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Multinational outbreak of Salmonella Enteritidis infection during an international youth ice hockey competition in Riga, Latvia, preliminary report, March and April 2015

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A multinational outbreak of salmonellosis linked to the Riga Cup 2015 junior ice-hockey competition was detected by the Finnish health authorities in mid-April and immediately notified at the European Union level. This prompted an international outbreak investigation supported by the European Centre for Disease Prevention and Control. As of 8 May 2015, seven countries have reported 214 confirmed and suspected cases, among which 122 from Finland. The search for the source of the outbreak is ongoing.

The event

On 14 April 2015, a general practitioner in a municipal public health authority in Finland notified the National Registry for Food and Waterborne Outbreaks (RYMY) [1] about a cluster of 30 cases of gastrointestinal disease. All had attended the Riga Cup on the 3 to 6 April weekend 2015 and six had laboratory-confirmed *Salmonella* serogroup D infection.

The Riga Cup is an annual international youth ice-hockey competition where mainly male teams, with occasional female players, participate. Players in the event are officially categorised in five age groups comprising individuals between 10 and 18 years-old. In 2015, the competition consisted of five tournaments lasting three to four days each, which took place over five consecutive weekends from 27 March to 26 April. The competition attracted 197 teams from Europe, including Belarus, Russia and Ukraine as well as 16 European Union and European Free Trade Association (EU/EFTA) countries. Finland contributed 50 teams. The total number of international and national participants was around 5,000. Tournaments were played according to age groups in four hockey arenas, which were used in parallel, with arena A being the main arena. A fifth arena was added on the last weekend of the tournament. Lunches and dinners for the teams were included in the competition package. The teams, including their male and female coaches and officials, were scheduled to have their meals at the arenas where they were playing. Breakfasts were taken at the hotels where the teams stayed.

Epidemiological and microbiological investigations in Finland

Following the notification of the first cluster of cases in mid-April, the Finnish National Institute for Health and Welfare (THL) monitored notifications reported to the National Infectious Disease Registry (NIDR) in order to capture *Salmonella* infections with a travel history to Latvia. Names were cross-checked against a list of the Finnish players in the Riga Cup obtained from the tournament website. Other cases of *Salmonella* sp. in NIDR with travel history to Latvia were contacted to determine whether they had participated in the tournament as an accompanying family member or official. Cases were identified according to the outbreak case definition (box).

Since this led to the finding of other possible clusters in different parts of Finland, THL invited on 21 April, the Finnish clubs participating in the Riga Cup 2015 to an online survey in order to describe and determine the magnitude of the outbreak. This survey asked about demographics, dates of participation in the competition and place of accommodation. Further, the respondents were requested to specify the tournament venues where they had eaten and on which dates, along with a description of what had been consumed. It was also

Box

Finnish case definition, multinational outbreak of *Salmonella* Enteritidis infection during an international youth ice hockey tournament in Riga, Latvia, March–April 2015

A confirmed case: any person with laboratory-confirmed infection with *Salmonella* sp. having participated in the Riga Cup 2015 between 27 March and 26 April 2015 and having had onset of diarrhoea with three or more loose stools a day on or after 27 March.

A suspected case: any person having participated in the Riga Cup 2015 between 27 March and 26 April 2015 and having had an onset of diarrhoea on or after 27 March.

enquired whether any foods and beverages had been taken outside the venues. Date of onset of diarrhoea with three or more loose stools a day, fever (≥38°C), headache, nausea, vomiting, or stomach ache were questioned as were any possible hospitalisations.

As of 8 May, 65 persons with *Salmonella* sp. infection, who had participated as players or officials in the Riga Cup 2015 between 27 March and 26 April 2015 and had onset of symptoms on or after 27 March were identified by the NIDR in Finland. By the same date, 315 persons had also responded to the online survey. Of these, 31 respondents were classified as confirmed, and were among the 65 confirmed cases identified by the NIDR. The survey additionally identified 57 suspected cases.

Of the 88 cases identified in the survey (Figure), 78 (87%) were male, the median age was 13 years (range: 8–66 years), 7 (8%) had been hospitalised, 86/87 (99%) had been playing at arena A and 39/88 (44%) participated during the tournament taking place over the second weekend of the competition.

Nine patient isolates were microbiologically characterised [2-4] at THL. The isolates were selected so that they represented clusters identified in different participating teams from different parts of Finland (Figure). All nine cases had been infected by *S.* Enteritidis phage type 1 (PT1) with multilocus variable-number tandem repeat analysis (MLVA) profile 3–10–6-4–1 (SENTR7-SENTR5-SENTR6-SENTR4-SE3). The isolates were susceptible to the standard panel of antibiotics tested (ampicillin, cefotaxime, chloramphenicol, gentamicin, mecillinam, meropenem, nalidixic acid, perfloxacin, streptomycin, sulfonamide, tetracycline, and trimethoprim). *S.* Enteritidis PT1 is rare in Finland as well as in Norway and Sweden (Lin Thorstensen Brandahl and Cecilia Jernberg, personal communications, May 2015), but common in Baltic countries, Russia and Korea [5,6].

International investigations

On 16 April, THL launched an urgent inquiry through the Epidemic Intelligence Information System for Food and Waterborne Diseases (EPIS-FWD) [7] to identify cases in other countries. Potentially affected non-EU countries were informed of the outbreak via the World Health Organization and were given access to EPIS-FWD.

On 24 April, a Rapid Risk Assessment of the outbreak by the European Centre for Disease Control and Prevention (ECDC) [8] was distributed through the Early Warning and Response System (EWRS). On the request from Latvia, ECDC deployed a fellow from the European Programme for Intervention Epidemiology Training (EPIET) from Finland to support the investigation in Riga on 26 April 2015.

On 29 April, the Latvian Centre for Disease Prevention and Control (LCDC) contacted the leaders of all teams participating in the Riga Cup, except the Finnish and Norwegian teams, which had already been contacted by their national public health authorities, by email and asked if they were aware of cases of gastroenteritis with onset during or after the tournament.

An ECDC expert on food and waterborne disease outbreaks joined the investigating team in Riga on 4 May 2015. As of 8 May, seven countries (Estonia, Finland, Hungary, Lithuania, Norway, Sweden, and the United Kingdom (UK)) have reported a total of 214 cases with gastrointestinal symptoms. Identical MLVA profiles to the Finnish cases were confirmed in one Norwegian and three Swedish patient isolates. The MLVAs were performed at the Department of Food-borne Infections at the Norwegian Institute of Public Health, and Public Health Agency of Sweden, respectively. The results were communicated through EPIS and at telephone conferences concerning the outbreak (Lin Thorstensen Brandahl and Cecilia Jernberg, personal communications, May 2015).

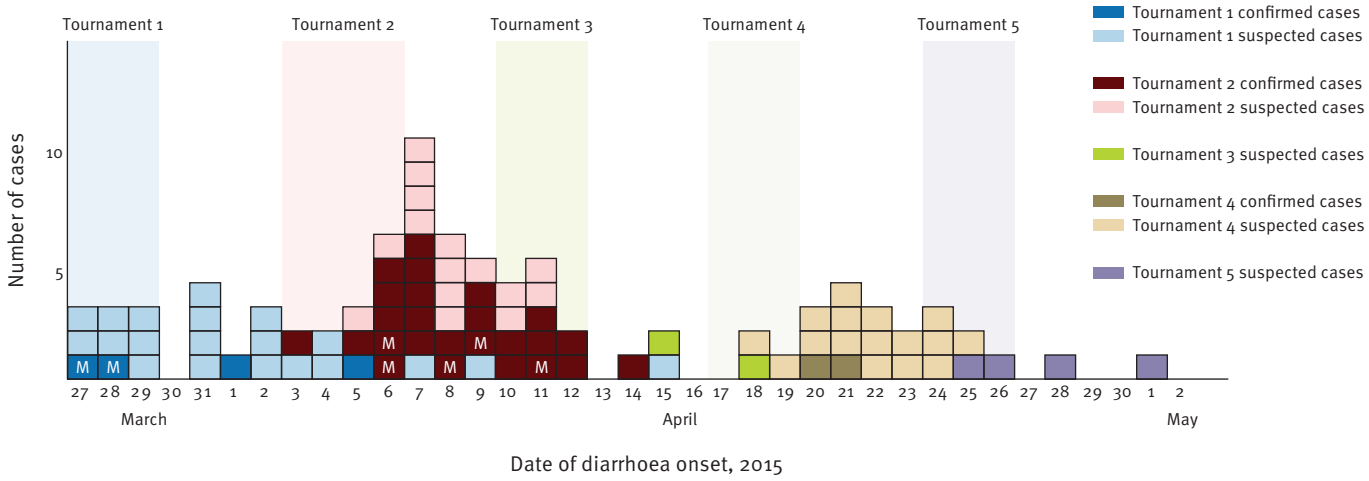
Food and environmental investigations in Latvia

Based on the information gained from the first detected cluster in Finland, the cafeteria at arena A was suspected as the source of outbreak [8]. On 16 April, the THL and the Finnish Food Safety Authority, Evira, alerted their respective counterparts in Latvia through the EWRS [9] and the Rapid Alert System for Food and Feed [10]. On the same day, the LCDC in collaboration with the Latvian Food and Veterinary services inspected the kitchen at arena A, interviewed all kitchen staff and their family doctors, and collected faecal samples and environmental and food samples. None of the kitchen staff reported gastrointestinal symptoms during the Riga Cup and all the samples were negative for *S.* Enteritidis. No cases of *S.* Enteritidis related to Riga Cup were registered in Latvia and the number of *S.* Enteritidis cases did not exceed the average level in March and April 2015.

Follow-up controls took place at arena A on 24 and 27 April. Members of one team playing at the tournament

Figure

Number of cases by date of onset of diarrhoea among participants attending an international youth ice hockey competition in Riga, Latvia, March–April 2015 (n=88)



MLVA: multilocus variable-number tandem repeat analysis

The letter M indicates cases that participated in the online survey related to the Finnish epidemiological investigation, and whose isolates were microbiologically characterised by MLVA.

on the second weekend of the competition, with five suspected cases, had only eaten their meals in arena B, so arena B was inspected on 29 April. Menus were retrieved from all arenas and wholesale suppliers were identified via the purchase receipts. The arena restaurants had been contracted by the organiser and advised to serve the same lunch and dinner menus to the participating teams. The meals were mainly prepared at the tournament arenas. The caterers at the venues served the team members a separate menu than the public during the weeks in between the tournament weekends. Leftovers of the food served to the teams were discarded at the end of each day. Latvian teams did not take lunch and dinner at the venues.

Control measures

Physicians in Finland were alerted about the outbreak by news bulletins (on 15, 20 and 30 April) [11]. The Finnish ice-hockey teams scheduled to play on the last tournament weekend were prior advised to take standard hand hygiene and food safety precautions, such as eating only cooked foods and drinking bottled beverages.

In Latvia, two other junior ice hockey events took place in Riga, from 28 April to 2 May 2015 and from 14 to 16 May 2015 respectively. As some Finnish teams were to take part in these two events, the Finnish public health authorities informed these teams and the Finnish Ice Hockey Association on 27 April about the Riga Cup outbreak and ongoing investigation. Standard hand hygiene and food safety precautions were recommended and participants were also advised to notify THL in case gastrointestinal symptoms appeared during or after the competition.

On 30 April and 8 May the Latvian public health authorities contacted the organisers of both events and recommended to consume only well-cooked foods and maintain proper hand hygiene. In addition, a phone number to a specialist from the Latvian public health authority was provided in case any of the event participants would develop gastroenteritis symptoms.

Conclusions

This outbreak was first detected by municipal public health authorities in Finland. Due to budget cuts at THL since 2015 *Salmonella* isolates of foreign origin are no longer typed nor are the cases monitored in real time and outbreaks of foreign origin can only be identified at a local level. This will delay detection of *Salmonella* outbreaks among travellers. Cross-border outbreaks and outbreaks related to mass gatherings pose special problems [12-15] and cooperation between national authorities and ECDC is needed. The investigation to determine the source of the outbreak is ongoing and a retrospective cohort study among event participants from several EU/EFTA countries is underway in order to identify the source.

Acknowledgments

We would like to thank experts from THL, The Centre for Disease Prevention and Control of Latvia, Latvian Food and Veterinary Services, national focal points of affected countries and ECDC which are continuously contributing to the outbreak investigation.

Conflict of interest

None declared.

Authors' contributions

Anne-Katrine Pesola, Triin Pärn, Sari Huusko, Jurijs Perevoščikovs, Jukka Ollgren, Saara Salmenlinna, Taru Lienemann, Celine M. Gossner, Niklas Danielsson and Ruska Rimhanen-Finne reviewed and approved the manuscript. Anne-Katrine Pesola acted as outbreak coordinator, Anne-Katrine Pesola, Triin Pärn, Sari Huusko, Jurijs Perevoščikovs, Jukka Ollgren, Celine M. Gossner, Niklas Danielsson and Ruska Rimhanen-Finne carried out the epidemiological investigation. Saara Salmenlinna and Taru Lienemann, carried out the microbiological investigation, Anne-Katrine Pesola, Triin Pärn and Ruska Rimhanen-Finne drafted the manuscript and data analysis. All co-authors critically reviewed the draft of the paper and approved the final version.

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Genome sequence analysis of Ebola virus in clinical samples from three British healthcare workers, August 2014 to March 2015

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We determined complete viral genome sequences from three British healthcare workers infected with Ebola virus (EBOV) in Sierra Leone, directly from clinical samples. These sequences closely resemble those previously observed in the current Ebola virus disease outbreak in West Africa, with glycoprotein and polymerase genes showing the most sequence variation. Our data indicate that current PCR diagnostic assays remain suitable for detection of EBOV in this epidemic and provide confidence for their continued use in diagnosis.

Monitoring of the evolution of the viral genome during the ongoing outbreak of Ebola virus disease (EVD) in West Africa is crucial for the early detection of mutants that may evade sequence-based diagnostics and for monitoring efficacy of therapeutic options. We present here our analysis of Ebola virus (EBOV) sequences obtained from blood samples from three British healthcare workers (HCWs) who were infected with EBOV in Sierra Leone.

Assessing sequence variation in Ebola virus

Between August 2014 and March 2015, three HCWs (Cases 1, 2 and 3) from the United Kingdom (UK) were infected with EBOV (Ebola virus/H.sapiens-wt/GBR/2014/Makona-UK1, Ebola virus/H.sapiens-wt/GBR/2014/Makona-UK2 and Ebola virus/H.sapiens-wt/GBR/2014/Makona-UK3, respectively; hereafter referred to as UK1, UK2 and UK3) in Sierra Leone.

Two were repatriated from Sierra Leone and the third became symptomatic upon return to the UK. All were transferred to the specialist isolation ward at the Royal Free Hospital in London, where they subsequently recovered. Informed consent was sought and received from each of the patients for viral whole genome sequencing and publication of the findings.

Viral genomes from pre-intervention whole blood and EDTA plasma samples were sequenced and analysed to provide a baseline for any subsequent transmission of EBOV in the UK and to identify and monitor mutations that may affect the sensitivity of treatment and diagnostics (Table 1).

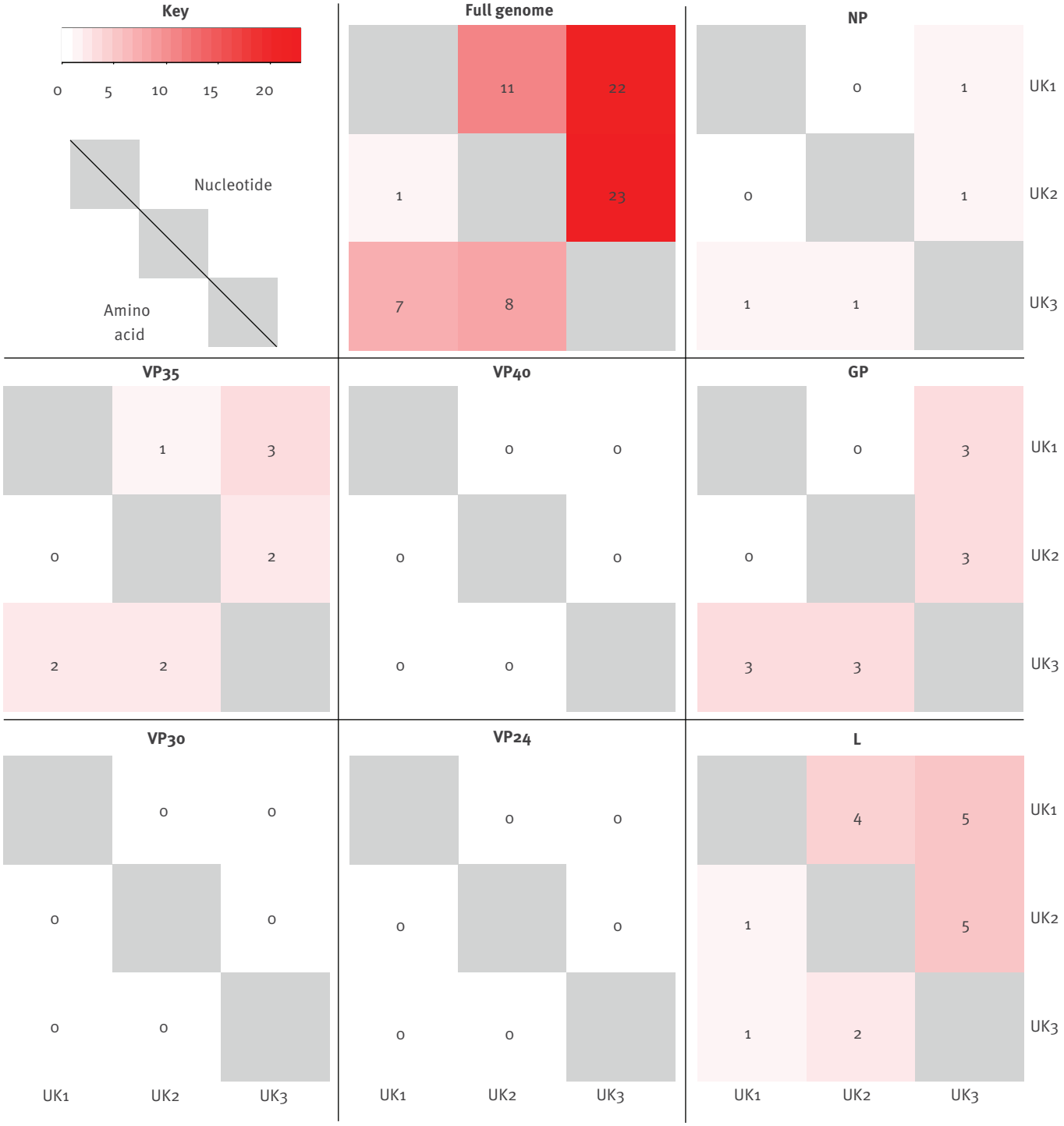
Sequence analysis

RNA was extracted from patient samples using the EZ1 RNA Universal Tissue Kit (QIAgen). Confirmation of EVD diagnosis in all three patients was performed using PCR assays targeting the NP gene [1]. Samples for sequencing were treated with DNase I (Life Technologies) and purified using an RNA Clean and Concentrator kit (Zymo). Single primer isothermal linear amplification (SPIA) cDNA was prepared from total RNA following the Ovation RNA-seq V2 (NuGens) protocol [2], with the exception that RNA was denatured for 5 min at 85 °C before first-strand synthesis. Samples were purified using a MinElute column (QIAgen). Following amplification, paired-end libraries were prepared for Illuminia MiSeq sequencing following the Nextra XT protocol using 1.5 ng of SPIA cDNA. Reads were trimmed to a minimum of Q30. Genomes were mapped to KM233113.1 using BWA 0.7.5 and consensus called with Quasibam 1.0 using a local instance of The Galaxy Project [3-5]. Consensus sequences were produced at a minimum depth of five reads and single nucleotide polymorphisms (SNPs) at a minimum depth of 20. Ambiguous bases were included when present in 20% of reads.

Full viral genome sequences were obtained from samples from all three infected HCWs patient samples and were submitted to GenBank (accession numbers are listed in Table 1). Sequence analysis showed that

Figure 1

Heatmaps showing nucleotide and amino acid variation between three Ebola virus isolates from three British healthcare workers infected in Sierra Leone, August 2014–March 2015



GP: glycoprotein; L: viral polymerase; NP: nucleoprotein; VP: virion protein.

Heatmaps showing the number of nucleotide and amino acid substitutions between Ebola virus isolates from three British healthcare workers infected in Sierra Leone, August 2014, December 2014 and March 2015, across the whole genome and in individual genes. Each heatmap is split in two diagonally, with the top right showing nucleotide changes and the bottom left amino acid differences between the three isolates. The number of differences are displayed in each box.

across the length of the EBOV genome, UK3 showed the most nucleotide variation (22 and 23 SNPs), but no insertions or deletions, compared with UK1 and UK2, respectively (Figure 1). These gave rise to seven and eight amino acid changes, respectively.

No nucleotide changes within the open reading frames (ORFs) for the virion protein (VP) 40, VP30 and VP24 genes were observed. Within the coding region for the nucleoprotein (NP) gene, no SNPs were seen between UK1 and UK2, although UK3 showed one non-synonymous SNP (P to S at position 1,957). One synonymous

Table 1

Sample details from three British healthcare workers with Ebola virus disease infected in Sierra Leone, August 2014–March 2015

Case	Medical centre worked at in Sierra Leone	Date sampled	Sample type	Genome length (bp)	Isolate name	GenBank accession number
1	Kenema Government Hospital	26 Aug 2014	Whole blood	18,920	Makona-UK1	KP120616
2	Kerry Town treatment centre	29 Dec 2014	EDTA plasma	18,929	Makona-UK2	KP658432
3	Kerry Town treatment centre	12 Mar 2015	EDTA plasma	18,921	Makona-UK3	KR025228

bp: base pairs.

SNP was seen between UK1 and UK2 in the VP35 ORF, while UK3 showed two non-synonymous SNPs to UK1 and UK2 (S to R at position 3,371 and E to G at position 3,380).

The GP gene showed no SNPs between UK1 and UK2, and three non-synonymous SNPs from UK3 to UK1 and UK2 (R to K at position 6,932, R to S at 7,265 and L to E at 7,352). The most SNPs within an ORF were found to be in the viral polymerase (L) gene, with UK1 and UK2 showing four nucleotide changes, and UK3 showing five changes in respect to UK1 and UK2. These SNPs total less than one third of SNPs found, for a gene that comprises 36% of the total genome. These data suggest that the L gene is conserved, with only two non-synonymous SNPs. One amino acid change is seen from UK2 to UK1 and UK3 (A to T at 17,848) and one amino acid change from UK3 to UK1 and UK2 (T to A at 16,894) (combined, UK3 differs in one position from UK1 and two positions from UK2).

A phylogenetic tree based on sequences from the three UK samples and all available published sequences was generated using a heuristic maximum likelihood algorithm (Figure 2). Analysis shows that the three UK sequences fall within one large Sierra Leonean clade, with UK2 and UK3 in a different subclade from UK1. UK3 appears to share a common ancestor with the group that UK2 sits within. Sequences from Mali and Liberia form a distinct outgroup from the Sierra Leonean clade.

Discussion

The ongoing EVD outbreak in West Africa is the largest known, with over 25,000 recorded cases up until April 2015 [6]. In response to the outbreak, a large number of international civilian and military aid teams have been deployed alongside local workers at multiple treatment and diagnosis centres in Guinea, Sierra Leone and Liberia. Over 860 HCWs are known to have been infected [6]. Monitoring of the evolution of the viral genome during outbreaks is crucial for the early detection of mutations that may have an impact on disease virulence or transmissibility or affect the sensitivity of sequence-based viral genome detection assays in widespread use. The high viral loads seen in individuals infected with Ebola virus shortly after symptom onset favours the development of whole genome sequencing using next generation sequencing. More than 450 EBOV genome sequences derived using whole genome sequencing have been reported from samples isolated in Guinea, Sierra Leone, Mali and Liberia [7-9]. Analysis of 78 genomes isolated from samples from patients in Sierra Leone between May and June 2014 suggested an observed evolutionary rate double that seen in previous EVD outbreaks [10]. The importance of tracking sequence variation in relation to molecular detection strategies was highlighted in that analysis. More recent analysis, however, identified an observed evolutionary rate equivalent to that of past outbreaks [11].

In our study presented here, sequence analysis of the NP gene, the target for widely used diagnostic detection assays [1], identified no SNPs within the regions where diagnostic primers bind. The GP gene product is the viral receptor, and the target of neutralising antibodies. Synonymous SNPs are present in locations where primers and probe bind for real-time detection methodologies based on the GP gene [1] (Table 2).

The observation of SNPs within the primer/probe binding sites of the GP gene is consistent with other sequences obtained from this outbreak in West Africa (data not shown). These SNPs are not expected to affect primer binding, although this is yet to be formally determined, but this reinforces the necessity of regular review of diagnostic detection strategies

Table 2

Ebola virus real-time PCR assay primers and probes designed by Trombley et al. [1]

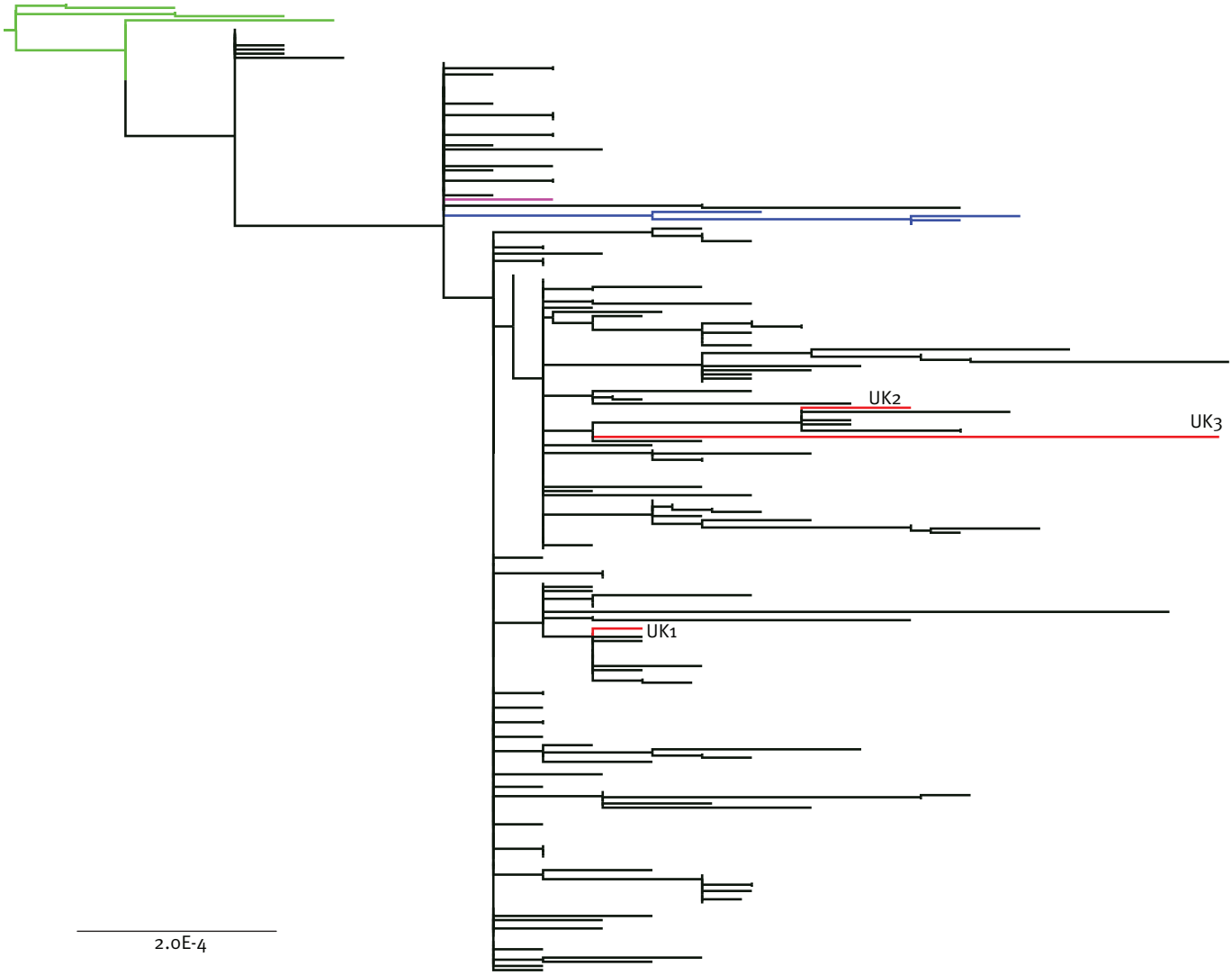
Gene target	Primer/ probe name	Sequence[a]	Base change
NP	F565	TCTGACATGGATTACCACAAGATC	None
	R640	GGATGACTCTTTGCCGAACAATC	None
	p597S	6FAM-AGGTCTGTCCGTTCAA-MGBNFQ	None
GP	F2000	TTT TCA ATCCTCAACCGTAAGGC	None
	R2079	CAG TCC GGT CCC A**G**A ATG TG	G to A
	p2058A	6FAM-CAT GTG CC**G** CCC CAT CGC TGC-TAMRA	G to A

GP: glycoprotein; NP: nucleoprotein.

[a] Single nucleotide polymorphisms in Ebola virus sequences from three patients from the United Kingdom infected in Sierra Leone are shown in bold.

Figure 2

Phylogenetic subtree of 233 near full-length Ebola virus genomes from the West African outbreak that started in 2014



The subtree is taken from a larger tree containing 258 sequences, which includes sequences from earlier outbreaks of Ebola virus disease.

Ebola virus genomes from patients outside the United Kingdom (UK) were obtained from GenBank (n = 255). The tree was generated using a heuristic maximum likelihood algorithm (FastTree – version 2.1.8) and the HKY model of nucleotide substitution.

The position of sequences from three patients repatriated to the UK from Sierra Leone are shown in red and labelled (UK 1–3). Sequences from patients in Guinea are shown in green, those from Mali are shown in blue and those from Liberia in purple. The remaining sequences (black) are from patients in Sierra Leone.

against available sequence information. A recent analysis of sequences from nine EBOVs from Mali and other available sequences also indicated no effect of SNPs on PCR-based detection assays [12,13].

Cases 2 and 3 from whom UK2 and UK3 were obtained, respectively, worked at the same treatment centre before infection and this is reflected in the close nature of the isolates' phylogeny. The patient from whom UK1 was obtained worked elsewhere: the UK1 sequence more closely resembles those reported by Gire et al. [10], who sampled from the same location.

During the intensive and widespread EVD epidemic in West Africa, the evolution of EBOV in Sierra Leone has been driven through person-to-person transmission in community settings, with a high number of HCW infections. HCW infections are less likely, because of rapid ascertainment through strict infection control and health monitoring, to lead to further transmission events. Currently, widely and increasingly used diagnostic detection strategies based on the NP gene have remained suitable for use. Molecular detection strategies based on the GP gene require close attention to ensure that SNPs occurring in this gene, perhaps as a result of host selective pressure, are evaluated for their impact on detection strategies. Viral sequences from any further cases of EVD in UK nationals or those imported into the UK will continue to be sequenced and analysed to ensure continued effectiveness of EVD diagnosis and monitoring of viral genome evolution.

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Conflict of interest

None declared.

Authors' contributions

Andrew Bell - planned experiments, sample preparation, sequence analysis, wrote the manuscript. Kuiama Lewandowski - planned experiments, sample preparation, sequence analysis, wrote the manuscript. Richard Myers - sequence and phylogenetic analysis. David Wooldridge - sequence library preparation. Emma Aarons -clinical input. Andrew Simpson - clinical input. Richard Vipond - scientific management. Michael Jacobs - lead clinician. Saheer Gharbia - conceived study and scientific management of samples. Maria Zambon - conceived and coordinated the study of sequence comparison of the three UK clinical cases, clinical input, manuscript preparation.
Andrew Bell and Kuiama Lewandowski contributed equally and are joint first authors.

Authors' correction

M Jacobs was inadvertently left out of the author list. This was corrected on 22 May 2015 at the request of the authors.

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Post-vaccine measles in a child with concomitant influenza, Sicily, Italy, March 2015

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We describe the occurrence of measles in an 18 month-old patient in Sicily, Italy, in March 2015, who received the first dose of a measles-containing vaccine seven days before onset of prodromal symptoms. Measles virus infection was confirmed by PCR and detection of specific immunoglobulin; viral genotyping permitted the confirmation of a vaccine-associated illness. The patient had a concurrent influenza virus infection, during a seasonal epidemic outbreak of influenza.

Case description

In early March 2015, measles-mumps-rubella-varicella zoster (MMRV) vaccine was administered to an apparently healthy 18-month-old child living in Sicily, Italy. Seven days later, the child presented to the family paediatrician with fever (40.1°C), catarrhal cough, runny nose and eyelid oedema. Macular rash appeared over the body two days later, starting on the trunk and then spreading to the neck and face. By day 13, the rash was fading, but due to the persistence of symptoms, the child was admitted to a children's hospital and reported as a possible case of vaccine-related measles to the Epidemiology Department of the Regional Public Health.

The local health authority carried out an epidemiological investigation: a standard measles notification form was sent to the regional health authorities and immediately forwarded to the Ministry of Health and to the Infectious Diseases Epidemiology Unit of the National Institute of Health. No direct link was identified with other measles cases in the community and the family had no history of travel outside Sicily. Moreover, contact investigation revealed no household members or pre-school contacts with symptoms consistent with measles. One of the child's parents developed influenza-like illness (ILI) symptoms (fever (›38°C) and cough, which lasted for three consecutive days) one day after administration of MMRV vaccine to the patient.

Urine and throat swab specimens were collected from the child and submitted to the Regional Reference Laboratory in Palermo for nucleic acid-based testing for measles, mumps, rubella and varicella zoster viruses and genotyping of any detected viruses. Given that this patient with suspected vaccine-associated measles developed symptoms during a seasonal epidemic outbreak of influenza viruses, and taking into account reports of morbilliform rash associated in patients with influenza B who tested negative for measles virus infection [1,2], testing was also requested for influenza and other respiratory viruses.

While no viruses could be detected in the urine specimen, measles, influenza A(H3N2) and respiratory syncytial viruses were detected in the throat swab.

On day 17, the patient's symptoms resolved without complications and the patient was discharged from hospital (Figure).

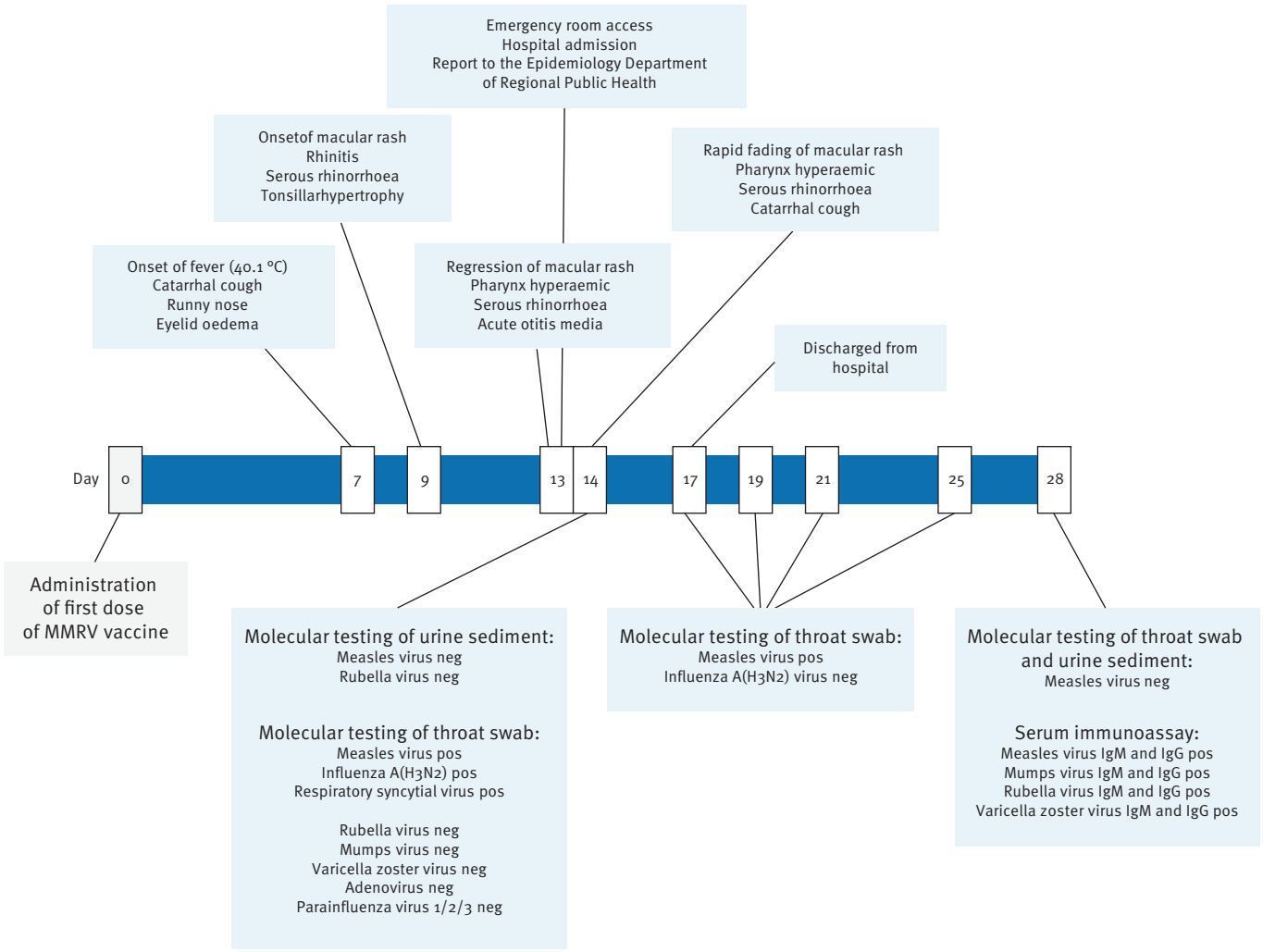
Measles virus was detected in throat swabs taken on days 17, 19, 21 and 25, but no influenza or other respiratory viruses were detectable in these specimens.

Measles virus was not detected on day 28 from a throat swab and urine specimen. A blood sample was taken at this time for serological testing for measles, mumps, rubella and varicella zoster viruses. A time line of events is shown in the Figure.

Seroconversion following MMRV immunisation was evaluated through the detection of specific measles, rubella, mumps and varicella zoster IgM and IgG antibodies by chemiluminescent immunoassay (CLIA) (measles virus: IgM = 3.1 arbitrary units (AU)/mL, IgG ›300

Figure

Time line of symptoms and physical signs in a child with post-vaccine measles and concomitant influenza, case management, specimen collection and laboratory results, Sicily, Italy, March 2015



MMRV: measles-mumps-rubella-varicella zoster; neg: negative; pos: positive.

AU/mL; mumps virus: IgM = 1.3 AU/mL, IgG = 78.9 AU/mL; rubella virus: IgM = 1.97 AU/mL, IgG = 18.0 international units (IU)/mL; varicella zoster: IgM = 0.71 AU/mL, IgG = 271.8 mIU/mL).

The measles virus was determined to be the Schwarz vaccine strain, genotype A, MVs/Palermo.ITA/12.15 [A] (VAC) [3] by sequence analysis of the genome.

Laboratory investigations

Serological and nucleic acid-based tests were performed for surveillance of measles and rubella, and genotype determination at the Regional Reference Laboratory of Palermo, formerly a member of the national network for influenza surveillance and genotyping (INFLUNET).

For the detection of specific measles, rubella, mumps and varicella zoster IgM and IgG antibodies, commercial CLIA tests were used (LIAISON (DiaSorin) and VITROS (Ortho Clinical Diagnostics)), which have the following cut-off values: measles IgM ≥ 1.0; measles IgG ≥ 13.5; mumps IgM ≥ 1.0; mumps IgG ≥ 10.0; rubella IgM ≥ 1.2; rubella IgG ≥ 15.0; varicella zoster IgM ≥ 1.0; varicella zoster IgG ≥ 100.0.

Throat swabs and the sediment of urine samples were tested using a real-time PCR instrument (QuantStudio 7 Flex Real-Time PCR system, Applied Biosystems), using specific primer/TaqMan probe sets for measles [4], mumps [5], rubella [4] and varicella zoster [6,7] viruses after extraction of total RNA using QIAmp Viral RNA Mini Kit (Qiagen).

Measles genotyping was conducted to distinguish wild-type from vaccine-associated measles viral strains. PCR products, targeting either the N gene or the H gene [8], were obtained from throat swab and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

SuperScript One-Step RT-PCR kit with Platinum Taq (Invitrogen) were used for both endpoint reverse transcription RT-PCR and real-time RT-PCR reactions.

Sequences were confirmed as measles virus following comparison with the BLAST algorithm and they were phylogenetically analysed to assign genotype and cluster. The sequences were identified as Schwarz vaccine strain (genotype A) and were submitted to GenBank (accession numbers KR262162 (gene N) and KR262161 (gene H)).

Background

In Italy, vaccination against measles is included in the national vaccination schedule. Two doses of measles-mumps-rubella (MMR) vaccine have been recommended in all regions since the early 1990s [9], sometimes in association with varicella vaccination. The first dose is given at 13–15 months-old and the second at the age of 5–6 years [10].

In accordance with the national measles elimination plan [11], an enhanced surveillance system was introduced in 2007 [12] with the aim of improving timeliness, completeness of case reporting and case investigation, including laboratory confirmation of diagnosis and viral genotyping.

As the incidence of wild-type measles decreases in countries with high levels of vaccination coverage, vaccine-associated cases could be misreported [13,14], suggesting that there is a need to improve the ability to distinguish between vaccine-associated measles and 'true' wild-type measles virus infection [15].

Post-marketing surveillance of vaccines is mandatory in Italy and adverse reactions observed after the administration of vaccines are reported through the national pharmacovigilance network. According to the latest data available [16], these are mainly represented by fever, skin rash and febrile seizures, while post-vaccination viral shedding is a very uncommon event, which has been rarely documented so far [17,18].

Discussion

With an estimated more than 500 million doses administered in over 60 countries since the 1970s, the benefit of measles vaccination in preventing illness, disability and death appear unchallengeable [19,20].

Moreover, vaccine safety is annually validated by accurate post-marketing surveillance of adverse reactions conducted by the Italian Medicines Agency (AIFA). As for other live attenuated vaccines, adverse reactions following MMR or MMRV immunisation rarely present with clinically significant illness [16]: such illness is indistinguishable from wild-type measles. In this context, the reference laboratory for molecular surveillance plays a fundamental role in measles virus characterisation, through viral sequencing and genotyping, in order to promptly differentiate between wild-type and vaccine-related strains [14,18].

In this report, we documented the pharyngeal excretion of the Schwarz measles vaccine virus in an apparently healthy child with a febrile rash after measles vaccination and with laboratory-confirmed influenza A(H3N2) coinfection.

On the basis of our data, some points can be noted.

Firstly, although unlikely, measles after MMRV vaccination is possible, and this can mimic wild-type infection, leading to potential measles case misclassification. The application of molecular techniques for viral genotyping is helpful to correctly classify a case and to drive the decisions of public health authorities at the local level.

Secondly, this is the first report of a measles case with concurrent influenza and respiratory syncytial virus detection: we cannot exclude the possibility that the co-presence of other viral natural infections in a very young child, showing a slight hypogammaglobulinaemia in serum protein electrophoresis, may have favoured, or even determined, the occurrence of vaccine-related measles virus in pharyngeal secretions. Unfortunately, the parent showing ILI symptoms was not tested for influenza virus, making us unable to assess, although very likely, an intrafamilial transmission of influenza virus infection.

Notably, virus excretion was demonstrated over a 25-day period after vaccination, which is longer than previously reported [17,21,22]. Interference with other coinfecting viruses or a defective host immune response could play a role in this unexpected persistence of measles virus, although this hypothesis will require further investigation.

Thirdly, virus excretion was repeatedly detected in the throat, but not in urine sediment. This finding partially contrasts with World Health Organization (WHO) guidance for laboratory diagnosis for measles virus infection, which suggests to test preferentially for the virus in the sediment of urine samples that have been collected within at least five days after the onset of rash [23]. In the case presented here, in accordance with WHO guidance, matched urine and throat specimens were collected on the fifth day after the onset of macular rash.

Detection of measles virus in respiratory samples up to 16 days after the onset of rash suggests that other host cell pathways or viral mechanisms, potentially related to other concomitant viral infections, might be responsible for such an event. However, also in this case, further studies are necessary to better explain such an anomaly.

In conclusion, development of measles in individuals who have received MMR or MMRV vaccine is a possible,

although extremely rare, event. Therefore, especially in geographical areas with a low incidence of measles, maintenance of efficient molecular surveillance systems and the improvement of the timeliness of both case reporting and virus genotyping is of paramount importance, to ensure correct differentiation between vaccine-related illness and natural measles infection [24].

Conflict of interest

None declared.

Authors' contributions

Conceived and designed the study: FT, FV. Collected clinical and epidemiological data: PD, CD, NC. Analysed data: FT. Wrote the paper: FT, FV.

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Genetic diversity of highly pathogenic H5N8 avian influenza viruses at a single overwintering site of migratory birds in Japan, 2014/15

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We isolated eight highly pathogenic H5N8 avian influenza viruses (H5N8 HPAIVs) in the 2014/15 winter season at an overwintering site of migratory birds in Japan. Genetic analyses revealed that these isolates were divided into three groups, indicating the co-circulation of three genetic groups of H5N8 HPAIV among these migratory birds. These results also imply the possibility of global redistribution of the H5N8 HPAIVs via the migration of these birds next winter.

In January 2014, newly discovered highly pathogenic H5N8 avian influenza viruses (H5N8 HPAIVs) caused outbreaks in poultry and wild birds in South Korea [1], although their ancestor had been isolated in China in 2013 [2]. Thereafter, these viruses have been circulating in both avian populations in South Korea [3,4] and sporadically in neighbouring countries, including China and Japan. Since November 2014, H5N8 HPAIVs have also appeared in poultry and wild birds in Europe [5,6]. Genetic analyses revealed that these isolates were closely related to the H5N8 viruses circulating in Korean birds. More recently, genetically similar HPAIVs also caused outbreaks in various avian species in North America [7]. These findings suggest that the H5N8 viruses have circulated and evolved in migratory birds.

Characteristics of the study area

The Izumi plain, which is located at the southern tip of Japan's mainland, is a major overwintering site of the white-naped crane (*Grus vipio*) and hooded crane (*Grus monacha*), both of which are categorised as vulnerable species on the International Union for Conservation of Nature Red List (Figure 1).

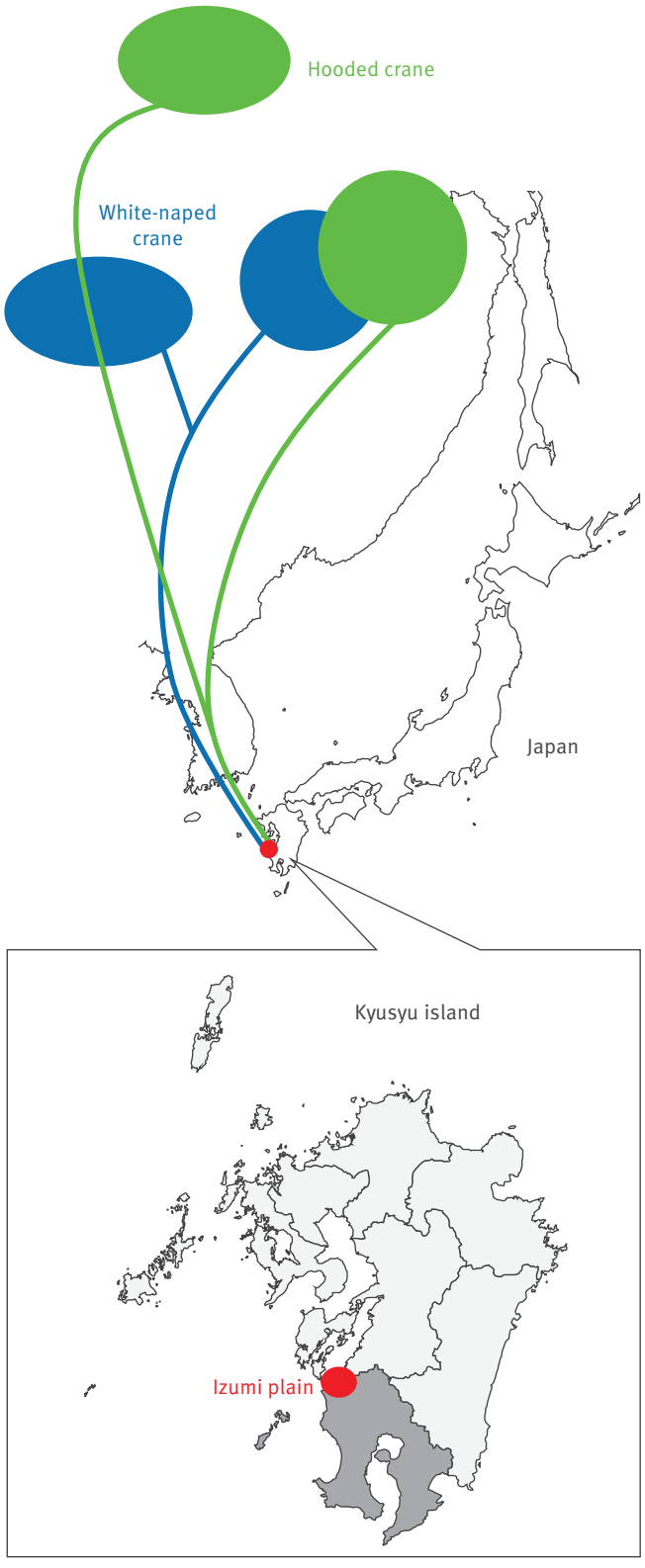
Over 10,000 cranes visit this plain in the winter season (arriving around November to December and leaving around February to March). For the purpose of protecting these endangered bird species, the local government creates artificial wet paddy areas for roosting cranes every winter. In addition to the cranes, many other migratory birds including wild ducks, a natural reservoir of influenza A viruses [8], also overwinter at this plain and share the wet paddies. Avian influenza viruses are therefore likely to be transmitted among the migratory birds, including the endangered cranes, at the Izumi plain. In fact, H5N1 HPAIVs were isolated from seven dead cranes in the 2010/11 winter season [9]. We have also isolated low pathogenic avian influenza viruses from duck faeces and the cranes' roost water collected at this area over the last two winter seasons (data not shown).

Influenza isolates from birds in the study area

On 23 November 2014, a debilitated white-naped crane was captured at the Izumi plain. Tracheal and cloacal swabs were collected and subjected to RNA extraction for the detection of influenza A viral genes and inoculation into embryonated chicken eggs for virus isolation. Influenza A viral M gene was detected in the RNA from the tracheal swab by conventional reverse transcription PCR. The allantoic fluids of the inoculated eggs showed haemagglutination activity. Further genetic analyses of the allantoic fluid revealed that the isolate was the H5N8 subtype influenza A virus. The infected white-naped crane died on 29 November 2014; investigations are under way into the cause of death. The partial sequence of the haemagglutinin (HA) gene revealed that the isolate encoded RERRRKR↓G at the

Figure 1

Map of crane flyways around the Izumi plain, Japan, 2014/15



The location of the Izumi plain is indicated in red circles. Breeding grounds (circles) and flyways (lines) of the white-naped crane (blue) and hooded crane (green) are also shown

HA cleavage sites, suggesting their potential to cause systemic infection, subsequently leading to high pathogenicity.

Since 23 November 2014 when the infected crane was found, wild birds within a 10 km radius of the point where the infected crane was found have been placed under active surveillance for HPAIVs. Local government staff searched for sick and dead wild birds in the area, and sent us the swab specimens and/or dead bodies to test for avian influenza viruses. As of 21 March 2015, eight H5N8 HPAIVs have been isolated from six debilitated or dead cranes, two dead mallard ducks (*Anas platyrhynchos*) and a water sample collected from the cranes' roost at the Izumi plain (Table 1). These virus isolations were reported in a timely manner to the World Organisation for Animal Health via the Ministry of Agriculture, Forestry and Fisheries of Japan [10].

To genetically characterise these H5N8 HPAIV isolates, we determined the complete genome sequences of the eight H5N8 HPAIV isolates, and deposited the sequences in the Global Initiative on Sharing Avian Influenza Data (GISAID) database (Table 2). Overall sequence data show that each gene segment of these isolates was genetically similar to the counterpart H5N8 HPAIVs recently isolated elsewhere in the world, suggesting that these isolates had not experience gene reassortment since their parental viruses caused outbreaks in South Korea in early 2014.

Phylogenetic analysis

To understand the genetic relationship between our isolates and related viruses, the HA and neuraminidase (NA) genes were phylogenetically analysed with counterparts from the representative avian influenza H5 (Figure 2A) and N8 (Figure 2B) subtypes, respectively. We found that the H5 genes from our eight isolates belonged to clade 2.3.4.4 and were genetically divided into three groups. The water isolate, A/environment/Kagoshima/KU-ngr-H/2014(H5N8), fell into a phylogenetic cluster together with the European isolates and was closely related to two wild duck isolates in Japan (Group A, indicated in green in the Figures). The first and second crane isolates, A/crane/Kagoshima/KU1/2014(H5N8) and A/crane/Kagoshima/KU13/2014(H5N8), were genetically similar to the North American isolates (Group B, blue in the Figures). The HA genes of the rest of our isolates (Group C, red in the Figures), as well as a poultry isolate from Japan were clearly distinct from those of the other recent H5N8 isolates. These findings suggest that three genetically distinct groups of H5N8 HPAIVs were independently circulating among the migratory birds at the Izumi plain. Intriguingly, the genetic grouping of our isolates matched broadly the dates of sampling; the forth to eighth isolates were categorised into Group C, while earlier isolates were categorised into Group A or B. To determine whether this virus group has genetic characteristics that become predominant among the migratory birds over the remaining virus groups, further investigation would be needed.

To further characterise the three genetic groups of H5N8 HPAIVs, the nucleotide sequences of the remaining six

genes were phylogenetically analysed with their counterparts from the representative avian viruses of various subtypes (Figure 2).

The bootstrap values between the isolates in Groups A and C and among the isolates in Group B in the phylogenetic trees of the PB2 (Figure 3A) and PB1 (Figure 3B) genes were 100%. Similarly, the bootstrap value between the isolates in Group A and the isolates in Groups B and C in the phylogenetic tree of the NS genes (Figure 3F) were 99%. These results support our findings in the phylogenetic trees of the HA and NA genes.

No mutations were found that are known to confer the ability to infect mammalian hosts or to provide resistance against anti-influenza drugs to avian influenza viruses, with the exception of an asparagine at position 31 in the M2 protein, which confers resistance to the M2 ion channel blocker amantadine [11].

Conclusion

We isolated eight H5N8 HPAIVs from migratory birds and the water in their environment at the Izumi plain in southern Japan. Based on their genome sequences, these isolates were genetically divided into three groups. These results indicate the co-circulation of at least three genetic groups of H5N8 HPAIVs among the migratory birds overwintering at a single site in Japan. These H5N8 HPAIVs are most likely to be derived from wild ducks [12], rather than from cranes whose flyways were restricted to East Asian countries (Figure 1A). These findings also imply the possibility of global redistribution of the H5N8 HPAIVs via migration of these ducks next winter.

Table 1

H5N8 influenza A viruses isolated in this study, Izumi plain, Japan, 2014/15 (n = 8)

Isolate	Collection date	Host	Specimen source
A/crane/Kagoshima/KU0.5014(H5N8)	23 November 2014	Sick white-naped crane	Tracheal and cloacal swabs
A/environment/Kagoshima/KU-ngr-H/2014(H5N8)	1 December 2014	NA[a]	Water sample
A/crane/Kagoshima/KU13/2014(H5N8)	7 December 2014	Dead hooded crane	Tracheal and cloacal swabs
A/crane/Kagoshima/KU21/2014(H5N8)	17 December 2014	Dead hooded crane	Tracheal and cloacal swabs
A/crane/Kagoshima/KU41/2014(H5N8)	24 December 2014	Dead hooded crane	Tracheal and cloacal swabs
A/crane/Kagoshima/KU53/2015(H5N8)	3 January 2015	Dead hooded crane	Tracheal and cloacal swabs
A/mallard duck/Kagoshima/KU70/2015(H5N8)	14 January 2015	Dead mallard duck	Conjunctival swab
A/mallard duck/Kagoshima/KU116/2015(H5N8)	13 February 2015	Dead mallard duck	Conjunctival swab

[a] NA, not applicable.

Table 2

Nucleotide identity of the H5N8 influenza A isolates and their closest relatives, Izumi plain, Japan, 2014/15 (n = 8)

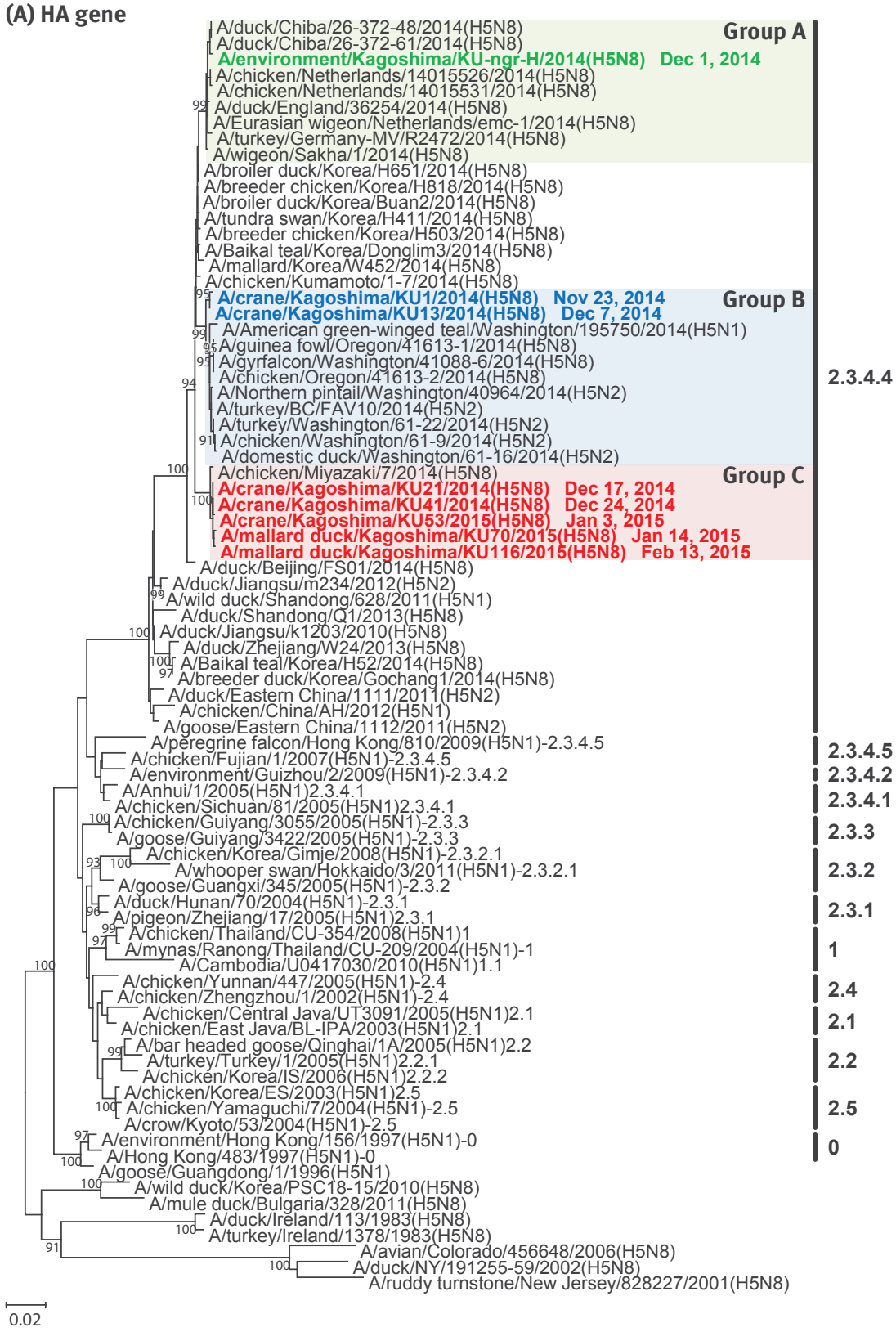
Isolate	Gene	Accession number[a]	Closest relative[b]	Identity (%)
A/crane/Kagoshima/KU0.5014(H5N8)	PB2	EPI553205	A/gyrfalcon/Washington/41088–6/2014(H5N8)	99.65
	PB1	EPI553206	A/gyrfalcon/Washington/41088–6/2014(H5N8)	99.43
	PA	EPI553207	A/Northern pintail/Washington/40964/2014(H5N2)	99.87
	HA	EPI553208	A/Northern pintail/Washington/40964/2014(H5N2)	99.29
	NP	EPI553209	A/Northern pintail/Washington/40964/2014(H5N2)	99.53
	NA	EPI553210	A/guinea fowl/Oregon/41613–1/2014(H5N8)	98.94
	M	EPI553211	A/Baikal teal/Korea/Donglim3/2014(H5N8)	100.00
	NS	EPI553212	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.76
A/environment/Kagoshima/KU-ngr-H/2014(H5N8)	PB2	EPI553359	A/duck/Chiba/26–372–61/2014(H5N8)	99.78
	PB1	EPI553360	A/duck/Chiba/26–372–61/2014(H5N8)	98.86
	PA	EPI553361	A/duck/Chiba/26–372–61/2014(H5N8)	99.81
	HA	EPI553362	A/duck/Chiba/26–372–61/2014(H5N8)	99.76
	NP	EPI553363	A/duck/Chiba/26–372–61/2014(H5N8)	99.87
	NA	EPI553364	A/turkey/Germany-MV/R2472/2014(H5N8)	98.86
	M	EPI553365	A/duck/Chiba/26–372–61/2014(H5N8)	100.00
	NS	EPI553366	A/duck/Chiba/26–372–61/2014(H5N8)	99.76
A/crane/Kagoshima/KU13/2014(H5N8)	PB2	EPI573635	A/gyrfalcon/Washington/41088–6/2014(H5N8)	99.52
	PB1	EPI573636	A/gyrfalcon/Washington/41088–6/2014(H5N8)	99.60
	PA	EPI573637	A/Northern pintail/Washington/40964/2014(H5N2)	99.44
	HA	EPI573638	A/Northern pintail/Washington/40964/2014(H5N2)	99.53
	NP	EPI573639	A/gyrfalcon/Washington/41088–6/2014(H5N8)	99.67
	NA	EPI573640	A/guinea fowl/Oregon/41613–1/2014(H5N8)	98.65
	M	EPI573641	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.80
	NS	EPI573642	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.76
A/crane/Kagoshima/KU21/2014(H5N8)	PB2	EPI573643	A/chicken/Miyazaki/7/2014(H5N8)	99.82
	PB1	EPI573644	A/chicken/Miyazaki/7/2014(H5N8)	99.69
	PA	EPI573645	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.86
	HA	EPI573646	A/chicken/Miyazaki/7/2014(H5N8)	99.71
	NP	EPI573647	A/breeder duck/Korea/H158/2014(H5N8)	99.73
	NA	EPI573648	A/chicken/Miyazaki/7/2014(H5N8)	99.79
	M	EPI573649	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NS	EPI573650	A/chicken/Miyazaki/7/2014(H5N8)	99.76
A/crane/Kagoshima/KU41/2014(H5N8)	PB2	EPI573651	A/chicken/Miyazaki/7/2014(H5N8)	99.82
	PB1	EPI573652	A/chicken/Miyazaki/7/2014(H5N8)	99.64
	PA	EPI573653	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.81
	HA	EPI573654	A/chicken/Miyazaki/7/2014(H5N8)	99.71
	NP	EPI573655	A/breeder chicken/Korea/H250/2014(H5N8)	99.73
	NA	EPI573656	A/chicken/Miyazaki/7/2014(H5N8)	99.72
	M	EPI573657	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NS	EPI573658	A/chicken/Miyazaki/7/2014(H5N8)	99.88
A/crane/Kagoshima/KU53/2015(H5N8)	PB2	EPI573661	A/chicken/Miyazaki/7/2014(H5N8)	99.78
	PB1	EPI573662	A/chicken/Miyazaki/7/2014(H5N8)	99.60
	PA	EPI573663	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.81
	HA	EPI573664	A/chicken/Miyazaki/7/2014(H5N8)	99.65
	NP	EPI573665	A/chicken/Miyazaki/7/2014(H5N8)	99.73
	NA	EPI573666	A/chicken/Miyazaki/7/2014(H5N8)	99.65
	M	EPI573667	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NS	EPI573668	A/chicken/Miyazaki/7/2014(H5N8)	99.88
A/mallard duck/Kagoshima/KU70/2015(H5N8)	PB2	EPI573669	A/chicken/Miyazaki/7/2014(H5N8)	99.60
	PB1	EPI573670	A/mallard/Korea/H297/2014(H5N8)	99.60
	PA	EPI573671	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.53
	HA	EPI573672	A/chicken/Miyazaki/7/2014(H5N8)	99.59
	NP	EPI573673	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NA	EPI573674	A/chicken/Miyazaki/7/2014(H5N8)	99.72
	M	EPI573675	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NS	EPI573676	A/chicken/Miyazaki/7/2014(H5N8)	99.76
A/mallard duck /Kagoshima/KU116/2015(H5N8)	PB2	EPI573677	A/chicken/Miyazaki/7/2014(H5N8)	99.56
	PB1	EPI573678	A/mallard/Korea/H297/2014(H5N8)	99.52
	PA	EPI573679	A/Baikal teal/Korea/Donglim3/2014(H5N8)	99.39
	HA	EPI573680	A/chicken/Miyazaki/7/2014(H5N8)	99.59
	NP	EPI573681	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NA	EPI573682	A/chicken/Miyazaki/7/2014(H5N8)	99.72
	M	EPI573683	A/chicken/Miyazaki/7/2014(H5N8)	99.80
	NS	EPI573684	A/chicken/Miyazaki/7/2014(H5N8)	99.88

[a] Accession numbers in the GISAID (http://platform.gisaid.org/) database are listed.

[b] Representative viruses with the highest nucleotide identity found in the GISAID and/or GenBank (http://www.ncbi.nlm.nih.gov/genbank/) databases on 23 March 2015 are listed. We thank the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based.

Figure 2A

Phylogenetic trees of the HA and NA genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)



HA: haemagglutinin; HPAIV: highly pathogenic avian influenza viruses; NA: neuraminidase.

The nucleotide sequences of the HA (A) and NA (B) genes from our H5N8 isolates were phylogenetically analysed with counterparts from other H5 and N8 subtype viruses, respectively, using the neighbour-joining method with a bootstrapping set of 1,000 replicates. Our isolates in Groups A, B and C are indicated in green, blue and red, respectively (see main text for details), with the dates of sampling. Bootstrap values of>90% are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

Figure 2B

Phylogenetic trees of the HA and NA genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

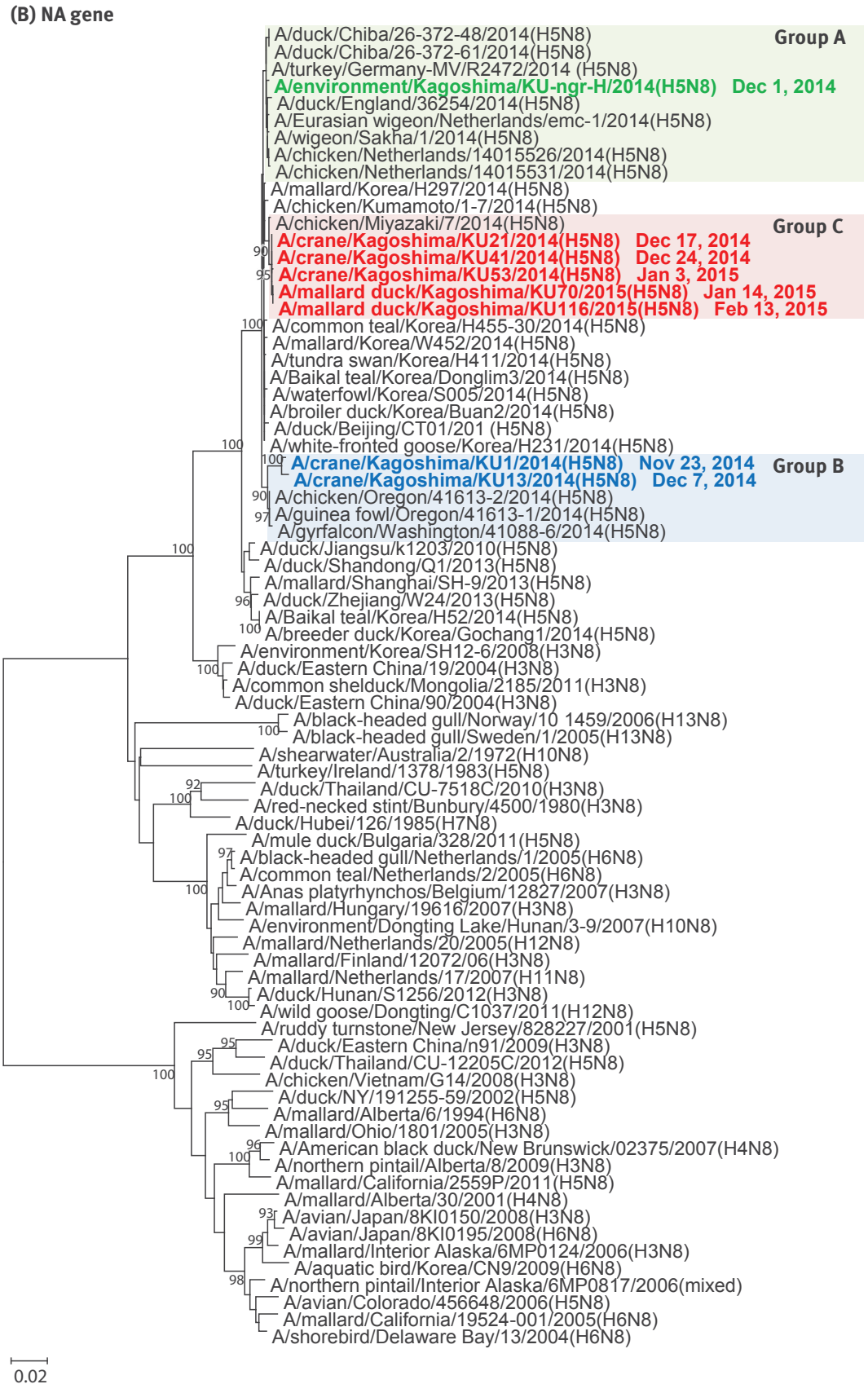


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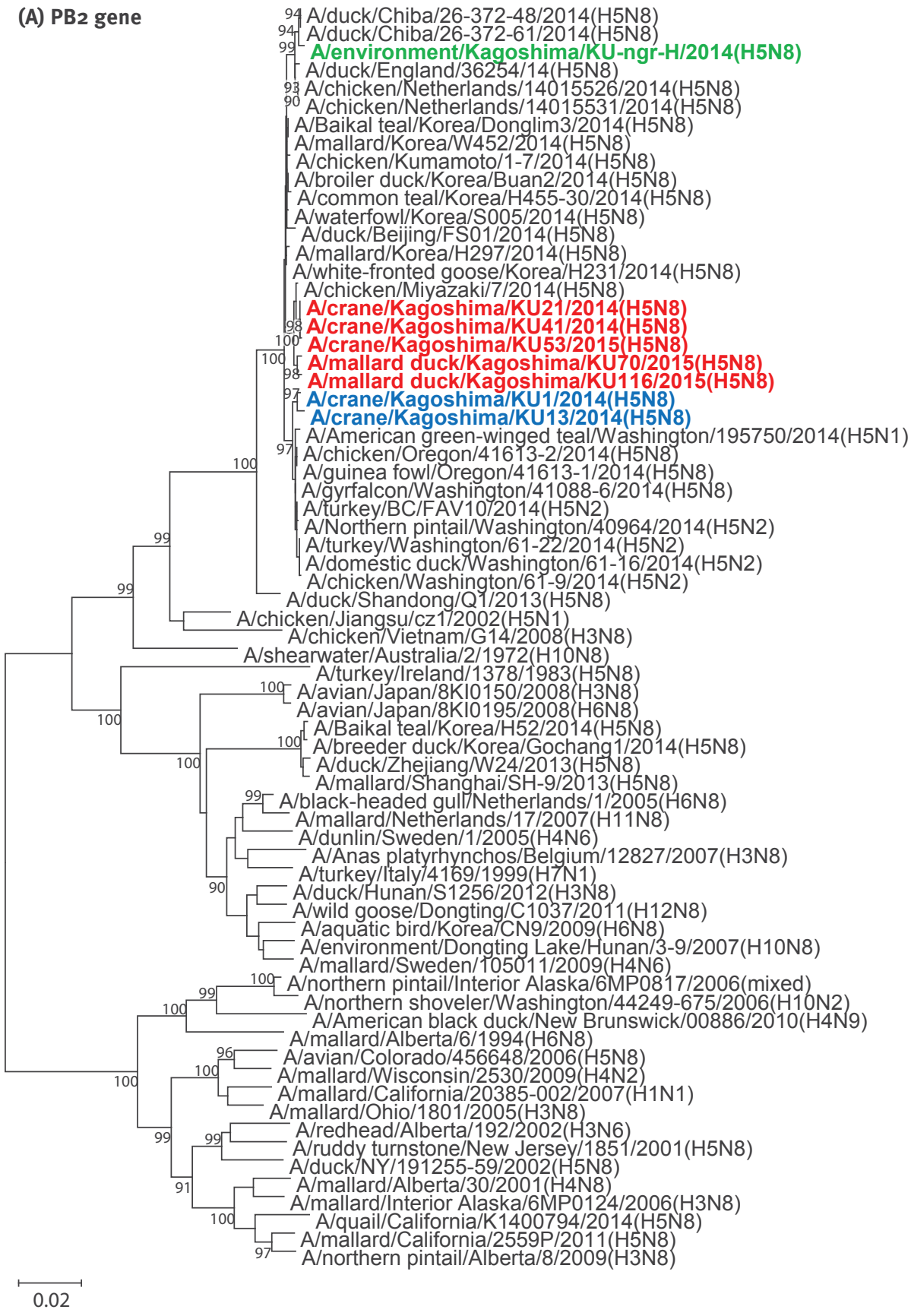
Figure 3A

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

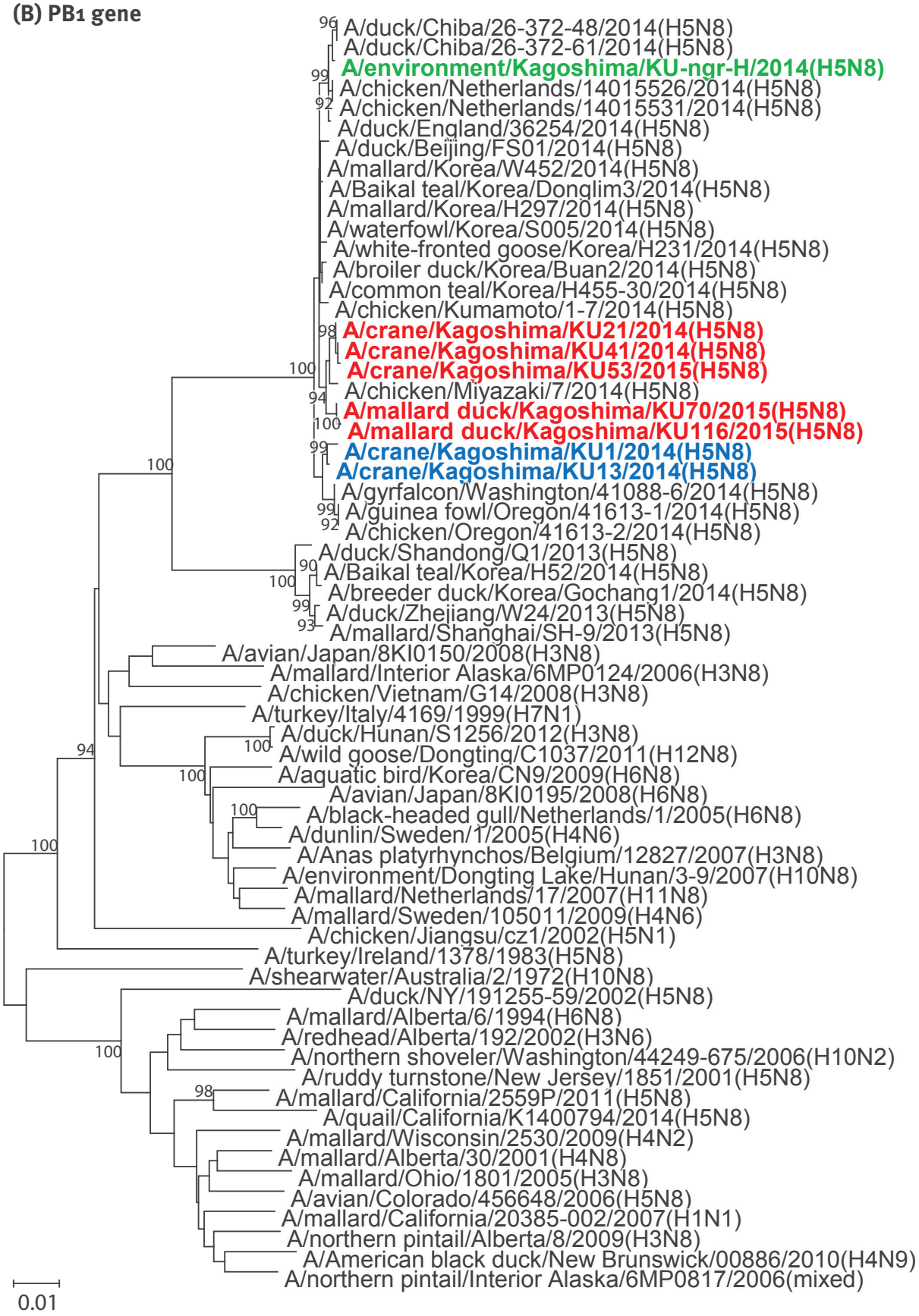


HPAIV: highly pathogenic avian influenza viruses.

The nucleotide sequences of the PB2 (A), PB1 (B), PA (C), NP (D), M (E) and NS (F) genes from our H5N8 isolates were phylogenetically analysed with counterparts from the representative avian viruses of various subtypes by using the neighbour-joining method with a bootstrapping set of 1,000 replicates. Our isolates in Groups A, B and C are indicated in green, blue and red, respectively (see main text for details). Bootstrap values of >90% are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

Figure 3B

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

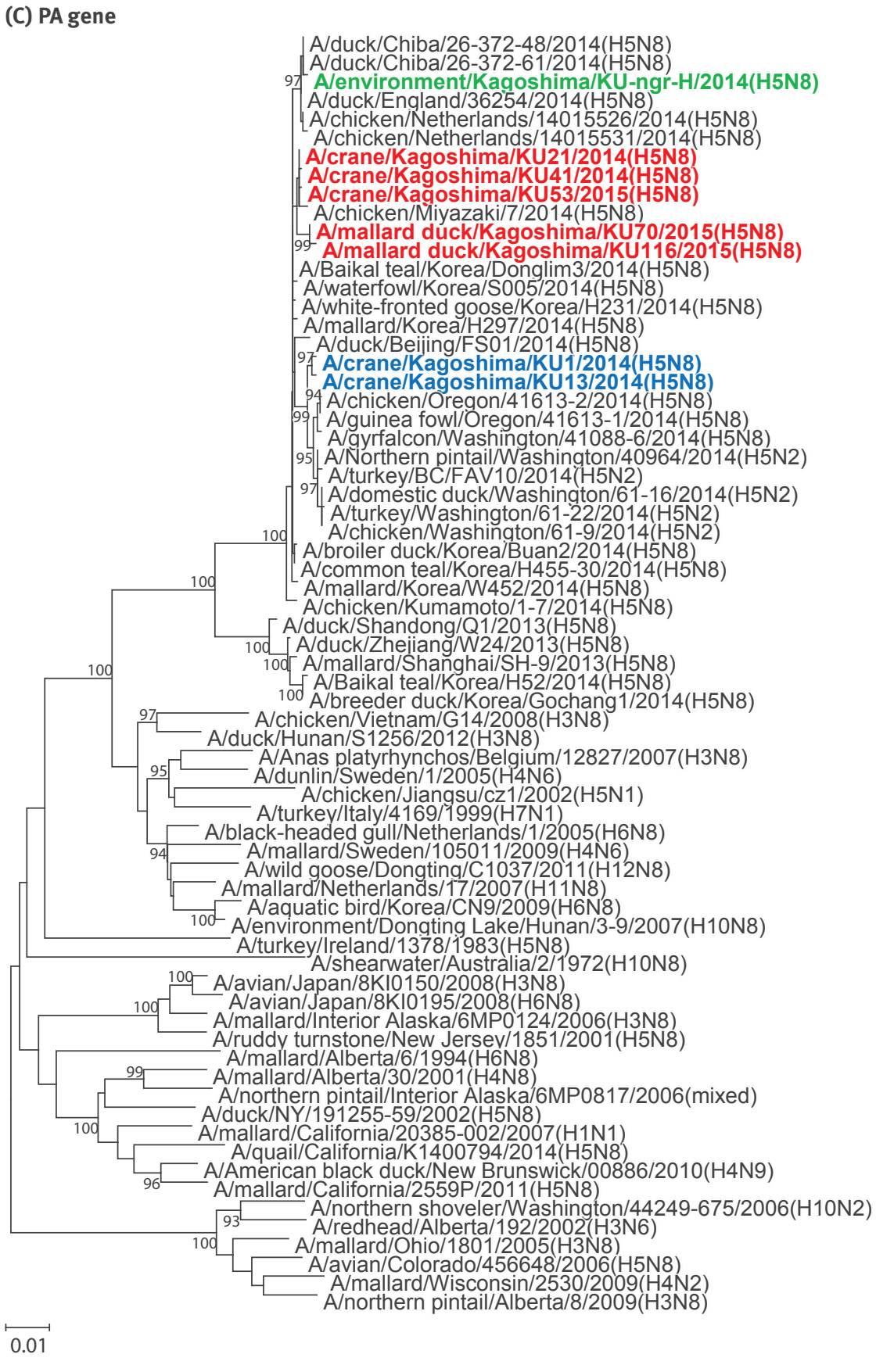


HPAIV: highly pathogenic avian influenza viruses.

The nucleotide sequences of the PB2 (A), PB1 (B), PA (C), NP (D), M (E) and NS (F) genes from our H5N8 isolates were phylogenetically analysed with counterparts from the representative avian viruses of various subtypes by using the neighbour-joining method with a bootstrapping set of 1,000 replicates. Our isolates in Groups A, B and C are indicated in green, blue and red, respectively (see main text for details). Bootstrap values of >90% are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

Figure 3C

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

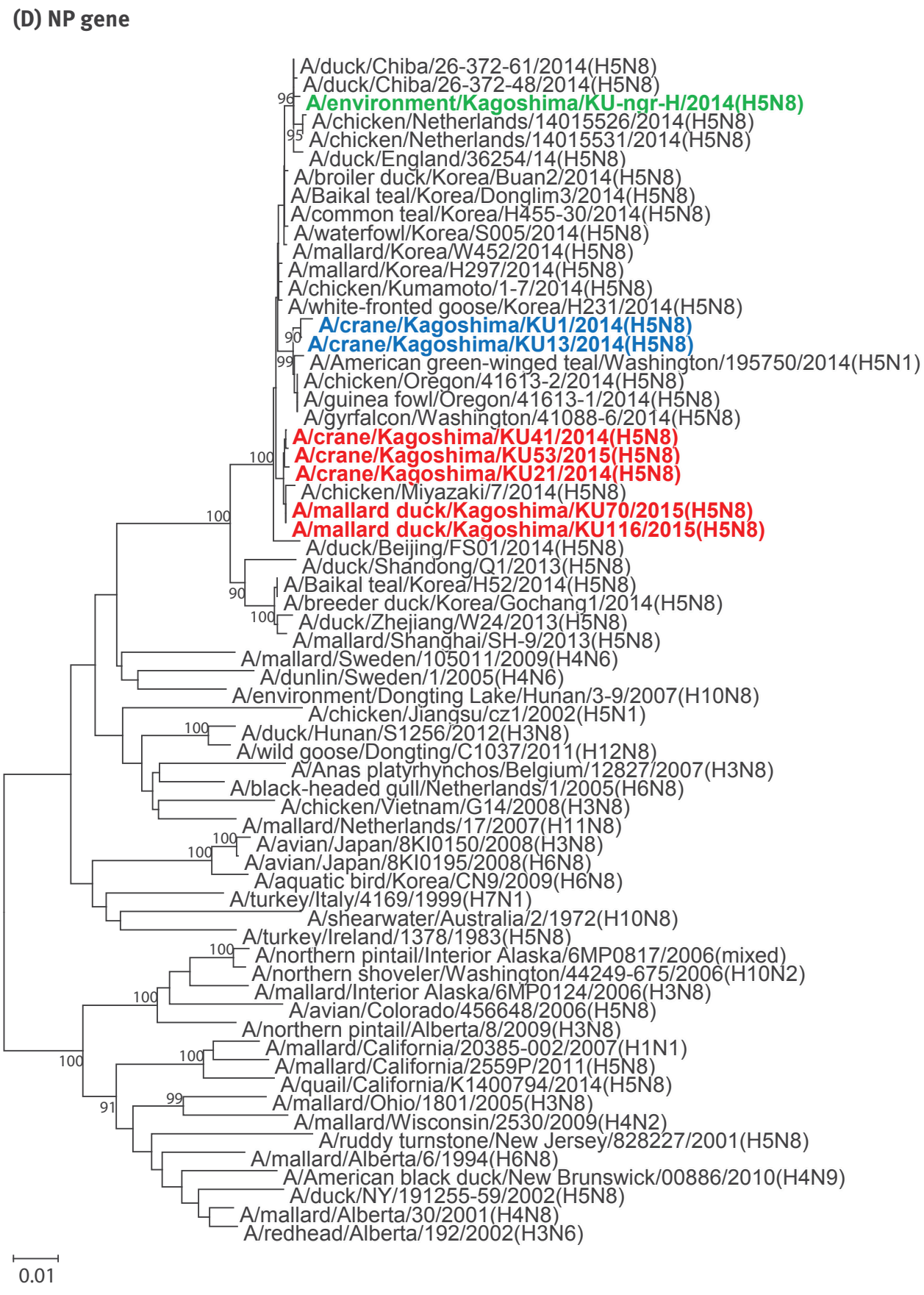


HPAIV: highly pathogenic avian influenza viruses.

The nucleotide sequences of the PB2 (A), PB1 (B), PA (C), NP (D), M (E) and NS (F) genes from our H5N8 isolates were phylogenetically analysed with counterparts from the representative avian viruses of various subtypes by using the neighbour-joining method with a bootstrapping set of 1,000 replicates. Our isolates in Groups A, B and C are indicated in green, blue and red, respectively (see main text for details). Bootstrap values of >90% are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

Figure 3d

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

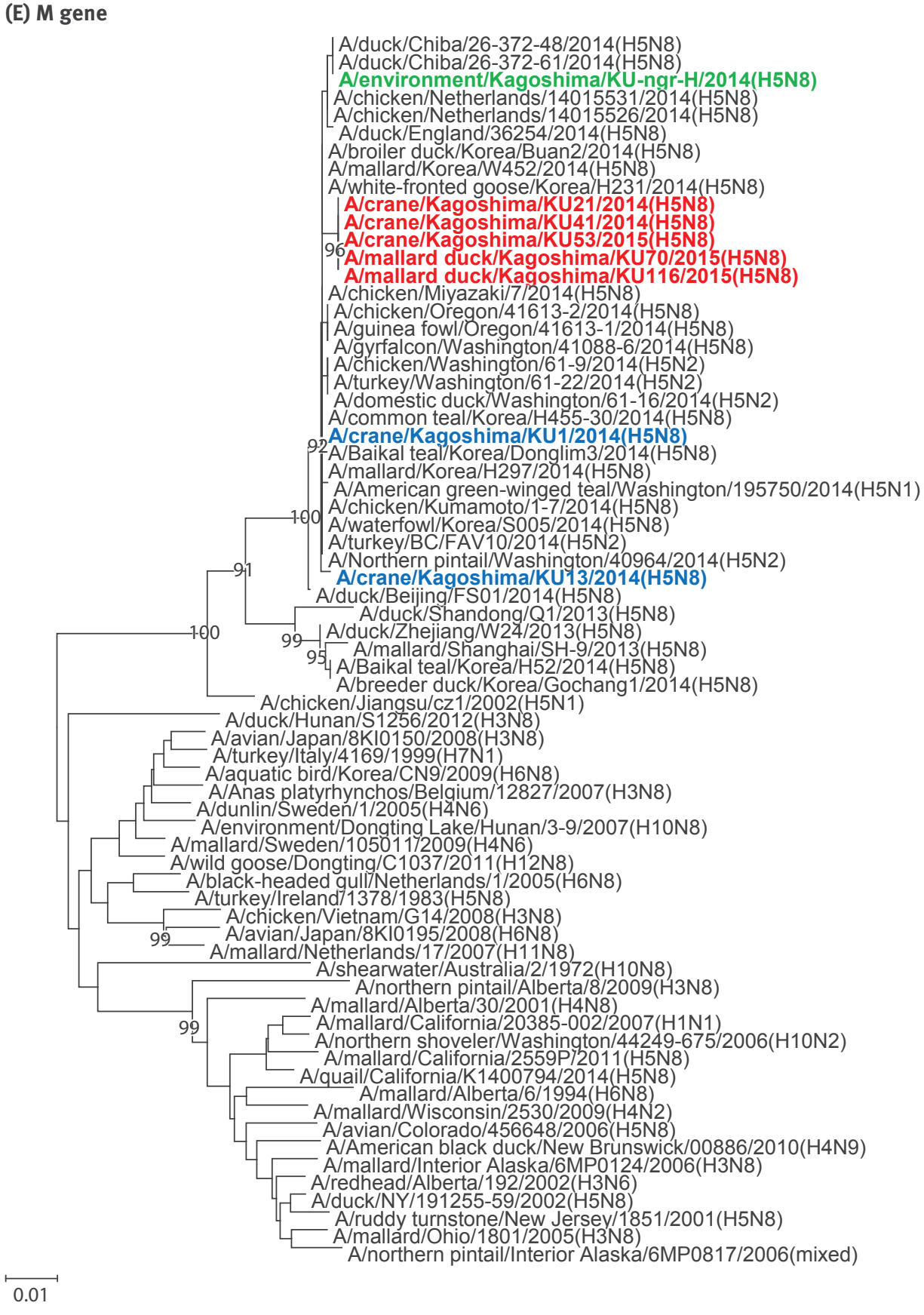


HPAIV: highly pathogenic avian influenza viruses.

The nucleotide sequences of the PB2 (A), PB1 (B), PA (C), NP (D), M (E) and NS (F) genes from our H5N8 isolates were phylogenetically analysed with counterparts from the representative avian viruses of various subtypes by using the neighbour-joining method with a bootstrapping set of 1,000 replicates. Our isolates in Groups A, B and C are indicated in green, blue and red, respectively (see main text for details). Bootstrap values of >90% are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

Figure 3E

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)



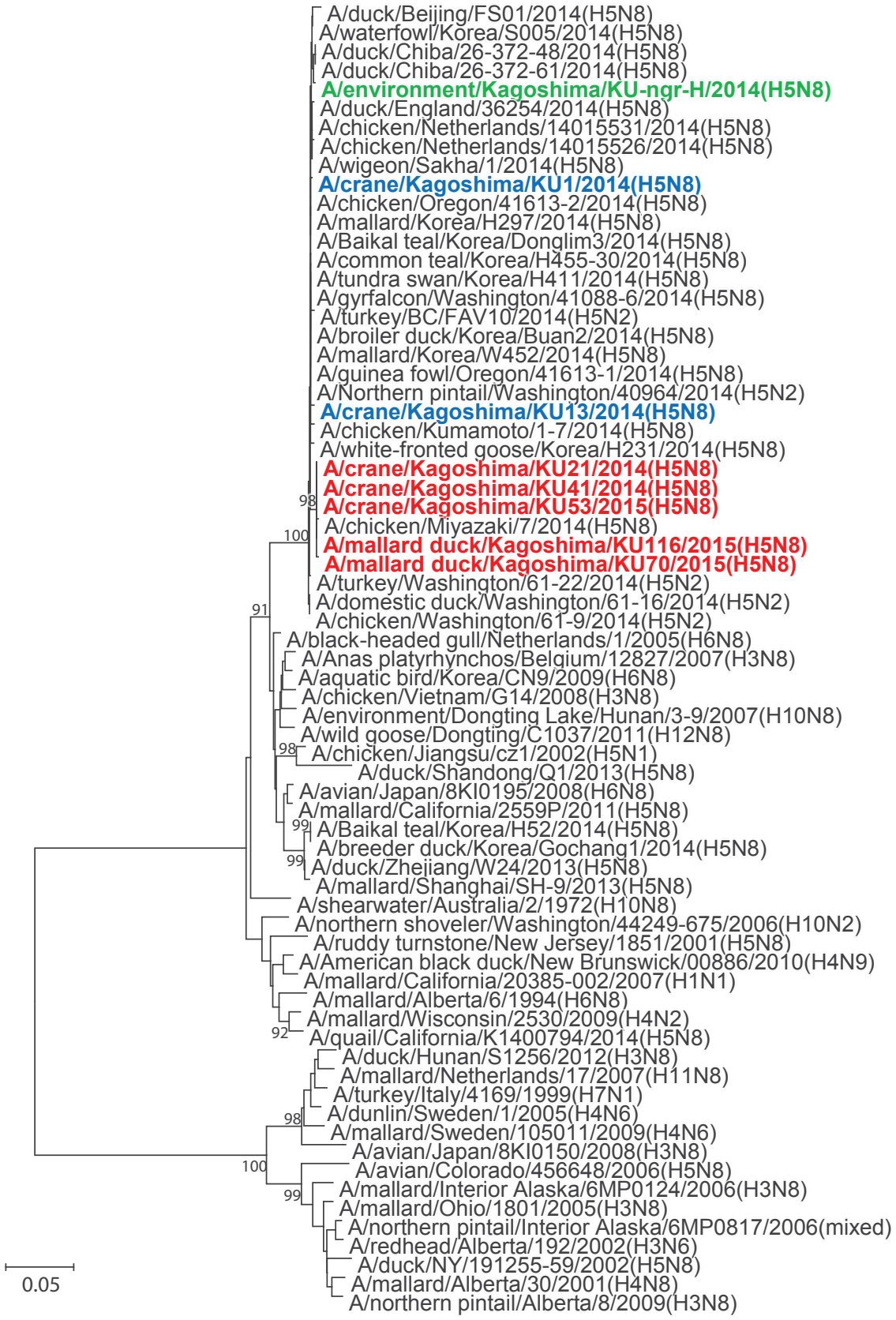
HPAIV: highly pathogenic avian influenza viruses.

The nucleotide sequences of the PB2 (A), PB1 (B), PA (C), NP (D), M (E) and NS (F) genes from our H5N8 isolates were phylogenetically analysed with counterparts from the representative avian viruses of various subtypes by using the neighbour-joining method with a bootstrapping set of 1,000 replicates. Our isolates in Groups A, B and C are indicated in green, blue and red, respectively (see main text for details). Bootstrap values of >90% are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

Figure 3F

Phylogenetic trees of six non-envelope genes of the H5N8 HPAIVs isolated at the Izumi plain, Japan, 2014/15 (n = 8)

(F) NS gene



HPAIV: highly pathogenic avian influenza viruses.

The nucleotide sequences of the PB2 (A), PB1 (B), PA (C), NP (D), M (E) and NS (F) genes from our H5N8 isolates were phylogenetically analysed with counterparts from the representative avian viruses of various subtypes by using the neighbour-joining method with a bootstrapping set of 1,000 replicates. Our isolates in Groups A, B and C are indicated in green, blue and red, respectively (see main text for details). Bootstrap values of >90% are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

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Conflict of interest

None declared.

Authors' contributions

Makoto Ozawa and Aya Matsuu designed the study; Makoto Ozawa, Aya Matsuu, Kaori Tokorozaki, Masayuki Horie, Tatsunori Masatani, Hiroko Nakagawa, Kosuke Okuya, Toshiko Kawabata, and Shigehisa Toda performed the experiments; Makoto Ozawa drafted the manuscript; Makoto Ozawa, Aya Matsuu, Kaori Tokorozaki, Masayuki Horie, and Tatsunori Masatani reviewed the manuscript.

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The challenge of West Nile virus in Europe: knowledge gaps and research priorities

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West Nile virus (WNV) is continuously spreading across Europe, and other continents, i.e. North and South America and many other regions of the world. Despite the overall sporadic nature of outbreaks with cases of West Nile neuroinvasive disease (WNND) in Europe, the spillover events have increased and the virus has been introduced into new areas. The high genetic diversity of the virus, with remarkable phenotypic variation, and its endemic circulation in several countries, require an intensification of the integrated and multidisciplinary research efforts built under the 7th Framework Programme of the European Union (FP7). It is important to better clarify several aspects of WNV circulation in Europe, including its ecology, genomic diversity, pathogenicity, transmissibility, diagnosis and control options, under different environmental and socio-economic scenarios. Identifying WNV endemic as well as infection-free areas is becoming a need for the development of human vaccines and therapeutics and the application of blood and organs safety regulations. This review, produced as a joint initiative among European experts and based on analysis of 118 scientific papers published between 2004 and 2014, provides the state of knowledge on WNV and highlights the existing knowledge and research gaps that need to be addressed with high priority in Europe and neighbouring countries.

Introduction

West Nile virus (WNV) is an African flavivirus originally maintained in sylvatic cycles mostly between mosquitoes and birds. Since the 1950s, there has been evidence of circulation outside its original ecological niches [1]. Relatively large outbreaks of West Nile neuroinvasive disease (WNND) have been recorded in humans and/or horses in an increasing number of areas. WNV infections have been identified in many European and Mediterranean countries (Figure 1) [1,2]. The increasing incidence of WNND, the appearance of new foci, and the endemic virus circulation in temperate areas have promoted research and innovation efforts financially supported by the European Commission (EC) under the 7th Framework Programme (FP7). However, knowledge gaps remain on several important aspects of virus ecology, biology, and pathogenicity. Together with the lack of safe vaccines and specific therapeutic treatments for humans, this limits our ability to efficiently predict, prevent and control WNV infections, with increasing costs due to the needs of guarantee the safety of blood transfusion and organ donation [3]. There are also rising problems associated with the loss in efficacy and increased resistance to many commercial insecticidal products used to suppress mosquito populations [4]. This requires the intensification of research efforts for emerging vector-borne infections, such as those caused by WNV and other flaviviruses, and the maintenance of the existing research capacity.

Therefore, the coordinators and representative members of four EC FP7 European funded projects on WNV and other mosquito-borne viral diseases (EuroWestNile, EDENext, Wings, Vectorie) met in Madrid on several occasions to review and discuss WNV knowledge advances and identify research gaps to be addressed, under a 'One Health' perspective, with high priority.

Methods

The expert group integrated their expert knowledge and opinion with a scientific literature review. Following a search in the databases ISI Web of Knowledge and PubMed, using defined qualifiers for the viral infection [(WNV)], the disease [(WNND)], host [(humans], [birds], [horses)], geographical location [(Africa], [Europe], [Mediterranean basin)] and issue [(virology], [genomics], [epidemiology], [ecology], [pathology], [diagnostics], [vaccine], [control], [review]). In the search, the period of publication was limited to publications from January 2004 to October 2014. Inclusion criteria were based on title, abstract and year of publication, leading, if relevant to the above mentioned qualifiers, to retrieval and analysis of the full paper. Exclusion criteria were based on the year of publication and on the novelty of the information provided choosing those most up to date on the same subject and those including the previous in the reference list. Only articles with the latest findings on a specific subject were included and those older than the period covered were discarded.

A total of 118 scientific publications were then chosen among the 513 initially retrieved.

Epidemiology of West Nile virus in Europe and neighbouring countries

Circulating West Nile virus strains

The re-emergence of WNV in Europe and neighbouring countries after 1990 led to an intensified surveillance for WNV infection not only in humans, but also in horses, birds, and mosquitoes in several areas. This has resulted in the detection and/or isolation of many different strains of WNV, eventually classified as up to seven (nine) different genetic lineages [5-10] (Table 1, Figure 2 A-C). There is a growing number of WNV lineages and to address inconsistent numbering in the literature we suggest in Table 1 and Figure 2 a harmonised WNV lineage numbering.

The most widespread WNV lineages include lineage 1, clade 1a, belonging to the Mediterranean and former eastern European subtype, and lineage 2, which emerged in central Europe in 2004, and dispersed from Hungary to the eastern part of Austria and to southern European countries [11,12]. Following an independent introduction, another lineage 2 strain was detected in 2004 in Rostov Oblast, southern Russia [13]; subsequently, this virus strain has been responsible for outbreaks of WNND in Volgograd Oblast southern Russia since 2007, and in Romania since 2010 [14,15]. Phylogenetic analyses revealed that all European WNV lineage 1 and 2 strains are derived from a limited number of independent introductions, most likely from Africa, followed by local spread and evolution [5,15-20]. Other lineages identified but not associated so far with human or animal diseases include WNV lineage 3, also known as Rabensburg virus, first isolated in 1997 in South Moravia (Czech Republic) in *Culex pipiens* and *Aedes rossicus* mosquitoes in Czech Republic, and named after the nearby Austrian city of Rabensburg [6], WNV lineage 4a, Krasnodar virus, first detected in a *Dermacentor* tick and then in mosquitoes and frogs in southern Russia [7], putative WNV lineage 4b, identified in 2010 mosquitoes in southern Spain [8], and lineage 4c detected in *Uranotaenia unguiculata* mosquitoes in Austria in 2013 [9].

In recent years, the availability of whole WNV genome sequences of European origin, necessary for diagnostic and molecular epidemiology and for better refining WNV taxonomic classification, has improved. A WNV strain bank and associated large genomic database has been built by the EuroWestNile consortium to facilitate this availability. The biobank and database are now available for the scientific community (www.eurowestnile.org).

Analyses of full genome sequence data have shown that the virus, notwithstanding its high genetic stability, can adapt to new ecological niches through mutation and selection events [21]. In addition to this plasticity, the presence of other flaviviruses infecting mosquitoes, birds and humans, may have important consequences not only for WNV ecology, epidemiology and pathogenicity, but also for diagnostics, surveillance and control strategies [22].

West Nile lineages and human cases

Most humans infected with WNV remain asymptomatic and only approximately 20–40% develop symptoms. The vast majority of clinical manifestations are a mild influenza-like illness, defined as West Nile fever (WNF); severe neuroinvasive disease (WNND) occurs only in <1% of the infected patients. WNND usually encompasses three different syndromes: meningitis, encephalitis, and acute flaccid paralysis [2 and references herein]. According to the European Centre for Disease Prevention and Control (ECDC), the total number of WNND cases reported from 2010 to 2013 was particularly high in Greece, with 262 cases of WNND notified in 2010 (incidence 2.34/100,000), 100 in 2011 (incidence 0.90/100,000), 161 in 2012 (incidence 1.45/100,000), and 86 in 2013 (incidence 0.78/100,000) (unpublished data).

WNV lineage 2 strains belonging to the Hungarian clade were responsible for the outbreaks in Greece [23,24] and in other central European countries, including Serbia [25-27], where 71 (incidence 0.98/100,000) and 302 (incidence 4.91/100,000) human infections were notified in 2012 and 2013, respectively. Autochthonous

human cases of infection with this WNV strain were identified in recent years in Hungary, Austria [28], Croatia [29,30], Albania, the former Yugoslav Republic of Macedonia, Kosovo* and Montenegro. Closely related WNV lineage 2 strains of the same Hungarian clade have also been detected in Italy since 2011 [31-33], leading to a large outbreak with 69 notified human infections (incidence 0.12/100,000) in 2013 [34], and in the Czech Republic in 2013 [35]. The epidemiological situation in Italy is, however, more complex than in other countries [36]. WNV lineage 1 strains of the western Mediterranean subtype, including the Livenza strain that caused an outbreak in the area in northern Italy in 2012 [19,37] that was later affected by the WNV lineage 2 strain in 2013, have been autochthonous in Italy and co-circulate with WNV lineage 2. WNV lineage 1 strains classified as western Mediterranean subtype circulated also in southern Spain [20], where sporadic human infections were reported in 2010.

In Turkey, human cases of WNV infection were reported in 2010 (47 cases, incidence 0.06/ 100,000) and in 2011 (five cases, incidence 0.01/100,000) [38]. WNV strains obtained between 2011 and 2013 from humans, horses and mosquitoes across Turkey proved to be closely related to 'old' lineage 1 strains from sub-Saharan Africa [39]. Israel, a highly WNV-affected Mediterranean country [40], noted over 100 cases diagnosed in 2010, 33 in 2011, 83 in 2012, and 63 in 2013 (incidence rates per 100,000 not available) [40]. Finally, the southern Russian/Romanian WNV lineage 2 strain [15] has led to large outbreaks of WNND in humans in southern Russia in each season from 2010 to 2013 (419 cases in 2010, 137 in 2011, 447 in 2012, and 177 in 2013, incidences rates per 100,000 not available), and in 2010 Romania, when 57 human infections (incidence 0.28/100,000) were identified [14].

Ecology

Mosquito vectors

Several mosquito genera are competent for WNV transmission; however, mosquitoes belonging to the *Culex pipiens* complex and their hybrids play a central role in modulating the virus circulation and the seasonal shifts among birds and humans [41]. In addition to *Cx. pipiens*, *Cx. perexiguus* and *Cx. modestus* have been identified as important WNV vectors [42], while the role of other species, including the *Aedes albopictus*, needs to be better evaluated.

Introduction, transmission and spread

Introduction of WNV into new areas is generally considered to be initiated by migratory birds while residential and synanthropic birds may contribute to virus dispersal into larger areas, as well as to the following establishment and spread in certain areas [43,44]. However, the relative importance of dispersal of WNV through infected mosquitoes rather than by infected birds is unknown. The introduction event may or may not be followed by an amplification phase depending on several coincidental factors, ranging from the birds' and vectors' competence, abundance and community assembly, to the local environmental condition including climate and landscape features [45-47]. One recognised important factor for WNV amplification and the following risk of transmission to humans is the feeding behaviour and host selection by the mosquito vectors. Based on preliminary research carried out in Italy and Spain, only few bird species seem to play a major role as blood donor for the mosquitoes [42,48,49]. Unfortunately, the reservoir competence for many European bird species is still unknown. Furthermore, mass mortality of highly susceptible species (such as corvids or other species) is less frequently observed in the Old than in the New World although some species, as the jackdaws (*Corvus monedula*) or other could potentially function as sentinel [50].

Virus persistence, silent circulation and spillover

Mechanisms of virus persistence in animal hosts in Europe are still unknown, but they could possibly lead to the development of persistent, chronic infections in certain individuals as seen in studies carried out in the United States [43,51]. In fact, in the New World, WNV, once established, circulates in enzootic cycles among few most competent mosquito species and their preferred feeding hosts [43,48,51]. The circulation of WNV in Europe may occur silently for several months, or even years, before the spillover event occurs [52]. One or more bird species may be involved in the amplification chain, some being important in the maintenance of the sylvatic cycles, while others (that might not be the same) may be involved in the periurban and urban cycles. Following spillover and following outbreak a subsidence phase may occur as a consequence of the rise of herd immunity in the reservoir birds, that is dependent on host longevity and the rate of recruitment of young individuals into the host population or the depopulation of highly susceptible bird hosts [43,51]. Unfortunately, the knowledge on herd immunity to WNV for many bird species of the Old World is still lacking.

WNV diversity and interactions with other flaviviruses

A wide spectrum of WNV lineages and strains with different potential pathogenicity and virulence thrive among other related flaviviruses with overlapping ecology such as Bagaza virus (BAGV) and Usutu virus (USUV)[52,53]. In this scenario, a previous infection of the WNV amplifying bird hosts with a low pathogenic strain of WNV or with a closely related flavivirus may confer some degree of cross-immunity which might reduce the amplification of any new WNV strain introduced into the same area [22]. This may explain, for instance, the scarcity of human WNV cases detected in Spain, where rates of WNV infection in birds are high and that may be due to the co-circulation of WNV lineage 6 [8] or other flaviviruses, that induce the production of potentially cross-protective antibodies in both

humans and birds, as reported for example for USUV [54].

However, co-infection may occur also in the mosquito vectors and therefore WNV replication could be in some way affected by previous infection with other co-circulating flaviviruses of the same antigenic group or even more distant viruses so that the epidemiological picture may change from one site area to another [55]. Although some investigations on viral interference have been started with one project (EuroWestNile), we are still at the beginning of our understanding of the consequences of viral co-infections both in the host and in the vectors, and therefore research in this field is now of high interest.

Consequences for WNV surveillance in Europe

Passive surveillance programmes focusing only on the analysis of dead birds may fail to detect ongoing WNV circulation in Europe. It is advisable to combine conventional surveillance activities with actively monitoring seroconversion in sentinel and/or wild resident birds and horses [56-59].

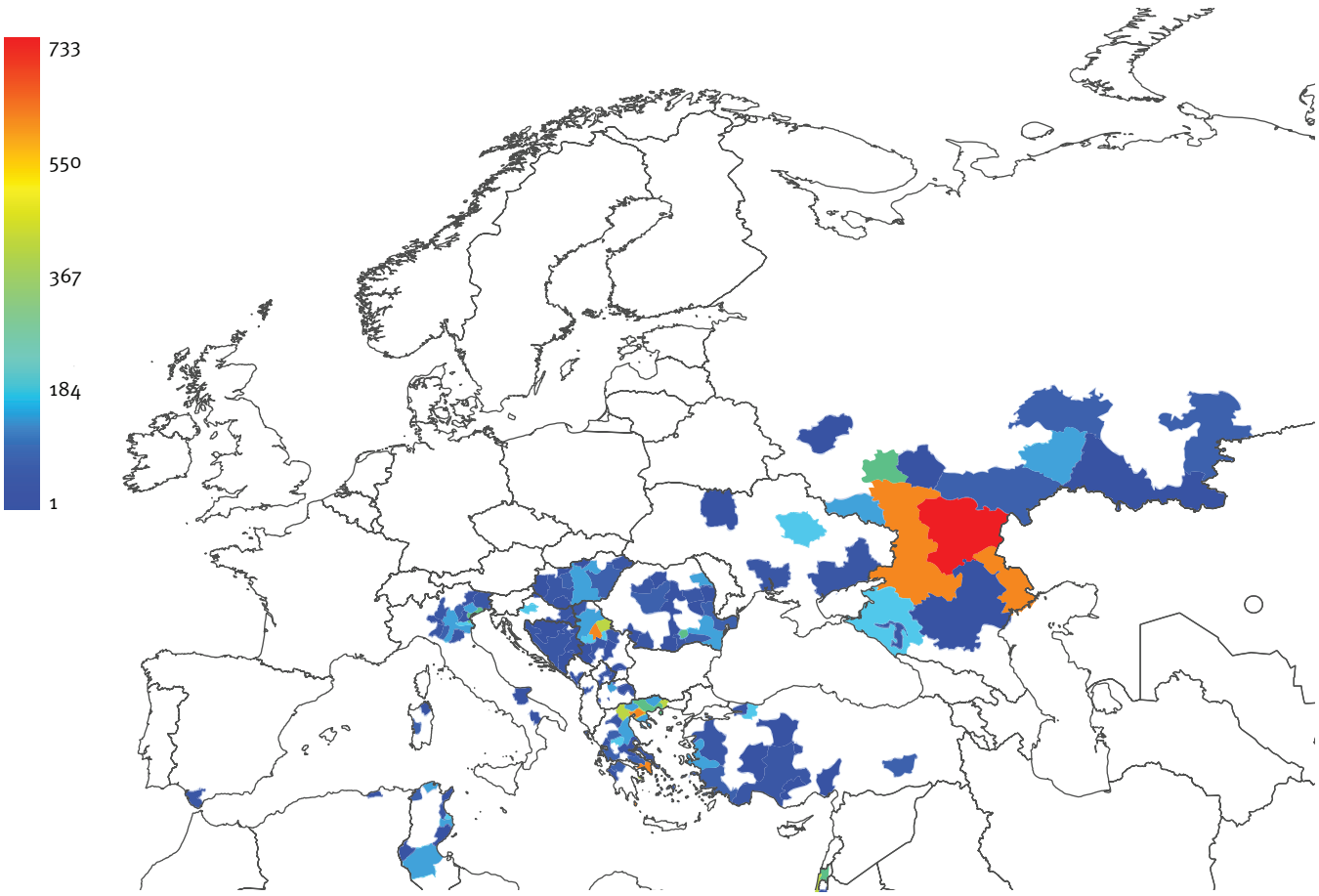
Pathogenicity and other phenotypic traits

Studies using animal models, mainly mice, have provided insights into WNV pathogenesis. Intra-peritoneal injection of WNV into mice generally leads to encephalitis and other neurological signs resembling those observed in humans with WNND [60]. This model provides researchers with a simple method enabling phenotypic characterisation of WNV strains for neuropathogenicity and neuroinvasiveness. Through the integration of this information with analysis of genetic changes occurring in field WNV isolates and reverse genetics using infectious clones, molecular chimeras or other strategies, it is possible to identify determinants of virulence [61-63]. However, the mouse model has important limitations and thus alternative animal models are highly desirable. An attempt of this kind, using one day old chickens, has recently been reported [64]. In addition, bird models of WNV infection provide information about the course of the infection in natural hosts [65 and references herein] and, consequently, about key aspects of WNV epidemiology.

Genetic changes in different geographic WNV variants might allow to identify the link with phenotypic traits related to virulence, amplification, transmissibility to

Figure 1

Cumulative number of human cases of West Nile virus infection in Europe and neighbouring countries, 2010 to 2013



Data source: European Centre for Disease Prevention and Control (ECDC).

Table 1

Overview of West Nile virus lineages and suggested lineage numbering

Suggested lineage numbering	Other lineage labelling in the literature	Representative strain	GenBank accession number	Note	Reference
Lineage 1a	Lineage 1	NY99-flamingo382-99, New York, 1999	AF196835	Most widespread WNV lineage	Lanciotti et al., 1999 [115]
Lineage 1b	Lineage 1	Kunjin MRM61C, Australia, 1960	D00246	Kunjin virus strains, Australia	Coia et al., 1988 [116]
Lineage 1c	Lineage 5	804994, India 1980	DQ256376	Only found in India	Bondre et al., 2007 [117]
Lineage 2	No	B956, Uganda 1937 (oldest WNV strain; WNV prototype strain)	AY532665	Second most widespread WNV lineage	Smithburn et. al, 1940 [118]
Lineage 3	No	Rabensburg virus 97-103, Czech Republic 1997	AY765264	Only found in central Europe	Bakonyi et al. [6]
Lineage 4a	Lineage 4	LEIV-Krnd88-190, Russia 1998	AY277251	Originally isolated from Dermacentor ticks	Lvov et al. [7]
Lineage 4b	Lineage 6 / Lineage 7	HU2925/06, Spain	GU047875	Only partial sequence available	Vázquez et al. [8]
Lineage 4c	Lineage 9	WNV-Uu-LN-AT-2013, Austria 2013	KJ831223	Identified in Uranotaenia mosquitoes	Pachler et al. [9]
Lineage 5	Lineage 6	Kunjin virus KUN MP502-66, Malaysia 1966	GU047874, HQ840762	Only partial sequences available	Vázquez et al. [8]
Lineage 6	Lineage 7	Dak Ar D 5443, Senegal	EU082200	Koutango virus	NA
Lineage 7	Lineage 8	ArD94343, Senegal 1992	KJ131502	Only partial sequence available	Fall et al. [10]

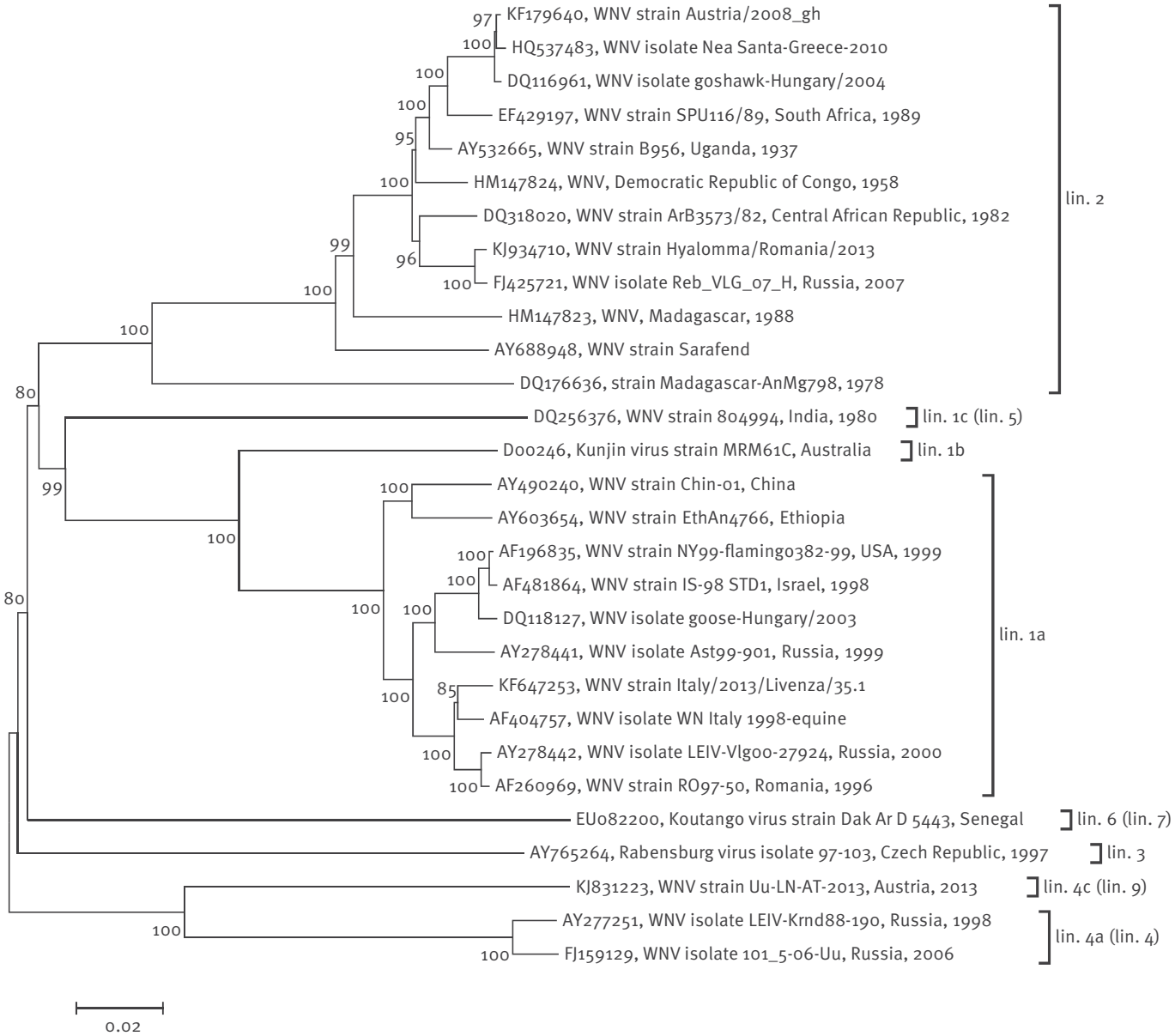
NA: not available; WNV: West Nile virus.

mosquitoes, and/or persistence, expressed in infected birds. Studies in avian models of WNV infection began in the US shortly after the first occurrence of WNV cases in 1999, and identified passerines as the group of birds developing higher and longer viraemias [66]. In contrast, studies testing the effect of European WNV strains in bird species indigenous to Europe have only been started recently [67-69].

Table 2 lists widely recognised genetic changes associated with virulence/attenuation, confirmed by viral cDNA clone mutagenesis/chimeras and using animal models of WNV infection [70-78]. Point mutations leading to amino acid changes either in structural (E) or non-structural (NS2A, NS3, NS4B) proteins result in attenuated phenotypes in different models. Pathogenicity appears also to be influenced by changes at both 5' and 3' non-coding regions. Interestingly, the WNV genome is relatively flexible as it can tolerate a number of changes in its sequence that do not seem to affect pathogenicity. However, generalisation of these findings could be misleading. The spectrum of WNV strains to which they can be applicable needs to be defined, as the phenotypic effect of each of the changes described for one given strain might not affect all WNV strains equally. Care should be exercised not to extend blindly the results observed in a given model to other susceptible species. For instance, the T249P mutation at the NS3 increases pathogenicity of a Kenyan WNV L1a strain for American crows (*Corvus brachyrhynchos*), and the opposite mutation reverts the high pathogenicity observed for the NY99 WNV strain in this species. However, this effect applies for American crows and not for house sparrows [79]. Moreover, Mediterranean WNV L1a strains with the $NS3_{249}P$ genotype did not show higher pathogenicity than their $NS3_{249}T$ counterparts either in mice [80], in the red-legged partridge (a bird species indigenous to southern Europe) [67], and in the house sparrow [68], although it has been recently shown that this mutation can modulate WNV pathogenicity of certain Mediterranean WNV strains for the European corvid, *Corvus corone* (carrion crow) [81]. Interestingly, the Greek WNV lineage 2 strains from the outbreaks in 2010 and 2011, involving human morbidity and mortality, have been shown to bear the $NS3_{249}P$, contrary to their likely precursors from central Europe and the WNV strains that caused outbreaks in Serbia and in Italy, which have a histidine residue instead of proline at this site [58]. Despite suggestive, the association between the high pathogenicity observed in human outbreaks and the presence of $NS3_{249}P$ in these Greek lineage 2 WNV strains need to be assessed experimentally. Also, phenotypic assessment of WNV strains should not be limited to pathogenicity but should take in consideration other phenotypic traits, such as host competence (capacity of a given host species to transmit the virus efficiently to a mosquito feeding on its blood)/transmissibility. Variations in host competence have been reported for different WNV strains in the house sparrow [68,69], which might help to explain, at least in part, the different epidemiological patterns observed in the New versus the Old World.

Figure 2A

Suggested harmonised West Nile virus lineage numbering based on phylogenetic analysis[a]

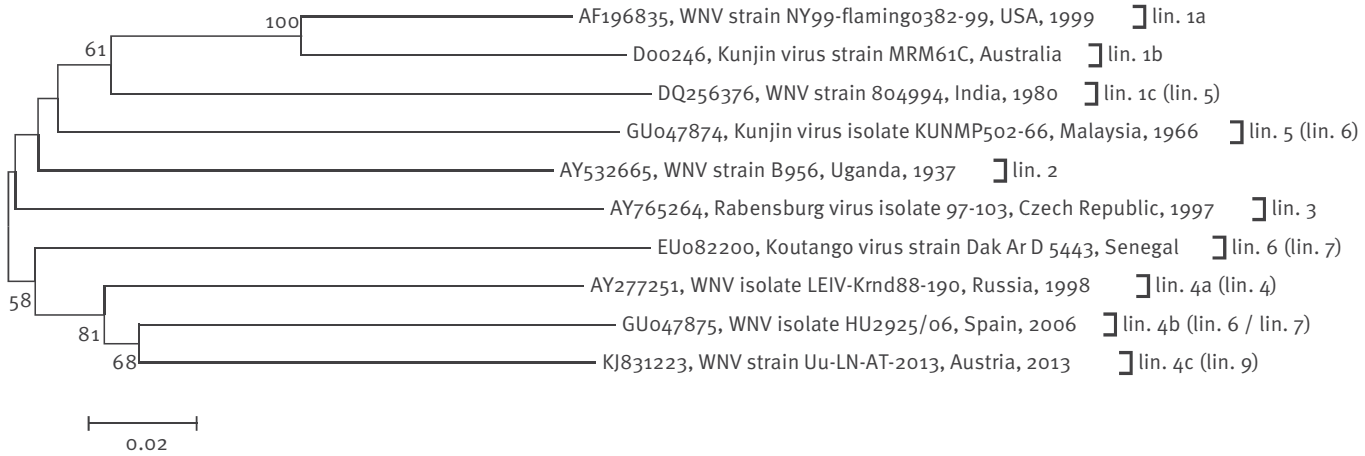


ClustalW alignments were conducted using BioEdit Sequence Alignment Editor (version 7.0.9.0). Phylogenetic neighbour-joining trees were generated with MEGA5 software [114], using 1,000 replicates for bootstrap testing, and evolutionary distances computation with the p-distance model. Bootstrap values less than 50% are hidden. The suggested new lineage numbering is indicated (in brackets other lineage designations previously used in literature are shown). In Figure 2A the phylogenetic tree was constructed on the basis of the complete polyprotein-encoding nucleotide sequences of 29 WNV strains including representatives of all WNV lineages for which complete polyprotein-encoding nucleotide sequences have been available. In Figure 2B the phylogenetic tree was constructed on the basis of 799 bp fragments within the NS5 gene, which enabled inclusion of the Malaysian Kunjin virus isolate GU047874 and the Spanish WNV strain GU047875, as well as eight selected WNV strains representing other WNV lineages. In Figure 2C the phylogenetic tree was constructed on the basis of 1502 bp fragments within the envelope glycoprotein gene, which enabled inclusion of the Senegalese WNV isolate KJ131502 as well as 8 selected WNV strains representing other WNV lineages.

[a] Other lineage labelling in the literature is given between brackets.

Figure 2B

Suggested harmonised West Nile virus lineage numbering based on phylogenetic analysis[a]

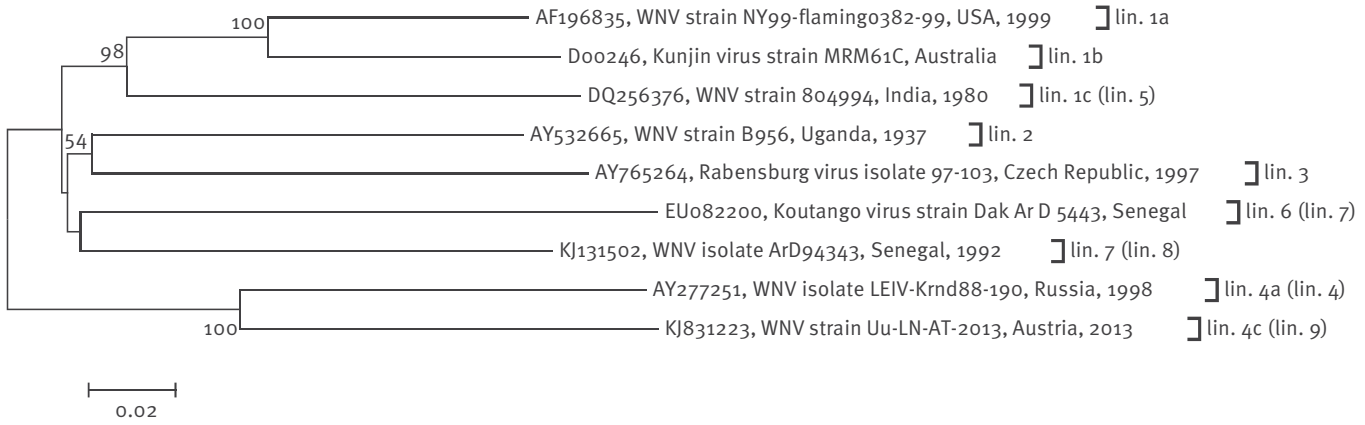


ClustalW alignments were conducted using BioEdit Sequence Alignment Editor (version 7.0.9.0). Phylogenetic neighbour-joining trees were generated with MEGA5 software [114], using 1,000 replicates for bootstrap testing, and evolutionary distances computation with the p-distance model. Bootstrap values less than 50% are hidden. The suggested new lineage numbering is indicated (in brackets other lineage designations previously used in literature are shown). In Figure 2A the phylogenetic tree was constructed on the basis of the complete polyprotein-encoding nucleotide sequences of 29 WNV strains including representatives of all WNV lineages for which complete polyprotein-encoding nucleotide sequences have been available. In Figure 2B the phylogenetic tree was constructed on the basis of 799 bp fragments within the NS5 gene, which enabled inclusion of the Malaysian Kunjin virus isolate GU047874 and the Spanish WNV strain GU047875, as well as eight selected WNV strains representing other WNV lineages. In Figure 2C the phylogenetic tree was constructed on the basis of 1502 bp fragments within the envelope glycoprotein gene, which enabled inclusion of the Senegalese WNV isolate KJ131502 as well as 8 selected WNV strains representing other WNV lineages.

[a] Other lineage labelling in the literature is given between brackets.

Figure 2C

Suggested harmonised West Nile virus lineage numbering based on phylogenetic analysis[a]



ClustalW alignments were conducted using BioEdit Sequence Alignment Editor (version 7.0.9.0). Phylogenetic neighbour-joining trees were generated with MEGA5 software [114], using 1,000 replicates for bootstrap testing, and evolutionary distances computation with the p-distance model. Bootstrap values less than 50% are hidden. The suggested new lineage numbering is indicated (in brackets other lineage designations previously used in literature are shown). In Figure 2A the phylogenetic tree was constructed on the basis of the complete polyprotein-encoding nucleotide sequences of 29 WNV strains including representatives of all WNV lineages for which complete polyprotein-encoding nucleotide sequences have been available. In Figure 2B the phylogenetic tree was constructed on the basis of 799 bp fragments within the NS5 gene, which enabled inclusion of the Malaysian Kunjin virus isolate GU047874 and the Spanish WNV strain GU047875, as well as eight selected WNV strains representing other WNV lineages. In Figure 2C the phylogenetic tree was constructed on the basis of 1502 bp fragments within the envelope glycoprotein gene, which enabled inclusion of the Senegalese WNV isolate KJ131502 as well as 8 selected WNV strains representing other WNV lineages.

[a] Other lineage labelling in the literature is given between brackets.

Diagnosis

The laboratory diagnosis of acute WNV infection is based on both the detection of WNV RNA in blood and cerebrospinal fluid (CSF) or virus isolation in cell culture from serum samples (direct diagnosis) and on the demonstration of a specific immune response against the virus (indirect diagnosis), recently reviewed by [82] and [83].

Detection of viral RNA

Detection of WNV RNA in biological specimens represents a rapid method to unambiguously prove the infection with WNV [84]. Different PCR-based protocols have been developed to amplify minimal amounts of WNV RNA [85]. However, viraemia is short-lived in dead-end hosts and already declining substantially once the symptoms begin. Hence, detection of viral genomes becomes increasingly challenging over time. As a consequence, WNV RNA is generally not detectable in the blood of patients with symptomatic infection [86]. Additionally highly sensitive nucleic acid amplification-based methods may rarely provide false-positive results by cross-reaction with other flaviviruses [87]. Figure 3 delineates a timeline of clinical and diagnostic markers during human WNV infection.

Recent data demonstrated that WNV RNA can be detected in urine much longer and at higher concentrations than in blood or CSF in individuals with WNF or WNND [86,88]. The duration of WNV RNA detection in urine seems dependent on the course of disease and could last for 20 days or longer [86].

Similar results were observed in experimentally infected monkeys and hamsters [89,90]. The virus excreted in urine is infectious, since it can be isolated in cell culture from urine specimens collected from patients with acute infection [91].

Implementation of WNV RNA testing and isolation from urine samples in routine protocols for WNV diagnosis demonstrated the utility of these tests for the confirmation of cases [30,86,88,91]. Although PCR methods have been developed to detect both major WNV lineages [92], a first external quality assessment (EQA) study in 2006, revealed that many laboratories had problems in detecting genomes of WNV lineage 2 [93], an issue of high concern since lineage 1 and 2 viruses are co-circulating in Europe. In a recent second EQA in 2011, the participating laboratories had improved significantly regarding this aspect [87]. PCR multiplexing can be useful in this respect, and recently a multiplex PCR method able to detect and differentiate WNV lineages 1 and 2 and USUV has been developed [94]. It is also essential that current PCR methods are constantly being checked and updated for their sensitivity and suitability to detect newly emerging WNV strains to cope with the observed genetic variability of European WNV strains.

Antibody detection

Antibodies against WNV start to appear about four to seven days following infection and IgM antibodies are already detectable at early time points after symptoms onset [86] (Figure 3). Thus, the diagnosis of WNV infection generally relies on the demonstration of specific antibodies against WNV in serum or CSF.

The major problem with most serological diagnostic tests for WNV is cross-reactivity with infections caused by other flaviviruses, and the last EQA on WNV serology organised by the European Network for Diagnostics of Imported Viral Diseases (ENIVD) also showed the limits and needs for improvement [95]. Therefore, considerable effort is being made to develop antigens and/or test formats which can be used for specific detection of anti-WNV antibodies [reviewed in 83]. These also have to take into consideration the heterogeneity of human antibody profiles to WNV infection [96]. Furthermore, several flaviviruses have been found in areas affected by WNV, including BAGV, tick-borne encephalitis virus TBEV , and USUV [22,53,97,98].

Taking into account these problems, the European Union case definition for WNV infections demands confirmation in cases with IgM detection in serum by virus neutralisation test (VNT) [99], which today is the gold standard diagnostic method for flavivirus serology (reviewed in [83]). However, VNTs are time-consuming and require a biosafety level (BSL)-3 laboratory. Monoclonal antibody panels with strong and specific reactivity to TBEV, USUV and WNV were recently developed [100], which will likely enable the development of improved immunoassays for the detection and differentiation of flavivirus infections. Furthermore, monoclonal antibodies could be used for the development of competitive tests for the detection of different types of anti-WNV antibodies suitable for the range of WNV host species, including many species of wild birds and susceptible mammals [101]. Alternatively, the use of mutant forms of viral proteins leads to a minimisation of antibody cross-reactivity and enables the serological differentiation of flavivirus infections [102].

Vaccines

The persistent long-term effects seen after resolution of acute WNV infection in humans, the emerging threat that WNV poses to Europe and the placement of WNV on the list of bioterrorism agents, makes the development of a safe and effective vaccine for humans an urgent priority [103]. In fact, while several vaccines are already available for horses [104-106] and other currently under development due to the impact of WNV infection on the horse industry, no vaccine is available for humans.

Towards the production of a human vaccine

The increasing understanding of WNV pathogenesis and correlates of protection in animal models pave the way to a more rational design of candidate vaccines. Studies performed in mice elucidated some of

Table 2

Non-exhaustive list of genetic determinants of West Nile virus pathogenicity identified in animal models

Genome region	Genetic change	Phenotypic effect(s)/mechanism(s) involved	Phylogenetic group	Animal model used	Experimental system	References
E	Various, on the 154-NYS(T)-156 (N-glycosylation) motif	N-glycosylation site abolished; unstable E-peptide fusion; decreased viral replication.	Lineages 1 (clades a and b) and 2	Mice	cDNA clone mutagenesis	[70,71]
NS2A	A30P	NS1' (NS1 extension) abolished; disruption of NS1-mediated immune evasion mechanisms.	Kunjin (Lineage 1, clade b)	Mice	cDNA clone mutagenesis	[72,73]
NS3	T249P	Increased virogenesis, efficient replication at higher temperatures.	Lineage 1, clade a	American crow	cDNA clone mutagenesis	[74]
NS4B	C102S P38G E249G	Abrogation of evasion from host innate immunity (IFN α/β response); decreased helicase activity (decreased virus replication).	Lineage 1 clade a	Mice	cDNA clone mutagenesis	[75,76]
3'UTR	Unknown	sfRNA abrogation.	Lineage 1, clade b	Mice	cDNA clone mutagenesis	[77]
5'UTR	5-AAT/TTG-52	Unknown	Lineage 1, clades a and b	Mice	cDNA clone mutagenesis and chimeras	[78]

WNV: West Nile virus.

The list represents widely recognised genetic changes associated with WNV virulence/attenuation that have been shown to occur in natural WNV isolates.

the mechanisms of protection, such as the role of the adaptive immune responses in mitigating and preventing development of disease. This is exemplified by the observation that transfer of WNV-specific antibodies to naïve mice protected the animals against development of severe neurological disease [107]. Several lines of evidence indicate that the B cell response after infection with WNV is predominantly directed to non-neutralising epitopes located in domain I and II of glycoprotein E. The most potent neutralising epitopes are located in domain III (the receptor-binding ligand). For the development of a WNV vaccine for humans it is important to understand how vaccination could increase the longevity of B cells that produce these potent neutralising antibodies. As the probability of neuroinvasion in the animal host appeared to correlate with the level and duration of viraemia, it is conceivable that persistence of neutralising antibodies is sufficient to protect against severe disease [108]. Although T cells play a role in eliminating an established infection, thereby reducing the antigen load, it is believed that antibodies are more important for vaccine-induced protection. It is worth noting that both CD4+ and CD8+ T cells have been shown to contribute to control of WNV infection in animal models but it cannot be excluded that T cells may also cause disease [109].

Several platforms have been used to develop candidate vaccines against WNV for humans [109]. Various vaccine candidates have been shown to be highly effective in preventing fatal disease in mice and horses [110-112]. Long-term immunogenicity and safety, however, are some of the most important problems in current veterinary vaccines against WNV and potential candidates for use in humans. The regulatory authorities as well as public opinion demand safe and well characterised vaccines. Therefore, an effective WNV vaccine should stimulate protective and long-term immunity, preferably after a single dose, and should be free of significant side effects, especially in the elderly and immunocompromised individuals. Research on the use of several platforms for development of a safe and effective vaccine against WNV lineages 1 and 2 was an important topic in several European consortia and yielded promising candidate approaches.

Knowledge gaps and research priorities

Although significant progress in the scientific knowledge on WNV in Europe has been made through projects funded by the FP7, several knowledge gaps still limit our ability to properly forecast changes in the risks of outbreaks to occur or to prevent and control virus transmission to humans and animals.

Knowledge gaps on the introduction and spread of WNV in Europe

Our knowledge on how, when and from where WNV is introduced into Europe is still very limited today. The role of migrating birds on WNV translocations is recognised, however, many important questions still remain unanswered e.g. the geographic origin of each introduction of WNV into Europe, the relative importance of the introduction of infected vectors rather than infected birds, and the detailed transmission mechanisms involved in virus establishment into new areas. For these reasons, better knowledge on the WNV ecology, vectors and virus strains circulating currently in Africa will be of utmost importance. Key factors leading to virus amplification and spread to neighbouring areas are also essentially unknown. Furthermore, we know that the virus may disappear from a previously infected area but we ignore the key factors leading to

its extinction. Spillover events are increasing in numbers and expand geographically over time in Europe, however, our knowledge on the factors triggering such events continues to be rather poor. We do not know in most cases which are the key amplifying bird host and the bridge vector species involved. Moreover, we do not know the consequences of interventions such as massive culling of species considered as 'pests' and the contribution of factors such as species community composition under variable climatic and environmental conditions at local level. With this level of eco-epidemiological uncertainty, building-up accurate predictive models of WNV outbreak risk is a complex undertaking, although progress has been made in this direction [46,47,113]. Epidemiological models rely on good knowledge on basic epidemiological information of the virus and the disease it causes, which is still lacking in many cases, as outlined above. Filling this gap will not be an easy task; for instance, competence for WNV transmission of each host and vector species must be calculated experimentally for each viral strain, but given the heterogeneity of WNV found in Europe, this work will require a huge effort even if restricted to few representative strains and host/vector species.

Knowledge gaps on the impact of the presence of other flaviviruses on WNV epidemiology

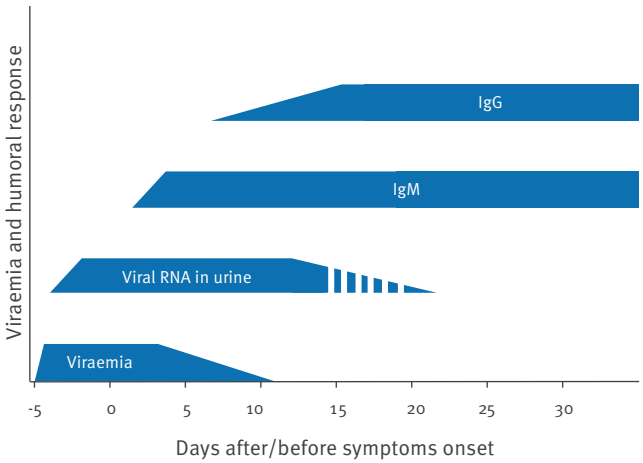
Another important knowledge gap is the impact that other co-circulating flaviviruses (and perhaps arboviruses) may have on WNV epidemiology in Europe. Cross-immunity between closely related flaviviruses might lead to some degree of cross-protection in the animal hosts, or, even enhance infection in certain cases. Further assessment of the consequences of viral interference on the immune response both in hosts and vectors in Europe is needed. Realistic WNV models should also incorporate these effects. The diversity of flaviviruses circulating in Europe, including mosquito-only flaviviruses as well as newly emerging flavivirus strains, represent another challenge for WNV research. Europe's preparedness to face emerging flavivirus threats depends largely on the availability of large datasets integrating genomic characterisation and analysis of all the strains isolated in Europe and neighbouring areas, including Africa. These datasets, together with the already started bank of viral strains, will be of paramount importance for the development of efficient molecular tools enabling proper flavivirus detection, identification and classification, along with studies on the origins of the different flaviviruses emerging in Europe and their evolutionary history.

Knowledge gaps on mosquito control

There is a need to strengthen the entomological capacity in Europe to improve surveillance, and risk assessment, and to identify the most appropriate and environmental friendly control strategies especially within hot spots of arbovirus emergence and spread in the Old World. The number of products available for mosquito control has been reduced in recent years. New insecticides and repellents are urgently needed since those available are losing efficacy. There is a clear need to define and evaluate the effectiveness of integrated mosquito control strategies and involve the public in mosquito source reduction. Models to support decision in mosquito control and management are also urgently required.

Figure 3

Viraemia and humoral immune responses against West Nile virus



Knowledge gaps on the identification of virulence factors

Virus strain characterisation, in particular, virulence studies and identification of virulence determinants demand proper experimental animal models. Rodents (mice, hamsters) used as surrogate incidental mammal host model still need some standardisation and the same applies to wild birds, used as susceptible, reservoir and amplification host models useful to obtain data on virus pathogenicity and transmission. Suitable animal models would not only constitute an invaluable tool for virus characterisation, but also enable studies on co-infection, cross-protection, vaccine efficacy, therapeutic testing, the effect of stress and immunosuppression during the course of infection, among others.

Knowledge gaps on laboratory detection

The challenge in diagnostics is the WNV diversity in Europe and the need to continuously check the effectiveness of existing laboratory tests for the detection of newly arising strains or antibodies to them. This requires a considerable effort from all WNV diagnostic laboratories involved. The main gap in WNV serology is to develop new generation immunoassays avoiding cross-reactions with different flaviviruses and enabling good flavivirus differentiation, minimising the need for confirmation by the gold standard VNT. For that, large panels of well-characterised monoclonal antibodies to different flaviviral epitopes would be invaluable. Development of markers of early/mature immune response is also desirable. In the veterinary field, serological tests differentiating infected from vaccinated

individuals are largely awaited. Where possible, inclusion of different types of antigens in the immunoassays would help to avoid false negative results due to heterogeneity of the humoral immune responses to WNV. In molecular diagnostics, new methods enabling rapid differential diagnosis of flavivirus infections, preferably in multiplex formats, are needed. New samples for virus detection and isolation (for instance, urine in humans, or feathers in wild bird surveillance) need further validation to assess their impact on diagnostic sensitivity and specificity, also, when possible, with different WNV strains.

Knowledge gaps on therapy and prevention

The lack of specific treatment and of human vaccines constitutes another gap in the mitigation of WNND. Together with the high susceptibility of the elderly, this renders the disease of great societal impact because of the ageing of the European population. The spread of WNV, even in the absence of clinical cases of WNND, imposes a significant economic burden for European public health due to the need to guarantee blood and organ transplantation safety.

Gaps in vaccine development include safety issues, especially in target susceptible populations such as the elderly and immunocompromised individuals. Due to the unpredictable regional spread of WNV outbreaks, clinical effectiveness testing of WNV vaccines in humans seems difficult. In combination with the yet limited market size, this has so far prevented further development of vaccine candidates by the pharmaceutical industry. Therefore, in order to move one or more of the existing (and promising) candidate vaccines towards clinical development, there needs to be a joint evaluation between industry, researchers and regulatory authorities. The possible interactions with other available flavivirus vaccines in Europe such as the ones against TBE and yellow fever virus (the latter only used for travellers to endemic areas outside Europe), and interactions with infections by other related flaviviruses, including cross-protection and/or antibody-dependent enhancement of infection, need further clarification.

Conclusion

The unpredictability of the West Nile virus risk for Europe and neighbouring countries is strongly linked to the knowledge gaps on many aspects of its complex ecology, genomic diversity, pathogenicity, transmissibility, diagnosis and control options, under different environmental and socio-economic scenarios. Therefore, there is an urgent need to intensify and continue the research efforts on WNV and other emerging vector-borne infections built under the FP7. In parallel, there is a growing need for a unified, harmonised, real-time epidemiological surveillance of WNV in vectors, humans and animals, especially within hot spots of virus circulation in Europe under the guidance of ECDC and other international organisations.

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Conflict of interest

None declared.

Authors' contributions

AT conceived and coordinated the various contribution to the manuscript; AR and MAJC coordinated, wrote, reviewed and edited the final version of the manuscript; AR, AT, LB, NN, and MAJC drafted the contribution on virology and epidemiology; AR and JF drafted the contribution on ecology; NP, MAJC and PK drafted the contribution on pathogenesis; LB, SU, PC and AM drafted the contribution on diagnosis; BM and NS drafted the contribution on vaccine; AR and MAJC drafted the contribution on knowledge gaps. All contributors participated in the final revision of the manuscript.

*Note

This designation is without prejudice to positions on status, and is in line with United Nations Security Council Resolution 1244/99 and the International Court of Justice Opinion on the Kosovo declaration of independence.

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West Nile virus circulation in south-eastern Romania, 2011 to 2013

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Lineage 2 West Nile virus (WNV), previously found only in sub-Saharan Africa and Madagascar, was identified in Hungary in 2004 and has rapidly expanded in Europe in the past decade. Following a significant outbreak of West Nile fever with neurological cases caused by lineage 1 WNV in Romania in 1996, scattered cases have been recorded in the south-east of the country in each transmission season. Another outbreak, affecting a larger area and caused by lineage 2 WNV, was recorded in 2010. We analysed human sera from neuroinvasive West Nile fever cases and mosquitoes, sampled in south-eastern Romania between 2011 and 2013, for the presence of WNV genome, and obtained partial NS5 and envelope glycoprotein sequences. Human- and mosquito-derived WNV sequences were highly similar (99%) to Volgograd 2007 lineage 2 WNV and differed from isolates previously detected in central and southern Europe. WNV was detected in one pool of *Culex pipiens* s.l. males, documenting vertical transmission. Lineage 4 WNV, of unknown pathogenicity to mammals, was found in the amphibian-feeding mosquito *Uranotaenia unguiculata* from the Danube Delta. Our results present molecular evidence for the maintenance of the same isolates of Volgograd 2007-like lineage 2 WNV in south-eastern Romania between 2011 and 2013.

Introduction

WNV is by far the most widely distributed arbovirus. It belongs to the Japanese encephalitis antigenic complex of the family *Flaviviridae*, transmitted in an avian cycle by ornithophilic mosquitoes, mainly of the genus *Culex*. Mammals can also be infected, but are considered dead-end hosts because viraemia is generally too low to infect mosquitoes. Eighty per cent of human infections are asymptomatic, and less than 1% of clinical cases lead to neuroinvasive disease [1,2].

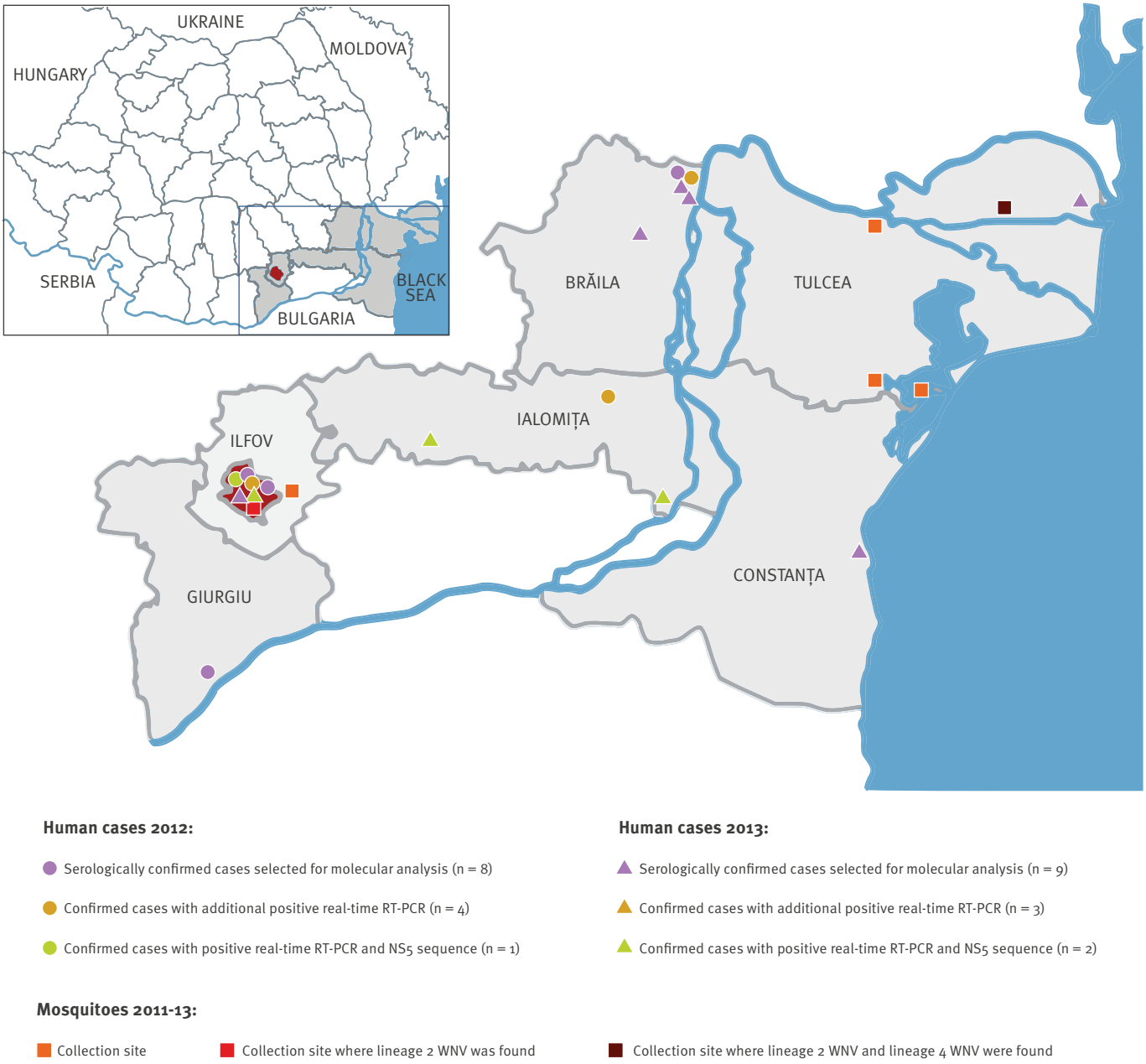
Evidence of WNV circulation in Europe dates back to the early 1960s [3], lineage 1 isolates being responsible for the major outbreaks. A recent retrospective study indicates the circulation of lineage 2 WNV in birds (*Sylvia nisoria*) in Cyprus as early as 1968 [4]. Starting in 2004, lineage 2 WNV was identified in Hungary in birds of prey and was subsequently found again in Hungary in 2005 and in Austria in 2008 and 2009 [5-7]. Erroneously considered to be non-pathogenic for humans and with a distribution restricted only to sub-Saharan Africa and Madagascar [8], lineage 2 isolates caused outbreaks of WNV infection in Russia (2007), Greece (2010–2013), Romania (2010), Italy (2011, 2012), Serbia (2012, 2013) and Croatia (2012, 2013) [9-21].

Following the unprecedented epidemic of West Nile fever in south-eastern Romania in 1996 [22], caused by a lineage 1 strain [23], scattered human cases were recorded every year until a second significant outbreak occurred in 2010 [14]. In the latter, the affected area also included counties in the north-east of the country as well as in Transylvania, beyond the Carpathian mountains. The WNV detected in one human serum was lineage 2, closely similar to an isolate from a patient during the outbreak in Volgograd, Russia in 2007 [14].

According to the data provided by the National Institute of Public Health, laboratory-based surveillance of neuroinvasive West Nile infection was carried out in 2012 as previously described [14], and detected one probable and 14 confirmed cases among 128 suspected cases. The majority of West Nile fever cases were recorded in south-eastern Romania: seven in Bucharest

Figure 1

Distribution of confirmed human cases of West Nile fever selected for molecular investigation and mosquito collection sites by county, Romania, 2011–2013



WNV: West Nile virus.
Counties shown at NUTS 3 level. Bucharest city is highlighted in russet.

city, two (one probable and one confirmed case) in the adjacent Ilfov county and in Ialomița (one case), Brăila (two cases), and Giurgiu (two cases) counties. A single case was also recorded in Iaşi, north-eastern Romania. Dates of onset were from 31 July to 11 September 2012. One death was recorded. In 2013, 22 confirmed and two probable cases were recorded among 142 suspected cases tested. Dates of onset were from 17 July to 19 September 2013. No deaths were reported (source: National Centre for Surveillance and Control of Communicable Diseases, National Institute of Public Health). The majority of West Nile fever cases in that year also occurred in south-eastern Romania: Ialomița (four confirmed cases), Brăila (one probable and four confirmed cases), Tulcea (one probable case and two confirmed cases), Constanța (two confirmed cases), Bucharest city (one confirmed case) and the adjacent Ilfov county (one confirmed case). Another five cases were recorded in the north-eastern part of the country: Iași (two confirmed cases), Galați (two confirmed cases) and Bacău (one confirmed case). Bacău county is a new affected area. Three cases were recorded in

Table 1

Mosquito pools tested for West Nile virus genome, Romania, 2011–2013

Collection area	2011 pools	2012 pools	2013 pools
	Tested/real-time RT-PCR-positive/sequenced		
Tulcea county	95/3/2	388/70/10	508/109/16
Bucharest and surroundings	9/0/0	87/5/1	98/2/1
Total number of mosquito pools/total number of mosquitoes	**104/3,291**	**475/12,159**	**606/15,405**

the central part of the country (Mureş and Sibiu counties). Sera from these three cases were also tested for the presence of tick-borne encephalitis virus-specific antibodies because this flavivirus had previously been found to be circulating in this area.

Here we present the molecular characterisation of WNV circulating between 2011 and 2013 in humans and mosquitoes in south-eastern Romania, an area of endemic WNV circulation as shown by previous human and animal host surveillance studies [24,25].

Methods

Human cases

Sera collected in 2012 and 2013 from patients with confirmed WNV neuroinvasive infection living in south-eastern Romania were included in this study (Figure 1). Only samples collected in the first seven days post onset were selected for molecular investigation. Viral RNA was extracted from sera using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany).

Mosquito collection and processing

Adult mosquitoes were collected from two main areas: Tulcea county and Ilfov county including Bucharest. Four sites were investigated in Tulcea county: Mila 26 (in the core of the Danube Delta), Sălcioara and Grindul Lupilor (on the Razim lagoon shore) and Tulcea city (Figure 1). In Tulcea county, mosquitoes were collected by overnight capture in cylindrical traps baited with birds (chickens) and small rodents (guinea pigs) or collected from vegetation with a backpack aspirator. In the Danube Delta and the lagoon shore, collections were performed for periods of five to eight days in August and September 2011 and from May to October in 2012 and 2013. In Bucharest and the periurban area of the city, mosquito captures were performed between July and September (2011–13) using CDC Gravid Traps [26] and BG Sentinel Traps (Biogents AG, Germany). Mosquitoes were also collected from resting sites such as hallways of buildings using hand aspirator.

For RNA extraction (QIAamp Viral RNA Mini Kit, Qiagen, Hilden, Germany), the mosquitoes were processed as follows: they were identified using an entomological key [27], and pooled by species, sex and physiological age in pools never exceeding 50 individuals.

Molecular analysis

All samples (mosquito pools and human sera) were screened for the presence of WNV genome by one-step real-time RT-PCR using a commercial kit (West Nile Virus Real-TM, Sacace Biotechnologies). Positive samples were further tested by RT-PCR using primers VD8, FU2, cFD3 and WNV9368f, targeting the NS5 genomic region [5,28,29]. When possible, a fragment spanning the envelope glycoprotein region (E) was amplified using primers WNVII 870f and WNVII 1630r [5]. All amplicons were sequenced (3130 Genetic Analyzer, Applied Biosystems) and the resulting sequences were aligned with ClustalW, BioEdit version 7.0.5.3 [30]. Maximum-likelihood phylogenetic analysis was conducted with Mega 6 software [31], which was also used for choosing the fittest nucleotide substitution model. The reliability of the phylogenetic trees was tested with 1,000 bootstrap replicates.

Results

Human samples

In 2012, serum samples from eight patients with serologically confirmed WNV infection, all in the acute phase of the disease, were tested by real-time RT-PCR.

Table 2

Mosquito species found to be positive for West Nile virus by sequencing and real-time RT-PCR, Romania, 2011–2013

Mosquito species	2011 pools	2012 pools	2013 pools
	Sequenced/real-time RT-PCR-positive/pools tested		
Culex pipiens s.l.	2[a]/2/68	5[a]/47[b]/343	13[a]/51/369
Culex modestus	0/1/21	3[a]/15/56	0/46/131
Coquillettidia richiardii	NA	0/5/30	2[a]/8/54
Anopheles hyrcanus	NA	1[a]/4/23	0/3/21
Uranotaenia unguiculata	NA	2[c]/3/4	2[c]/2/4
Ochlerotatus caspius	NA	0/1/10	NA
Anopheles maculipennis complex	NA	NA	0/1/18

NA: not applicable.

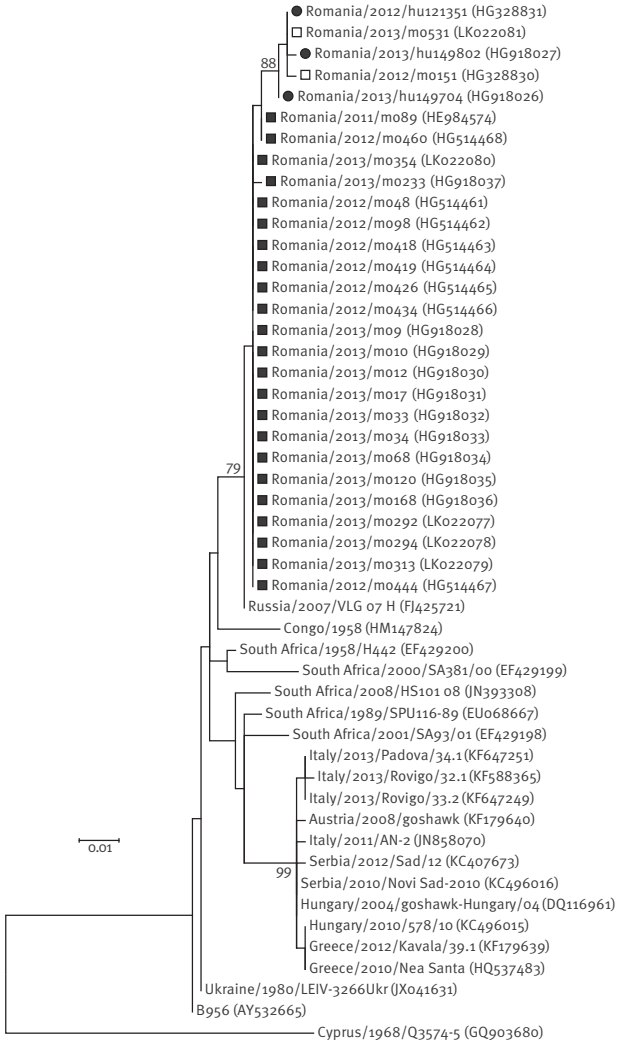
[a] West Nile virus lineage 2.

[b] 46 pools of females and one pool of males.

[c] West Nile virus lineage 4

Figure 2

Phylogenetic tree of lineage 2 West Nile viruses based on NS5 partial sequences, Romania, 2011–2013



Black squares: sequences obtained in this study from mosquitoes collected in Danube Delta (Mila 26); white squares: sequences obtained in this study from mosquitoes collected in Bucharest city; black circles: sequences obtained in this study from human sera. Numbers at nodes represent the bootstrap percentages (values<70% are not shown).

The analysis was conducted on a 466 nt sequence (nt positions 9,463–9,928 in isolate Reb_VLG_07_H, GenBank acc. no. FJ425721) using maximum-likelihood method, Kimura 2-parameter model, 1,000 bootstrap replicates. A sequence obtained from strain Q3574–5 (Cyprus, 1968; GenBank acc. no. GQ903680) was used as an outgroup.

All patients lived in south-eastern Romania: Bucharest city (four cases) and the counties of Brăila (two cases), Giurgiu (one case) and Ialomița (one case). WNV genome was detected by real-time RT-PCR (Ct values: 28.1–34.4) in four of these samples: Bucharest city (two cases), Brăila (one case) and Ialomița (one case) (Figure 1).

In 2013, nine serum samples fulfilled the inclusion criteria: Bucharest city (two cases) and Brăila (three cases), Constanța (one case), Ialomița (two cases), Tulcea (one case) counties. WNV genome was found in the samples from one patient living in Bucharest city and two from Ialomița county (Ct values: 29.85–33.65) (Figure 1).

In summary, during the two years of investigation, we detected seven positive serum samples in the PCR screening assay. Only one sample from 2012 and two from 2013 yielded an NS5 amplicon suitable for obtaining a DNA sequence. None of the above samples yielded an E amplicon suitable for sequencing.

Mosquito samples

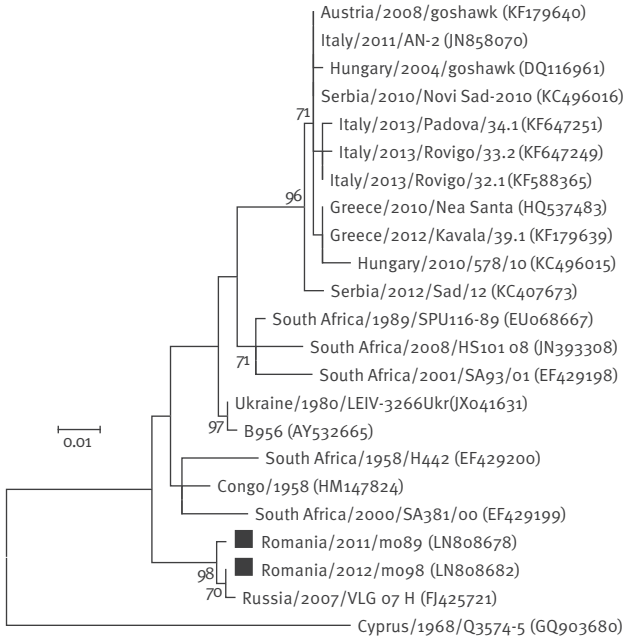
In 2011 to 2013, we collected and analysed 30,855 mosquitoes. About 75% of these insects were captured in the Danube Delta, and 189 of the 1,185 mosquito pools tested were real-time RT-PCR-positive for WNV genome (Table 1). As expected, the majority of these pools consisted of *Culex pipiens* s.l., followed by *Cx. modestus*. Other mosquito species were also found real-time RT-PCR-positive for WNV genome (Table 2). Of interest was the detection of WNV genome in a pool of *Cx. pipiens* s.l. males collected in 2012 in Bucharest. Unfortunately, no amplicon for sequencing could be obtained from this sample. During the three years of the study, we obtained 30 DNA sequences for WNV NS5 derived from mosquito pools containing *Cx. pipiens* s.l., *Cx. modestus*, *Anopheles hyrcanus*, *Coquillettidia richiardii* and *Uranotaenia unguiculata* species (Table 2). Two partial E sequences were also obtained from *Cx. pipiens* s.l. mosquitoes collected in the Danube Delta in 2011 and 2012.

In 2012 and 2013, we identified the WNV lineage 2 genome in sera collected from three patients with meningoencephalitis living in south-eastern Romania. The first detection was in August 2012 in the acute phase serum of a resident of Bucharest. In September 2013, similar WNV isolates were found in a patient living in Bucharest and in a resident of Ialomița county.

Our phylogenetic analysis based on NS5 partial sequences (Figure 2) indicated that the viruses in circulation in Romania between 2011 and 2013 were very similar to a lineage 2 WNV isolated during the outbreak in Volgograd, Russia, in 2007; all our mosquito and human-derived sequences were more than 99% similar to that isolate. The same strain of lineage 2 WNV, 99% identical to Volgograd 2007, has been circulating in Bucharest since 2010 [14]. Although the investigated genomic region is strongly conserved, a bootstrap value of 79% from the node relating the Romanian sequences to the Volgograd 2007 isolate indicated the robustness of the analysis. It is worth mentioning that the sequences obtained from human specimens clustered with two sequences derived from two pools of *Cx. pipiens* s.l. females collected in 2012 and 2013 in Bucharest area. All sequences derived from mosquitoes collected in the same ecosystem (Danube Delta, Mila 26 collecting site), regardless of the year of collection, clustered in a single subclade comprised of three

Figure 3

Phylogenetic tree of lineage 2 West Nile viruses based on envelope glycoprotein (E) sequences, Romania, 2011–2012



Black squares: sequences obtained in this study from mosquitoes collected in Danube Delta (Mila 26). Numbers at nodes represent the bootstrap percentages (values<70% are not shown).

The analysis was conducted on a 460 nt sequence (positions 934–1,393 in isolate Reb_VLG_07_H, GenBank acc. no. FJ425721) using maximum-likelihood method, Tamura-Nei model, 1,000 bootstrap replicates. A sequence obtained from strain Q3574–5 (Cyprus, 1968; GenBank acc. no. GQ903680) was used as an outgroup.

major groups that differed by a small number of synonymous and non-synonymous nucleotide substitutions.

Furthermore, the phylogenetic analysis based on E partial sequences confirmed the topology of the NS5 tree, placing the Romanian sequences in the same clade with the isolate obtained in 2007 in Volgograd, Russia (Figure 3).

An interesting finding was the detection of lineage 4 WNV in four pools of *Ur. unguiculata* mosquito collected in 2012 and 2013 (Figure 4). Similar strains have already been reported by Russian authors from this mosquito species, known to feed on amphibians, and from frogs collected in Volga Delta, as well as from *Dermacentor marginatus* ticks collected in the Caucasus [32].

NS5 and E partial sequences described in this study are available in GenBank under the following accession numbers: HE984574, HE984575, HG328830, HG328831, HG514461-HG514468,HG918026-HG918037, LK022077-LK022085, LN808678 and LN808682.

Discussion

As shown by sequencing, Volgograd 2007-like lineage 2 WNV isolates were found both in patients with neurological WNV infections (2012 and 2013) and in mosquito vectors (2011–13) in south-eastern Romania.

In 2011, Volgograd 2007-like isolates were detected in two pools of *Cx. pipiens* s.l. mosquitoes collected from the Danube Delta. In 2012, in this area, the same virus was found in nine mosquito pools consisting of specimens belonging to three species: *Cx. pipiens* s.l. (five pools) and *Cx. modestus* (three pools) mosquitoes, already known as WNV principal vectors in Europe [23,33], but also in *An. hyrcanus* (one pool). The results for 2013 in the Danube Delta indicated the presence of the same lineage 2 WNV in 15 mosquito pools belonging to two species: *Cx. pipiens* s.l. (13 pools) and *Cq. richiardii* (two pools). Also, in 2012 and 2013, we detected Volgograd 2007-like isolates in two pools of *Cx. pipiens* s.l. mosquitoes collected in Bucharest city. In Europe, WNV has been previously detected in *Cq. richiardii* and *An. hyrcanus* in the Volga Delta [32].

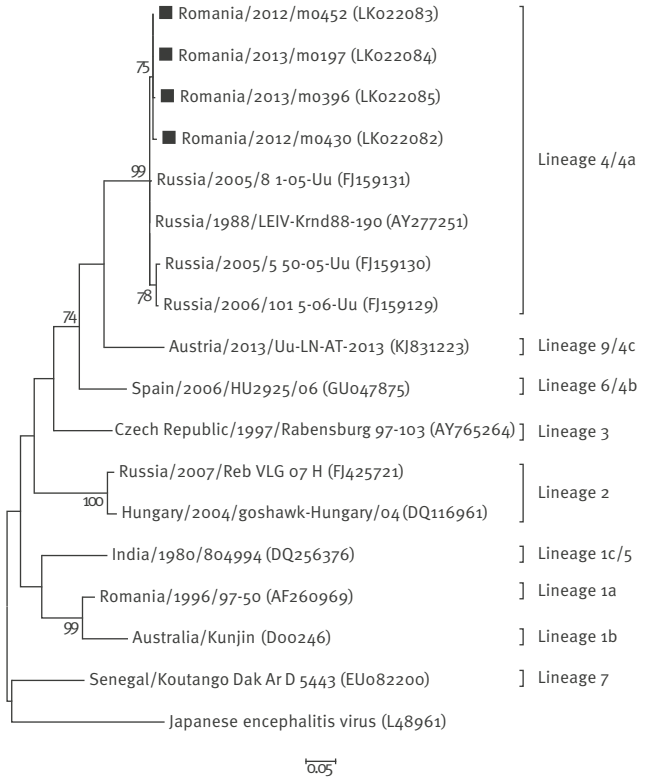
WNV genome was also detected in one pool of *Cx. pipiens* s.l. males collected in 2012 in Bucharest city. To our knowledge, this is the first direct field evidence of vertical transmission of WNV in Europe. WNV has previously been detected in *Culex* spp. males in Kenya [34] and in North America [35,36]. Vertical transmission has also been documented in overwintering *Culex* spp. females [37] and is thought to represent a mechanism for WNV maintenance [38]. The persistence of WNV in a temperate climate may be achieved by overwintering of infected arthropod vectors and by long–term infection in birds [39], and may explain endemic WNV circulation. Indeed, maintenance of this WNV lineage 2 Volgograd-like strain in the same area for three years has been documented by us.

Initially considered to be non-pathogenic for humans [40], lineage 2 WNV was detected in Europe in 2004, in a goshawk (*Accipiter gentilis*) in south-eastern Hungary. It then became established and caused sporadic cases of infection in wild birds, sheep, horses and humans [5,41]. In the following years, similar isolates were detected in birds of prey in eastern Austria [7] and in *Culex* sp. mosquitoes and collared doves (*Streptopelia decaocto*) in Italy [42]. During the period from 2010 to 2013, lineage 2 WNV isolates similar to those in central Europe caused major outbreaks in Greece [10-13]. The Greek isolates from 2010 and 2012 had unique amino acid substitutions (V119I in NS2B; H249P in NS3; S14G, T49A and V113M in NS4) compared with the isolates from Hungary and Austria, which might explain their high pathogenicity [43].

Although there is a high degree of similarity (96%) between our sequences and those obtained from other isolates circulating in central and southern Europe in recent years, our sequences clearly clustered with the Volgograd 2007 isolate. This distribution of a distinct strain of lineage 2 WNV in eastern Europe may be related to the Mediterranean/Black Sea flyway of northward migrating birds, as it is documented that

Figure 4

Phylogenetic tree of lineage 4 West Nile viruses based on NS5 partial sequences, Romania, 2012–2013



Black squares: sequences obtained in this study from mosquitoes collected in Danube Delta (Mila 26). Numbers at nodes represent the bootstrap percentages (values<70% are not shown).

Analysis was conducted on a 365 nt sequence (positions 9,479–9,843, in isolate LEIV-Krnd88–190, GenBank acc. no. AY277251) using maximum-likelihood method, Kimura 2-parameter model, 1,000 bootstrap replicates. A sequence obtained from a Japanese encephalitis virus isolate (GenBank acc. no. L48961) was used as an outgroup.

Lineages were defined as previously proposed [48].

birds play a crucial role in the spread of the virus [44]. The Volga and Danube deltas are also connected by autumn migration, and there is evidence that WNV may be introduced as birds travel to their owerwintering sites in Africa [45]. Our findings, together with other published data suggest that at least two independent introduction events of two different lineage 2 WNV strains occurred in Europe, followed by their subsequent endemisation.

The presence of WNV lineage 4 identified in two consecutive years (2012–13) in four pools of *Ur. unguiculata* mosquitoes captured in the Danube Delta is worth mentioning. Studies between 2002 and 2006 in the Volga Delta, an ecosystem similar to that of the Danube Delta, demonstrated the presence of WNV lineage 4 in *Ur. unguiculata* and in the lake frog *Rana ridibunda* [46]. Lineage 4 (isolate LEIV-Krnd88–190) was first identified in 1988 in *Dermacentor marginatus* ticks collected in the north-west Caucasus [32]. Attempts to propagate the virus in suckling mice or in mammalian or mosquito cell lines have failed. The strains of this lineage seem to be associated with arthropods and amphibians and their pathogenicity for vertebrates is not characterised [46]. Recent studies conducted in Spain and Austria [47,48] reported two WNV strains of unknown pathogenicity which can be assigned to new lineages closely related to lineage 4. The Spanish WNV was found in *Cx. pipiens* mosquitoes, while the Austrian virus was detected in *Ur. unguiculata* mosquitoes. It has been proposed based on NS5 partial sequences that WNV isolates previously found in *Ur. unguiculata* mosquitoes and *Dermacentor marginatus* ticks from Russia should be grouped in a clade designated lineage 4 or 4a, while the sequence from Spain should be classified in lineage 4b or 6, and the WNV sequence identified in mosquitoes from Austria should be comprised in lineage 4c or 9 [48]. As shown in Figure 4, WNV sequences derived from *Ur. unguiculata* mosquitoes collected in Romania clearly cluster in the proposed lineage 4/4a, along with the Russian isolates. It has been speculated that less or non-pathogenic WNV strains may infect birds, conferring them immunity, thus limiting the spread of pathogenic strains [47].

Conclusion

The neurovirulent strain Volgograd 2007-like of lineage 2 WNV has been circulating in Romania in mosquito populations and causing disease in humans since at least 2010, as shown by previous [14] and present findings. The distribution of this strain may be linked to the flyways connecting Africa to eastern Europe and the Danube and Volga deltas, followed by virus maintenance and endemic circulation in the region.

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Conflict of interest

None declared.

Authors' contributions

SD, IRPG and GO set up and performed molecular diagnostic, sequencing and phylogenetic analysis. IRPG, DB and CSC performed serological diagnosis of human cases. AIC and IRPG performed molecular detection tests in mosquitoes pools. AS managed West Nile fever surveillance program. FLP and EF performed field collection of mosquitoes, their identification and processing for molecular analyses. SD and CSC wrote the paper. PR coordinated the entomological work and provided a critical review of the manuscript.

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