA positive serum) neutralised USUV ($PRNT_{90}$=70), but it did not neutralise WNV.

A total of 81 pools of tissue samples from wild bird carcases belonging to 35 species were examined for the presence of WNV RNA and eight (10%) tested positive (Table). These correspond to three Northern Goshawks, one Bearded Parrot-bill, one Common Pheasant, one Legged Gull, one Hooded Crow, and one White-tailed Eagle, all of which died during winter-spring and summer of 2012.

In addition, one serum sample resulted infective in cell culture and was subsequently confirmed to be WNV RNA positive. This sample, which was from a

Figure 2

Phylogenetic analysis based on complete genome nucleotide sequences of a West Nile virus strain derived from a goshawk in Vojvodina province, Serbia 2012



The SRB-Novi Sad/12 isolate (GenBank accession number: KC407673) from the gowshawk recovered in this study is marked with an arrow. GenBank accession numbers, geographic origin and year of isolation of samples are shown. The scale bar depicts genetic distance. The Usutu virus USUV SAAR1776 strain was used as out-root.

White-tailed Eagle, had shown the highest P/N (11.2) and $PRNT_{90}$ (>160) titres, and was collected in July 2012 (Table).

The complete genome sequence was obtained from an isolate originating from a Northern Goshawk (SRB-Novi Sad/12) that was found dead in the Vojvodina province (the exact location is not known) during the spring of 2012 (Figure 1). Virus from this tissue sample was successfully amplified after a single passage on Vero cells. No anti-WNV antibodies had been detected in the serum.

Pairwise alignment of the SRB-Novi Sad/12 isolate sequence with representative WNV lineage 1 and 2 complete genomes sequences revealed that it belongs to lineage 2 (Figure 2) and clusters with recent WNV strains isolated in Hungary in 2004, Greece in 2010, and Italy in 2012, with which it presents the highest similarities, 99.51%, 99.47% and 99.45%, respectively. Percentage of similarity with the other lineage 2 sequences analysed varied between 93.03% (Israel 1999 isolate) and 97.65% (South African 1989 isolate), whilst homology with lineage 1 New York 1999 isolate was 79.53%. A total of 29 unique nucleotides scattered through the genome were found in the SRB-Novi Sad/12 isolate when compared to those circulating lately in Europe. Only one of these nucleotide substitutions (T5343A) results in an amino acid change in the NS3 region (H244Q).

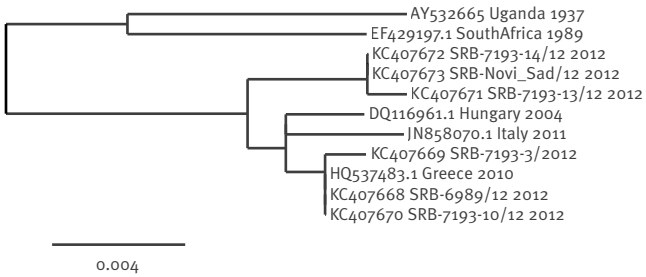
Further analysis of partial E sequences (863 nucleotides) from the other Serbian birds, which except for a pheasant collected in winter-early spring, were all collected during the summer of 2012, confirms that these sequences also belong to lineage 2; however, two clusters of sequences were clearly distinguishable (Figure 3), a first cluster with sequences (from a pheasant and an eagle) showing 100% similarity with SRB-Novi Sad/12 isolate, and a second cluster with sequences, showing four to five synonymous nucleotides variations compared with the SRB-Novi Sad/12 isolate. The latter isolates were from a crow and a parrot-bill (with four synonymous substitutions) as well as a goshawk, (with five synonymous substitutions). The Serbian sequences in the second cluster are highly similar, only one nucleotide variation (two in the case of the goshawk isolate) in the region analysed, with a strain identified from a mosquitoes pool in Greece in 2010 [14].

Discussion

The presence and circulation of WNV among wild resident and migratory birds in Serbia was serologically confirmed, for the first time, as seven (8%) of the 92 serum samples investigated, which belong to birds of 30 species within 21 families of 11 different orders,

Figure 3

Phylogenetic analysis of West Nile virus (WNV) strains detected in Vojvodina province based on partial genome nucleotide sequences from the E region of the WNV genome, Serbia, 2012



SRB: Serbian strains.

GenBank accession numbers geographic origin and year of isolation of samples are shown. The scale bar depicts genetic distance. SRB-7193-13/12 (Common Pheasant); SRB-7193-14/12 (White-tailed Eagle); SRB-Novi Sad/12 (Northern Goshawk); SRB-7193-3/12 (Northern Goshawk); SRB-7193-10/12 (Hooded Crow); SRB-6989/12 (Bearded Parrot-bill).

presented anti-WNV IgG. Birds testing positive belong to three orders and three species: four Mute Swans, two White-tailed Eagles, and one Common Pheasant. Five of the seven birds had neutralising antibodies with relatively low titres, except for one White-tailed Eagle which presented a $PRNT_{90}$ >160. None of these five sera neutralised USUV. Only one serum sample of a total of 12, which were tested for USUV, neutralised USUV, but this WNV-ELISA positive sample did not neutralise WNV, confirming the circulation of USUV in the region [22]. The prevalence of WNV seropositive birds found in the northern part of Serbia (8%) is similar to that previously described in other European countries, 1% to 10% [2-8], suggesting that, even though WNV entrance in Serbia has probably been a recent event, the virus is currently circulating with the same intensity than in surrounding countries.

Birds have been implicated in spreading WNV infection during their migration [11]. Anti-WNV antibodies are usually less frequently found among resident birds than migratory birds, suggesting that they can act as reservoirs of WNV and carry the virus over long distances, while resident wild birds can act as amplifiers of local WNV strains. Three of the seropositive birds found here were resident birds (two White-tailed Eagles and one Common Pheasant), while the other four (Mute Swans) are considered both migratory and resident birds in Serbia. Samples were collected from winter-early spring to late summer 2012, suggesting that WNV infects both wild resident and migratory birds and point to a possible overwintering and expansion of the virus in the province of Vojvodina. However, as the number of samples tested was limited, a more detailed surveillance analysis should be conducted as part of future investigations to clarify this point.

RNA amplification was achieved in tissue samples from eight birds, which were found dead: one Bearded Parrot-bill, one Common Pheasant, one Hooded Crow, one Legged Gull, three Northern Goshawks, and one White-tailed Eagle. Six of these birds died during the summer of 2012 while two (a pheasant and a goshawk) died during winter-early spring. Serum from another White-tailed Eagle (found dead in August 2012) was infective in cell culture, and subsequently confirmed to be WNV RNA positive. Notably, this infective serum showed the highest P/N and $PRNT_{90}$ titres, thus, pointing to a very recent infection. Eight of the nine WNV-RNA positive birds were strictly resident, suggesting that they became infected in the country. Moreover, isolation of WNV-RNA from dead predators (5 of the 9 WNV positive birds) provides more evidence that birds of prey play a key role in virus transmission [12,15,16].

Phylogenetic analysis of the complete genomic sequence of the virus recovered from a dead Northern Goshawk (SRB-Novi Sad/12) showed a lineage 2 strain (Figure 2) that clusters with the viruses responsible for the most recent human and animal outbreaks reported in neighbouring countries [13-17]. However, SRB-Novi Sad/12 isolate was unique, as it showed a total of 29 distinctive nucleotides when compared to those circulating in Europe, although this resulted in only one single amino acid change (H244Q) in the nonstructural protein 3 (NS3) region.

Comparison of partial sequences of the E region from five additional WNV sequences recovered from respective birds in this study shows that at least two different groups of lineage 2 strains, which simultaneously circulated during summer of 2012, can be distinguished (Figure 3). Those that exactly match that of SRB-Novi Sad/12 isolate and those showing four to five synonymous nucleotide variations in comparison to SRB-Novi Sad/12. Except for the presence of an additional nucleotide change (C171T) in the sequence recovered from a goshawk, the sequences in this second cluster present only a single nucleotide variation with a strain recently isolated in Greece in 2010 [14].

These results suggest that WNV has reached the country in, at least, two different events. Our results also suggest that the virus not only has become endemic in Serbia and surrounding countries, but that it is also evolving while circulating in the area. According to these findings, it seems plausible to think that since its original detection in Hungary, WNV lineage 2 has expanded southwards and reached Serbia recently. However, as until very recently WNV has been an almost neglected disease in the region, it cannot be ruled out that there had been prior sporadic human and animal cases that

have gone unnoticed. Nevertheless, re-introduction of the virus in the future by migratory birds should be monitored.

In August 2012, the first reported WNV outbreak in humans occurred in Serbia [23], with 58 West Nile fever cases. Even though, no WNV genomic sequences are available from these human cases, our data suggest that they were most likely caused by lineage 2 strains similar to the ones reported here.

In summary, the present study provides the first evidence for the presence of WNV infection among wild birds in Serbia, and reports the isolation and characterisation of the first WNV strains in the country. WNV recovered from Serbian birds represent two clusters of lineage 2 strains closely related to other lineage 2 strains currently circulating in neighbouring countries. It is reasonable to think that similar lineage 2 viruses have been responsible for the 2012 first human clinical outbreak reported in Serbia. The data reported here, and the fact that WNV is already endemic in other neighbouring countries, suggests that further WNV infections are likely to occur in Serbia in the future. Therefore, in our opinion, additional epidemiological studies and a state-of-the art surveillance system for the detection of incursions of WNV into Serbia deems mandatory.

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Dramatic change in public attitudes towards vaccination during the 2009 influenza A(H1N1) pandemic in France

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We investigated the potential impact of the 2009 influenza A(H1N1) pandemic on attitudes towards vaccination among people aged 18 to 75 years and living in metropolitan France. We used data from three national telephone surveys conducted on representative samples in 2000, 2005 and 2010 (n=12,256, n=23,931, n=8,573 respectively). In France, unfavourable attitudes towards vaccination in general dramatically increased from 8.5% in 2000 and 9.6% in 2005 to 38.2% in 2010. In 2010, among respondents who held unfavourable attitudes towards vaccination, 50% mentioned specifically their opposition to the influenza A(H1N1) vaccine. The sociodemographic profile associated with these attitudes also changed greatly. In particular, unfavourable attitudes towards vaccination in general became significantly more frequent among less educated people in 2010. These attitudes were also correlated with vaccination behaviours. For example, parents who were unfavourable towards vaccination in general were more likely to report that they had at least one child who did not get the measles-mumps-rubella vaccine. As this shift in attitude may have a significant impact on future vaccination coverage, health authorities should urgently address the vaccine confidence gap.

Introduction

Public concern about vaccine safety is as old as vaccines themselves [1,2]. Nevertheless, many public health experts consider that one of the greatest challenges currently facing vaccinology is the ongoing decline of public confidence in vaccines [3-6]. Such decline is illustrated by the so-called revival of anti-vaccination movements that may compromise immunisation programmes [7-9]. Another illustration is the suboptimum measles-mumps-rubella (MMR) vaccination coverage observed in many European countries, which has recently caused several measles outbreaks, especially in France [10-12]. This vaccine confidence gap is also illustrated by controversies surrounding specific vaccines during the last decades, including MMR vaccine in the United Kingdom, hepatitis B vaccine in France, and not least the 2009 influenza A(H1N1) vaccine [5].

A number of studies have been carried out to investigate factors associated with attitudes and behaviours towards the 2009 influenza A(H1N1) vaccine. Many of them found that the willingness to accept this vaccine was significantly associated with respondents' sociodemographic background (gender, age, household's composition, socioeconomic status), as well as with prior vaccination attitudes and behaviours, and especially seasonal influenza vaccination uptake [13-17]. Conversely, only a few studies have investigated the potential impact of the 2009 influenza A(H1N1) pandemic on public attitudes and behaviours towards vaccination in general [18-19].

In the present study, we investigated the potential impact of the 2009 influenza A(H1N1) pandemic on attitudes towards vaccination in general among people living in metropolitan France. In particular, we aimed at testing the following three hypotheses: (i) we expect a growing proportion of French citizens to express unfavourable attitudes towards vaccination in general during the 2009 influenza A(H1N1) episode; (ii) as a growing number of French citizens oppose vaccination, their sociodemographic profile should change; (iii) such opposition should be a significant predictor of vaccination behaviours. We used data from three national surveys conducted by the French National Institute for Prevention and Health Education (INPES) in 2000, 2005 and 2010. In order to test these

hypotheses, we first compared the French population's attitudes towards vaccination across the three surveys, from 2000 to 2010. As the data collection process took several months for the last survey, from October 2009 to June 2010, we also had the opportunity to observe how these attitudes changed during the influenza A(H1N1) pandemic. Secondly, we investigated the sociodemographic factors associated with attitudes towards vaccination in general and compared them across the three surveys. Thirdly, we examined the relationship between attitudes towards vaccination and self-reported vaccination behaviours.

Methods

Design and samples

We used data from the last three waves (2000, 2005, 2010) of the 'Health Barometer', a telephone survey on health perceptions, knowledge, attitudes and behaviours targeted at the general population and conducted by the INPES. Each wave was carried out on a representative random sample of the population aged 12 to 75 years (15 to 85 years in 2010) living in continental France by use of a computer-assisted telephone interview (CATI) system.

Design and protocol were identical for the three surveys and have been approved by the French Commission on Individual Data Protection and Public Liberties (CNIL). They were based on a two-stage random sample of French-speaking people. Residents of collective dwellings, hospitals and institutions were excluded from the target population. Private households with landline telephones were included in the sample (phone numbers were randomly generated, in order to include people with confidential numbers), as well as people owning only mobile phones. The first sampling step was household selection (by phone number), then an eligible subject was randomly selected to answer the questions, using the next-birthday method in 2000 and 2005 (the interviewer asked which member of the household of eligible age had their birthday coming up next and interviewed that person), and the Kish method in 2010 (the interviewer asked for the first names of all household members and for their birthdays, then selected the respondent whose birthday was most recent). All collected data were anonymised and self-reported. The study protocol included a formal request to participate, sent by postal mail, explaining the objectives of the study. This letter was sent before the first telephone call (or after for subjects with confidential numbers whose addresses were initially unknown).

Data collected

The sample sizes reached n=13,685 in 2000, n=26,672 in 2005 and n=9,761 in 2010, with similar cooperation rates (64%, 58% and 61%, respectively). We restricted the analysis to respondents aged 18 to 75, corresponding to n=12,256 in 2000, n=23,931 in 2005, n=8,573 in 2010.

Respondents were asked about their attitude towards vaccination in general ('very favourable', 'somewhat favourable', 'somewhat unfavourable', 'very unfavourable'). They were also asked whether or not they were unfavourable towards certain vaccines in particular, and if so, to which ones (with an open-ended question and multiple responses allowed). Regarding vaccination behaviour, respondents with children aged one to 15 years were asked in the three surveys if at least one of the children had not been vaccinated with the combined MMR vaccine. In 2005 and 2010, participants were also requested to report their own general immunisation status (up-to-date or not). Finally, in 2010 only, they were asked whether or not they had been vaccinated against seasonal influenza in 2008.

The questionnaire collected data on respondents' sociodemographic background: gender, age, educational level, household composition and income. For each respondent, we computed the equivalised household income (EHI). EHI involves a weighting scale that enables analysis of the relative well-being of households of different size and composition. We counted 1 point for the first adult in the household, 0.5 points for each additional person aged 15 years and older, and 0.3 points for each child younger than 15 years. EHI is computed by dividing total household income by the sum of points allocated to the household members.

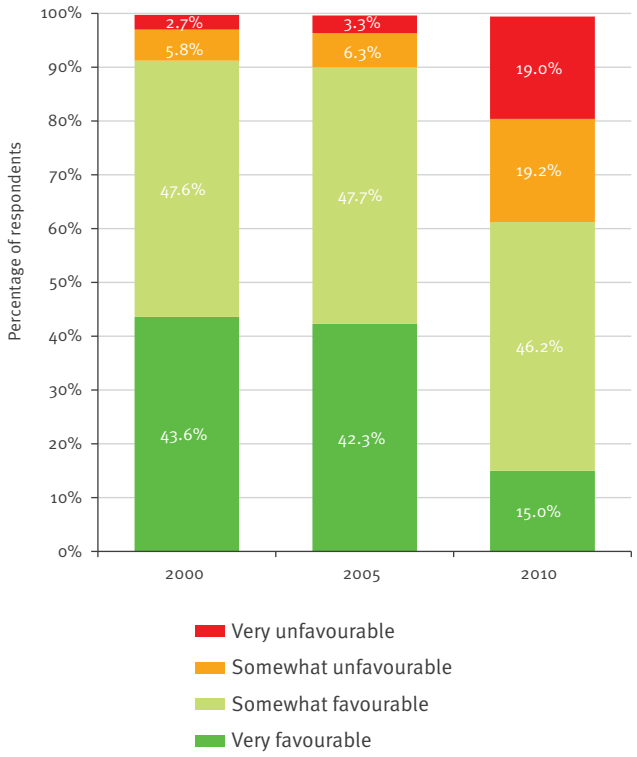
Statistical analysis

Data were weighted with respect to the inclusion probability. They were also adjusted to distributions in the French population according to gender, age, educational level, geographical region and urbanisation level. All statistical analyses were performed using the weighted data.

Firstly, we compared respondents' attitudes towards vaccination in general across the three waves of the Health Barometer, as well as their responses to the open question (people were asked to indicate, unprompted, toward which vaccines they were unfavourable). Concerning the 2010 wave, as data collection lasted from October 2009 to June 2010, we examined how the attitudes towards vaccination in general varied during this period. To do so, we collapsed the four response items into a binary outcome ('somewhat' or 'very' unfavourable, versus 'somewhat' or 'very' favourable and no response). As the sociodemographic structure of the monthly subsamples differed, we took this into account to make each month comparable to the others (with a weighting procedure based on gender and age distributions). The size of the monthly subsamples was quite small for October (n=272), June (n=279) and May (n=674), but above n=1,000 for the other months. For each month we computed the proportion of respondents who were unfavourable towards vaccination in general, and the corresponding 95% confidence intervals. We used the Pearson's chi-square test for bivariate analyses and Wald's chi-square for logistic

Figure 1

Attitudes towards vaccination in general in the population aged 18–75 years, INPES surveys, France, 2000, 2005, 2010



INPES: French National Institute for Prevention and Health Education.

Percentages do not add up to 100% as a few people refused to answer this question or answered 'don't know'

regressions to assess the statistical significance of observed variations.

Secondly, for each wave separately, we investigated the sociodemographic factors associated with attitudes towards vaccination in general (using the binary outcome). We performed bivariate analyses and multivariate analyses with logistic regression models. The following covariates were introduced into the models: gender, age, educational level, EHI and presence of at least one child aged under the age of four years in the household. For each of them, we computed adjusted odds ratios.

Finally, for each wave separately, we examined the relationship between attitudes towards vaccination in general and vaccination behaviours (with logistic regressions taking into account gender, age, educational level, EHI and presence of at least one child aged under the age of four years in the household as potential confounding factors).

Results

Attitudes towards vaccination from 2000 to 2010

Figure 1 displays reported attitudes towards vaccination in general in 2000, 2005 and 2010. Non-responses were very rare for the three waves (<1%). The distributions of attitudes towards vaccination in general were quasi identical in 2000 and 2005. Unfavourable attitudes were reported by 8.5% of respondents in 2000 and 9.6% in 2005, but this proportion dramatically increased in 2010, reaching 38.2%.

Among respondents who stated that they were unfavourable towards vaccination in general, in 2000, 22% reported that they were unfavourable towards all vaccines (16% in 2005), 24% mentioned specifically their opposition to the seasonal influenza vaccine (20% in 2005), another 24% mentioned the hepatitis B vaccine (37% in 2005), 9% the MMR vaccine (8% in 2005) and another 9% the tuberculosis vaccine (9% in 2005). In 2010, all these proportions decreased sharply: among respondents who were unfavourable towards vaccination in general, 5% opposed all vaccines, 11% mentioned the seasonal influenza vaccine, 12% mentioned the hepatitis B vaccine, 2% the MMR and 2% the tuberculosis vaccine. Moreover, among those opposing vaccination in general, 50% mentioned spontaneously their opposition to specifically the influenza A(H1N1) vaccine.

Looking more closely at data collected from October 2009 to June 2010, it appeared that the proportion of respondents who reported being unfavourable towards vaccination in general varied significantly during this period ($p<0.001$) (Figure 2): 31% of respondents opposed vaccination in general in October 2009, this proportion rose to 40–41% in December–January and began to decline only after March 2010 (31% in June).

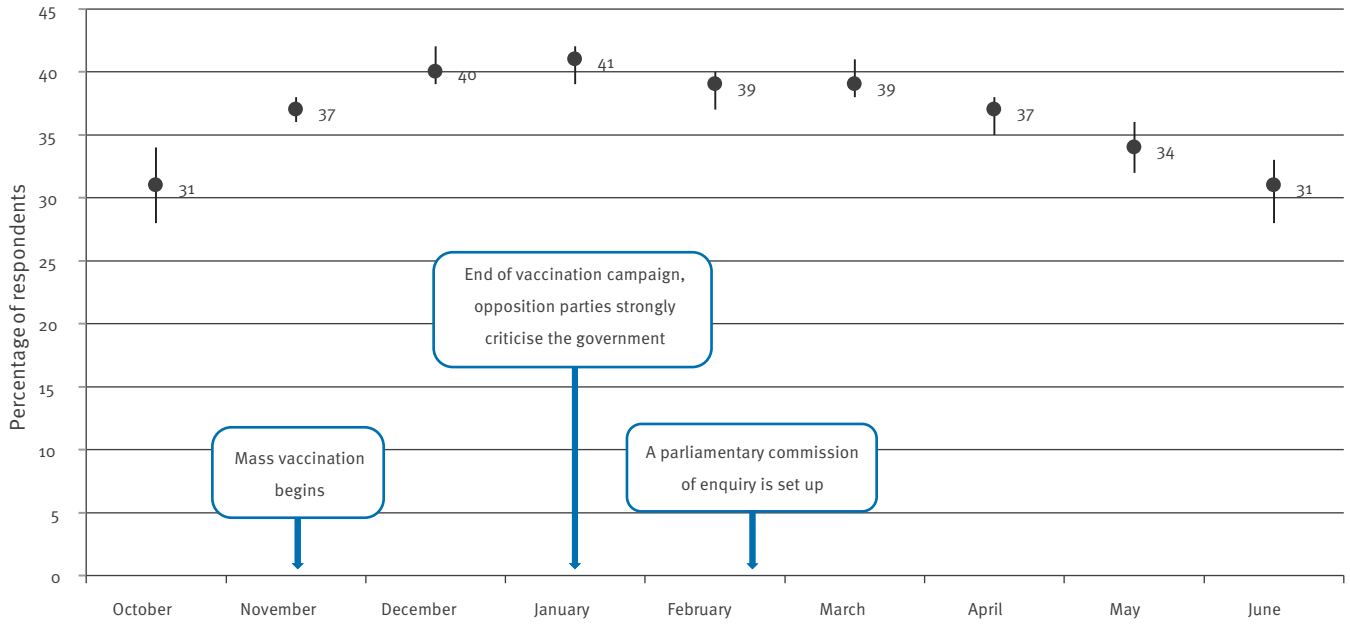
Sociodemographic factors associated with attitudes towards vaccination in general

The sociodemographic factors associated with unfavourable attitudes towards vaccination in general were quite similar in 2000 and 2005 (Table 1). Female respondents were more likely to express such attitudes (odds ratio (OR): 1.27 in 2000, 1.25 in 2005), as well as older respondents (OR for those aged 65 to 75 years: 3.32 in 2000, 3.25 in 2005, with 18 to 24 year-olds as the reference category). In multivariate analysis, for the surveys conducted in 2000 and 2005, educational level and presence of children under the age of four years in the household were not significant predictors of attitudes towards vaccination. Finally, concerning EHI, we observed a slightly significant ($p<0.05$) effect in 2000 (OR: 0.79 for the highest income level versus the lowest one), which became non-significant in 2005 (OR: 0.92).

The results were quite different in 2010. The gender effect reversed, as female respondents became less

Figure 2

Percentage of 18–75 year-olds who reported being 'very' or 'somewhat' unfavourable towards vaccination in general, INPES survey, France, October 2009–June 2010 (n=8,573)



INPES: French National Institute for Prevention and Health Education.
95% confidence intervals are represented by vertical segments.

likely to oppose vaccination in general (OR: 0.88). The age effect also changed, as opposition to vaccination proved more prevalent among respondents aged 50 to 64 years (instead of those aged 65 to 75 years in 2000 and 2005). Moreover, educational level and presence of children under the age of four years in the household became significant predictors of attitudes towards vaccination in general: the propensity to express unfavourable attitudes decreased as the educational level rose, and this propensity was also lower among respondents who had at least one child aged under the age of four years in their household. Finally, the income effect did not change: in 2010 as in 2000, wealthier people were less likely to oppose vaccination in general.

Attitudes towards vaccination and vaccination behaviours

In all three Health Barometers, among respondents who had at least one child aged one to 15 years in their household, attitudes towards vaccination in general were significantly correlated with their children's) immunisation status regarding the MMR vaccine (Table 2): those who endorsed unfavourable attitudes towards vaccination in general were more likely to report that they had at least one child who had not received the MMR vaccine (OR: 4.20 in 2000, 5.95 in 2005, 1.53 in 2010). In 2005 and 2010, respondents who were unfavourable towards vaccination in general were less likely to state that their vaccinations were up to date (OR: 0.27 and 0.41 respectively). Finally, in 2010, among respondents aged 65 years and older (French health authorities strongly recommend seasonal influenza vaccine for this age category), these unfavourable attitudes were negatively associated with vaccination against seasonal influenza in 2008 (OR: 0.13).

Discussion

Limits of the study

We have to acknowledge several limitations of the present study. First, our data might be biased, since a significant minority of contacted households/people refused to participate. These refusal rates were quite low, however, in comparison with similar telephone surveys, and we have no particular reason to suspect that vaccination attitudes and behaviours may have been correlated with refusal, as the letter announcing the survey did not detail the topics to be investigated. Secondly, our study shares the usual limitations of retrospective surveys based on self-reporting, including recall bias and social desirability bias, especially for vaccination behaviours. Thirdly, we do not know when unfavourable attitudes towards vaccination started to increase, as no data were collected before October

Table 1

Factors associated with unfavourable attitudes towards vaccination in general, INPES surveys, France 2000, 2005, 2010

	2000 (n=12,256)		2005 (n=23,931)		2010 (n=8,573)	
	Row %	OR	Row %	OR	Row %	OR
Gender						
Male (ref.)	7%	1	9%	1	39%	1
Female	10%***	1.27***	11%***	1.25***	37%*	0.88^{ns}
Age (years)						
18–24 (ref.)	5%	1	5%	1	27%	1
25–34	6%	1.19^{ns}	8%	1.81***	32%	1.49**
35–49	8%	1.68***	9%	2.01***	36%	1.54**
50–64	10%	2.22***	11%	2.47***	48%	2.45***
65–75	14%***	3.32***	14%***	3.25***	43%***	1.91***
Educational level						
No diploma (ref.)	11%	1	11%	1	48%	1
Below high-school graduation	9%	0.84^{ns}	10%	0.93^{ns}	42%	0.77*
High-school, first university degree	7%	0.95^{ns}	8%	0.94^{ns}	32%	0.60**
Three or four years completed at university	10%	1.28^{ns}	11%	1.16^{ns}	28%	0.49***
More than four years completed at university	6%***	0.78^{ns}	8%***	0.87^{ns}	23%***	0.37***
Equivalised household income						
<900 €/month (ref.)	9%	1	10%	1	40%	1
900–1,500 €/month	9%	0.97^{ns}	9%	0.90^{ns}	42%	1.06^{ns}
≥ 1,500 €/month	7%	0.79**	9%	0.92^{ns}	34%	0.85*
Don't know/refuse to answer	10%*	1.11^{ns}	11%*	1.13^{ns}	39%***	0.92^{ns}
Children under four years in the household						
None (ref.)	9%	1	10%	1	40%	1
At least one	7%*	1.13^{ns}	8%***	0.89^{ns}	28%***	0.72*

INPES: French National Institute for Prevention and Health Education; OR: adjusted odds ratio; Ref: reference category in logistic regression.

*** statistically significant at p<0.001; ** statistically significant at p<0.01; * statistically significant at p<0.05; ns not significant (Pearson's chi-square test for bivariate analysis, Wald's chi-square for logistic regressions).

The Table shows row percentages and adjusted odds ratios.

Table 2

Adjusted odds ratios measuring the impact of attitudes towards vaccination in general on vaccination behaviours, INPES surveys, France, 2000, 2005, 2010

Dependent variable	Year	OR [95% CI][a] unfavourable versus favourable
At least one child aged one to 15 years in the household did not get the MMR vaccine[b]	2000 2005 2010	4.20 [3.09–5.71] 5.95 [4.89–7.24] 1.53 [1.14–2.06]
Respondent's immunisation status up to date	2005 2010	0.27 [0.24–0.29] 0.41 [0.36–0.46]
Respondent aged 65 years or older and vaccinated against seasonal influenza in 2008	2010	0.13 [0.09–0.17]

CI: confidence interval; EHI: equivalised household income; INPES: French National Institute for Prevention and Health Education; OR: adjusted odds ratio.

[a] Odds ratios adjusted to gender, age, education and EHI levels, and presence of children under the age of four years in the household in a logistic regression.

[b] Among the subsample of respondents who had at least one child aged one to 15 years in their household.

2009. Finally, the comparison between data collected in 2000, 2005 and 2010 could have been biased since the corresponding sample sizes were quite heterogeneous. Such heterogeneity may induce lower levels of statistical significance for analyses conducted on the smaller samples (in this case the 2010 sample), but as the statistical relationships measured in 2010 were quite strong, we did not encounter this problem.

Impact of the 2009 influenza A(H1N1) pandemic on attitudes towards vaccination

Despite the third limitation mentioned above, our results strongly suggest that the 2009 influenza A(H1N1) episode had a dramatic impact on attitudes towards vaccination in general, at least among the French (hypothesis (i) confirmed). Beyond the increase in negative attitudes observed in 2009 to 2010, half of the respondents who endorsed these attitudes spontaneously mentioned their opposition to the influenza A(H1N1) vaccine. These attitudes reached a peak in December and January, when French health authorities and the World Health Organization began to be sharply criticised in the French media for exaggerating the influenza A(H1N1) threat. This attitudinal shift illustrates the proposition that many people who accept vaccines could change their mind [5], and it supports the hypothesis that the 2009 influenza A(H1N1) episode may have undermined public confidence in health authorities and vaccination [20]. In France, concerns about vaccine safety started to get media attention in November, but the level of negative attitudes towards vaccination in general had already reached 33% in October. Nevertheless, controversies regarding the seriousness of the pandemic threat and the massive purchase of vaccine began in July 2009. Consequently, the 2009 influenza A(H1N1) episode certainly contributed to the increase in negative attitudes towards vaccination in general, but it may not be the only cause. Furthermore, it is not possible to assess at this stage how long such negative attitudes will last.

Socioeconomic status and attitudes towards vaccination

The sociodemographic profile associated with unfavourable attitudes towards vaccination in general significantly changed in 2009/10 (hypothesis (ii) confirmed). The profile observed in 2009/10 also suggests a link between these attitudes and opposition to the influenza A(H1N1) vaccine. Indeed this profile was consistent with results from previous French studies that investigated factors associated with influenza A(H1N1) vaccine uptake or acceptance: Influenza A(H1N1) vaccination acceptance was found to be higher among more educated and wealthier people [21], its uptake was correlated with high educational level, high socioeconomic status and living in a household with a child under the age of five years [16].

In contrast, some previous studies found that highly educated parents were prone to refuse vaccination for their children [22-24]. However, these parents did not oppose vaccination in general, they rather want to balance the risks and benefits of each vaccination, and their hesitancy is often directed at specific vaccines [25,26]. More generally, studies on risk perceptions usually found that low socioeconomic status people are more sensitive to risks, especially for unfamiliar and controversial risks [27], and the 2009 influenza A(H1N1) episode may have contributed to shifting the perception of vaccine risks towards being more unfamiliar and controversial than they were perceived before. Of course, such change in risk perceptions may be temporary and reversible, at least partially, as suggested by the inverted 'U' shape in Figure 2.

Our results also suggest an increasing social differentiation of attitudes towards vaccination, as two usual markers of a low socioeconomic status, namely a low educational level and a low income level, became predictive of unfavourable attitudes in 2010. As trust in public health authorities is a key issue regarding vaccination acceptance in general and influenza A(H1N1) vaccination acceptance in particular [4-6,21,28], such disparities may result from the social differentiation of trust in health authorities and the pharmaceutical industry. This hypothesis is supported by a number of previous studies. For example, a German study dealing with information-seeking behaviour during the 2009 influenza A(H1N1) pandemic found that people with lower education were much less likely to use information material from official authorities [29]. An American study also found that people belonging to ethnic minorities (who often have a lower socioeconomic status) were more likely to distrust influenza vaccination, and this belief was associated with lower vaccination rates [30]. More generally, a low socioeconomic status is frequently associated with mistrust of health authorities [31]. The social differentiation of confidence in health authorities and vaccination programmes could significantly contribute to health inequalities in infectious diseases, which are public health priority [32].

Attitudes towards vaccination and vaccination behaviours

To our knowledge, only a few studies have investigated the potential impact of the 2009 influenza A(H1N1) pandemic on attitudes and behaviours. In a German study, a minority of healthcare workers stated that the pandemic had influenced their attitude towards vaccination in general [18]. A French study conducted in 2011 and based on self-reported data found no impact of the 2009 influenza pandemic on subsequent seasonal influenza vaccination coverage [19], but according to a later study (carried out in 2012), using data provided by the comprehensive social health insurance database, this coverage had decreased in 2010 [33].

In our study, attitudes towards vaccination and self-reported vaccination behaviours remained strongly correlated after adjustment on the respondents' sociodemographic background (hypothesis (iii) confirmed). Nevertheless, regarding children's MMR vaccination

status, the relationship was not as strong in 2010 (OR: 1.53 versus 4.20 in 2000 and 5.95 in 2005). As our data are cross-sectional, these relationships should be interpreted cautiously. Indeed, as the proportion of people who endorsed unfavourable attitudes towards vaccination greatly increased in 2010, we can expect that many of them reported vaccination behaviours (for themselves as for their children) that occurred several years before they changed their mind towards vaccination. Thus their behaviours were not necessarily determined by their attitudinal shift.

Nevertheless, our results showed that attitudes and behaviours are consistent with one another, and one could expect that this attitudinal shift may manifest in vaccination behaviours in coming years. Consequently, trends in children's immunisation should be carefully scrutinised in the next decade, as a significant proportion of future parents (27% of 18 to 24 year-old respondents, 32% of 25 to 34 year-olds) endorsed unfavourable attitudes towards vaccination in general in 2010.

Conclusions

In 2010, we observed a dramatic shift in the French population's attitudes towards vaccination in general: unfavourable attitudes have become far more frequent, and the corresponding sociodemographic profile has also changed. Such attitudes and sociodemographic profile should be closely monitored in the future, as this shift may either persist or vanish. Moreover, the 2009 influenza A(H1N1) pandemic certainly contributed to this upheaval. As attitudes and behaviours are generally consistent one with another, this phenomenon could have a considerable impact on future vaccination coverage. Consequently, health authorities should urgently address this increasing lack of confidence in vaccination.

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Conflict of interest

None declared.

Authors' contributions

CJ, AG and FB conceived and designed the surveys used in this article, PPW and AG conducted statistical analyses, PPW, PV, JR and AC contributed to the interpretation of data and wrote the first draft, and all authors revised the article critically and approved the final version.

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Plasmodium knowlesi infection imported to Germany, January 2013

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To the editor: We read with great interest the article by Orth et al. [1] in *Eurosurveillance* on a recent case of imported *Plasmodium knowlesi* infection in Germany. This case nicely illustrates the pivotal role of microscopy on thick and thin blood films by experienced microscopists for malaria diagnosis. The statement in the discussion section that only five cases imported to Europe have been published so far, underestimates the occurrence of this infection. Two more cases imported to the Netherlands have been described previously [2,3].

One case was a migrant worker from Malayan Borneo, positive in microscopy with 2% infected erythrocytes. The rapid BinaxNOW Malaria Test was positive for the pan-malarial aldolase band but negative for *P. falciparum* histidine-rich protein 2 (HRP-2). Retrospective analysis of the initial sample also showed positive results in the *P. falciparum*–specific lactate dehydrogenase (LDH) and pan-malarial LDH in the OptiMAL Rapid Malaria Test (DiaMed, Cressier, Switzerland). This patient was successfully treated with oral chloroquine for three days [2].

The other patient was a tourist who also visited Malayan Borneo and participated in a two-day jungle trek. At presentation, this case had a low parasitaemia (0.0005%) with microscopy and negative reactions for both HRP-2 and aldolase in the BinaxNOW Malaria Test. The patient was successfully treated with malarone, a combination of atovaquone and proguanil [3]. Both cases were confirmed as *P. knowlesi* infections by molecular methods after treatment had been started.

We agree with Orth et al that physicians should be aware of the possibility of imported *P. knowlesi* infections in travellers. This is particularly relevant, as *P. knowlesi* with its 24-hour replication cycle can result in a high parasitaemia and severe, life-threatening disease. It is safe to assume that the geographic range of *P. knowlesi* comprises all countries in south-east Asia, including the south of China.

Moreover, not only clinicians, but also laboratory personnel, traditionally only trained to identify the four more frequently observed *Plasmodium* species, *P. falciparum, P. vivax, P. ovale and P. malariae,* should be aware of this infection and its diagnostic challenges. *P. knowlesi* is morphologically very similar to *P. malariae* but can also be confused with P. *falciparum* in microscopy. As illustrated by the two cases described above, *P. knowlesi* infection causes variable results with commercially available rapid diagnostic tests, which do not seem to be reliable for diagnosis of *P. knowlesi* [3,4]. Although rapid diagnostic tests can complement microscopic diagnosis, they cannot replace microscopy, especially in patients with low parasite loads. For patients suspected of *P. knowlesi* infection, confirmation can be obtained either by specific PCR or by sequence analysis of generic *Plasmodium* PCR products, which are available in most specialised centres in Europe. While such confirmation is in progress, treatment should be installed based on positive blood smear results. From literature and our experience, it seems that oral treatment regimens suited for uncomplicated *P. malariae* and P. *falciparum* are also effective in clearing mild *P. knowlesi* infections, since resistance to antimalarial drugs has not been observed yet [5]. For more severe and complicated *P. knowlesi* infections, parenteral treatments associated with short parasite clearance times, such as artesunate, seem preferable.

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