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Decreased effectiveness of the influenza A(H1N1) pdm09 strain in live attenuated influenza vaccines: an observational bias or a technical challenge?

PM Penttinen [1] , MH Friede [2]
1. European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden
2. Initiative for Vaccine Research (IVR), World Health Organization (WHO), Geneva, Switzerland

Correspondence: Pasi M Penttinen (Pasi.Penttinen@ecdc.europa.eu)

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There are currently two types of approved influenza vaccines: inactivated or recombinant vaccines, and live attenuated vaccines. The live attenuated influenza vaccines (LAIV) constructed on a backbone of an A/Leningrad virus strain into which the seasonal haemagglutinin (HA) and neuraminidase (NA) selected for the vaccine were inserted by reassortment, were used in the former Soviet Union for over 50 years [1]. Since the early 2000s, a different attenuated virus strain based on the A/Ann Arbor strain, has been approved for vaccine manufacturing in the United States (US) and more recently in the European Union/European Economic Area (EU/EEA) [2,3]. The proposed advantages of the LAIV were that they had superior efficacy compared to inactivated vaccines in young children [4], they were programmatically more suited to immunisation of children [5] and improved cost-effectiveness could potentially be achieved with childhood LAIV programmes [5-7]. LAIV have also been shown to be of great use in pandemic response since the production yield (doses per egg) is much greater than for inactivated vaccines, and the time between production and release is shorter. In addition, the nasal route of delivery could facilitate rapid population-wide immunisation during pandemics.

The technology to produce pandemic LAIV based on the A/Leningrad backbone has been licensed to the World Health Organization (WHO) for manufacture and use in developing countries. It is estimated that a total production capacity of pandemic LAIV will be ca 500 million doses by 2018 (data not shown). A loss of seasonal LAIV production capacity would impact this pandemic response capacity, and is therefore of global concern.

The US Advisory Committee on Immunization Practices (ACIP) has recently withdrawn the recommendation for use of LAIV in the US for the season 2016/17 following an earlier withdrawal of a preferential recommendation [2]. These decisions were made mainly taking into account the lack of demonstrated vaccine effectiveness (VE) against influenza A(H1N1)pdm09 in observational studies conducted. The studies by the US Centers for Disease Control and Prevention (CDC), and the US Department of Defence, suggested a lower relative effectiveness in comparison to the inactivated influenza vaccine (IIV) [2]. However, two VE studies conducted in Europe and published in this issue of *Eurosurveillance*, reported moderate and reasonable, statistically significant VE in children aged two years and older [8,9]. Furthermore, data from a study funded by the manufacturer of FluMist (US)/Fluenz (Europe) showed similar effectiveness for LAIV in the 2015/16 season [2]. These data were also considered by the ACIP.

In Europe, two EU countries, Finland and the United Kingdom (UK), have introduced LAIV into their publicly-funded routine paediatric vaccination programmes [10]. The two National Immunization Technical Advisory Groups, the UK Joint Committee on Vaccination and Immunisation and the Finnish National Expert Group on Vaccines, considered the available evidence of effectiveness as sufficient to continue the roll-out of vaccination programmes in their countries [11], (personal communication, H Nohynek, September 2016).

Any issues related to LAIV effectiveness or future availability may impact seriously on the roll-out of current and future paediatric and adolescent influenza vaccine and they have potential to affect global pandemic preparedness.

The results from VE studies by Pebody et al. and Nohynek et al. done during the 2015/16 influenza season in the two EU/EEA countries rolling out paediatric and adolescent vaccination programmes including LAIV, document moderate effectiveness of LAIV against influenza

Table

Comparison of study designs and populations assessing vaccine effectiveness of live attenuated influenza vaccine, northern hemisphere countries, United States, United Kingdom and Finland, influenza season 2015/16

	CDC United States	DoD United States	ICICLE United States	PHE United Kingdom	THL Finland
VE against A(H1N1)pdm09 (95%CI)	**–21% (–108% to 30%)**	**15% (–48% to 51%)***	**50% (–2% to 75%)***	**41.5% (–8.5% to 68.5%)***	**47.9% (21.6–65.4%)**
Study design	Test-negative case–control	Test-negative case–control	Test-negative case–control	Test-negative case–control	Cohort
Source population / inclusion criteria	Children and adolescents aged 2–17 years*	Children and adolescents (Military dependents) aged 2–17 years presenting to participating facilities	Children and adolescents aged 2–17 years	Children and adolescents 2–17 years of age	Children 24–35 months of age
Inclusion criteria	MAARI, including cough, and onset of illness ≤ 7 days before enrolment	ILI (fever ≥ 38 °C AND cough and/or sore throat of < 72 hours duration)	ARI with fever ≥ 100.0°F (37.8 °C), duration < 5 days	ILI	Laboratory-confirmed influenza
Assessment of vaccination status	Current-season vaccination (at least one vaccine dose ≥ 14 days before illness onset; vaccine records obtained from electronic medical records and immunisation registries for children aged 2–8 years; with addition of reported vaccination for patients aged 9–17 years)*	Electronic medical records	Vaccination status was ascertained by medical record review and/or state or regional vaccine registries	Self-reported by patients to general practitioners	National immunisation registry
Case definition	RT-PCR-positive subjects*	RT-PCR-positive subjects	RT-PCR positive subjects	RT-PCR positive subjects	RT-PCR, multiplex RT-PCR, culture and/or antigen detection test
Final sample size (number of vaccinated with LAIV / number of non-vaccinated)*	133/1,078*	93/338*	101/594	111/514*	8,323/46,119
Adjusted for	Study site, age, self-rated general health status, race/hispanic ethnicity, interval (days) from onset to enrolment, and calendar time	Age groups, three time periods	Site, age group, visit date, outpatient visits in past 6 months, health insurance, and sex	Age group, sex, month, pilot area and surveillance scheme	Propensity scores, and adjusted by their quintiles
Source	ACIP presentation 22 June 2016 also cited in [2] and personal communication (J Clippard, September 2016)*	ACIP presentation 22 June 2016 also cited in [2] and personal communication (S Federinko, September 2016)*	ACIP presentation 22 June 2016 also cited in [2] and personal communication (H Caspard, September 2016)*	Pebody 2016 [9]	Nohynek 2016 [8]

ACIP: Advisory Committee on Immunization Practices; ARI: acute respiratory infection; CDC: Centers for Disease Control and Prevention; DoD: Department of Defence; ICICLE: Influenza Vaccine Effectiveness Influenza Clinical Investigation for Children; ILI: influenza-like illness; MAARI: medically attended acute respiratory infection; PHE: Public Health England; THL: Terveyden ja hyvinvoinnin laitos (National Institute for Health and Welfare).

A(H1N1)pdm09 in the UK (estimated VE: 41.5%*) and influenza A in Finland (estimated VE: 47.9%) (Table). Results from ongoing analysis of VE studies in Scotland are consistent with these results (personal communication, J McMenamin, September 2016). This contrasts with results from the US CDC studies which found no significant effectiveness against this strain. All the studies showed effectiveness against antigenically matched B viruses (even though numbers of influenza B cases were very low in the Finnish study) and in all of them low level circulation limited assessment of VE against influenza A(H3N2). Each of the studies report a lower effectiveness for LAIV against influenza A(H1N1) pdm09 in comparison with inactivated influenza vaccines, which was not the case in randomised controlled trials when FluMist/Fluenz was authorised.

All studies, with the exception of the Finnish one, use the test-negative case–control study methodology which has the potential to control for many of the

biases inherent with observational studies (Table) but lacks power when stratifying e.g. in strata with small sample sizes. This methodology was extensively evaluated in the past and can be considered the gold standard for observational VE studies [12-16]. Therefore the observed discrepancies between the conducted studies are surprising and deserve careful assessment.

Potential explanations for the discrepancies in the VE study results for LAIV during the 2015/16 influenza season could be related to study design, analytical methods to calculate the adjusted VE, or true differences in effectiveness due to properties of the virus or the target populations. Methodological and analytical differences should affect the effectiveness results for influenza B viruses and inactivated influenza vaccines in the same way. All of the studies agree on some LAIV effectiveness against B viruses. LAIV used in Europe and North America are produced in the same factory, therefore it is unlikely that differences in the composition of the vaccine explain the differences in VE.

The factors driving the lower effectiveness observed in the US over the past five years compared to that seen in the European studies are likely to be related to population or programme-specific effects. In this regard, the comparatively high coverage of influenza vaccination in children 6 months to 2 years of age in the US, before the age at which LAIV is given as part of the vaccination programme, may be a contributing factor. Other factors could include environmental issues such as storage and administration temperature particularly since an early formulation of this vaccine was shown to be thermolabile [17].

Nonetheless, a lower comparative (compared to IIV) effectiveness against the influenza A(H1N1) strains was observed in all the studies. The comparatively lower effectiveness is most likely related to the biological properties of the influenza A(H1N1)pdm09 strain used in the vaccines. Potential explanations include (i) the transition to quadrivalent formulations which occurred 5 years ago, and a potential competition between the B strains and the A(H1N1)pdm09 strain and (ii) a lower fitness of the A(H1N1)pdm09 strain in terms of sialic acid binding specificity, rate of cell entry, replication and budding.

Following the ACIP decision, the European Centre for Disease Prevention and Control (ECDC) and WHO have facilitated a series of discussions between relevant public health research groups in order to review available data and generate hypotheses to explain the differences in VE results and to develop a framework to test these hypotheses. To complement this, WHO organised a global consultation in Geneva on 20–21 September 2016 to discuss potential explanations for recent evidence of decreased performance of LAIV compared with IIV. At this meeting, the potential explanations outlined above were discussed and apart from the methodological constraints of observational studies, they were considered to be likely but requiring research to confirm. Gathering more data, testing the hypotheses and identifying corrective actions will require dedicated resources The manufacturer of the LAIV used in Europe and North America has embarked on a comprehensive virological research programme to study many of these hypotheses to improve and optimise the effectiveness of the 2017/18 vaccine formulation (personal communication, M Downham, 20 September 2016). The involved public health agencies are seeking to enhance their VE studies and have embarked upon better understanding drivers of the variability in the effectiveness estimates. Unfortunately, additional national or supranational funding sources do not appear to be available to rapidly fund adequately scaled operational public health research during the upcoming 2016/17 season.

The US Vaccines for Children Programme had ordered 14 million doses of LAIV for the upcoming 2016/17 influenza season, representing roughly two thirds of the global sales for 2016 [18]. They will now not be used due to the June ACIP decision. Difficult commercial decisions will now need to be taken in the coming months regarding the production for the 2017/18 northern hemisphere season. In a situation where all influenza vaccines used in Europe are produced by commercial manufacturers EU/EEA countries depend on commercial decisions by the manufacturers for availability of LAIV for continued immunisation programmes.

In addition to the LAIV currently used in Europe and North America, several manufacturers in developing countries have started the production of LAIV using the A/Leningrad backbone, and one Indian manufacturer produces pandemic and nationally approved seasonal LAIV vaccines. No data regarding the 2015/16 VE are available from these manufacturers. The policy decisions made in Europe and in the US have an impact on commercial decisions by all manufacturers and as mentioned above, on the global capacity to respond to influenza.

The US Food and Drug Authority (FDA) and the European Medicines Agency (EMA) consider that the benefit–risk ratio of the LAIVs licenced by them remains positive and no changes in market authorisation are envisaged [17]. In the coming months, EMA will introduce a new guideline requiring manufacturers to provide annual VE estimates as part of the market authorisation [19].

The VE results for LAIV 2015/16 clearly show the necessity of assessing VE on an annual basis. With core funding from ECDC, the European Influenza Monitoring Vaccine Effectiveness (I–MOVE) network has established a methodology and an EU/EEA-wide network to estimate seasonal VE [20]. The challenge of conducting these studies is to find study sites with sufficiently high uptake of influenza vaccines and the resources to recruit large enough sample sizes. The European Innovative Medicines Initiative has called for a proposal to prepare for a platform to enable these

studies, in particular to establish a governance model where such studies could be undertaken in a public-private partnership. Such partnership should include public health agencies recommending and assessing vaccination programmes and manufacturers producing the vaccines in an atmosphere of transparency and scientific independence [21].

The European seasonal influenza immunisation programmes of children are based on estimated healthcare cost savings (Finland) [7] and estimated reductions of transmission of influenza and indirect protection of the elderly and risk groups (UK) [22]. Both programmes are currently being rolled out, especially in the UK, in a step-wise fashion. Therefore full assessments of the impact of these programmes are only awaited within the next few years. Now these programmes are faced with two immediate risks, before such assessments can be made; on the one hand a low (or non-existent as in the US) effectiveness which would decrease the impact of the programmes and on the other hand the dependence on the commercial decisions of the manufacturers.

Virological, epidemiological and immunological studies are urgently needed to understand the reasons behind the decrease of the influenza A(H1N1)pdm09 component of LAIV to inform the vaccine strain selection decision for the northern hemisphere in February 2017, the public health decisions on the vaccines to be recommended for the 2017/18 season and to support sound commercial decisions by the vaccine manufacturers.

*Author's correction

The VE for 2-17 year-olds in the UK was corrected on request of the authors on 22 and 29 September 2016. In addition, figures for the final sample sizes for CDC, DoD and PHE and case definition for CDC were corrected in the Table on 29 September 2016.

Following publication, the exact confidence intervals for VE in DoD and ICICLE were provided to the authors in personal communications and specified in the Table on 29 September 2016. Exact age groups for the source population and information on vaccination status in the CDC study were provided to the authors in personal communications and specified in the Table on 29 September 2016.

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Conflict of interest

None declared.

Authors' contributions

Both authors contributed equally to conception and writing of this editorial.

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South-east Asian Zika virus strain linked to cluster of cases in Singapore, August 2016

S Maurer-Stroh [1 2 3 4], T Mak [4], Y Ng [4], S Phuah [4], RG Huber [1], JK Marzinek [1 2], DA Holdbrook [1], RT Lee [1], L Cui [4], RT Lin [4 5]

1. Bioinformatics Institute (BII), Agency for Science, Technology and Research (A*STAR), Singapore
2. Department of Biological Sciences (DBS), National University of Singapore (NUS), Singapore
3. School of Biological Sciences (SBS), Nanyang Technological University (NTU), Singapore
4. National Public Health Laboratory (NPHL), Ministry of Health (MOH), CDC2, Singapore
5. Department of Laboratory Medicine, National University Hospital, Singapore

Correspondence: Sebastian Maurer-Stroh (sebastianms@bii.a-star.edu.sg)

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Zika virus (ZIKV) is an ongoing global public health emergency with 70 countries and territories reporting evidence of ZIKV transmission since 2015. On 27 August 2016, Singapore reported its first case of local ZIKV transmission and identified an ongoing cluster. Here, we report the genome sequences of ZIKV strains from two cases and find through phylogenetic analysis that these strains form an earlier branch distinct from the recent large outbreak in the Americas.

Outbreak detection

On 22 August 2016, the Ministry of Health (MOH), Singapore, was informed by a general practitioner of a spate of cases presenting with non-specific symptoms, including rash, polyarthralgia and low grade fever. In addition, a number of cases had mild conjunctivitis. Four days later, one of the cases was referred to the Communicable Disease Centre, Tan Tock Seng Hospital. The patient's blood and urine samples tested positive for Zika virus (ZIKV), and the blood was negative for both dengue and chikungunya viruses by polymerase chain reaction (PCR). Since the patient had no travel history within the past month, the MOH announced the first locally transmitted ZIKV infection on 27 August, after laboratory confirmation on a second set of blood and urine specimens. For the purpose of outbreak investigation and public health measures, MOH then directed the collection of clinical specimens from cases working or living in the surrounding areas, with fever (>37.8°C), rash, conjunctivitis and/or joint pain in the preceding two weeks. All clinical information and samples pertaining to the outbreak investigation were collected under the provisions of the Infectious Diseases Act in Singapore [1].

Genome sequencing of outbreak samples

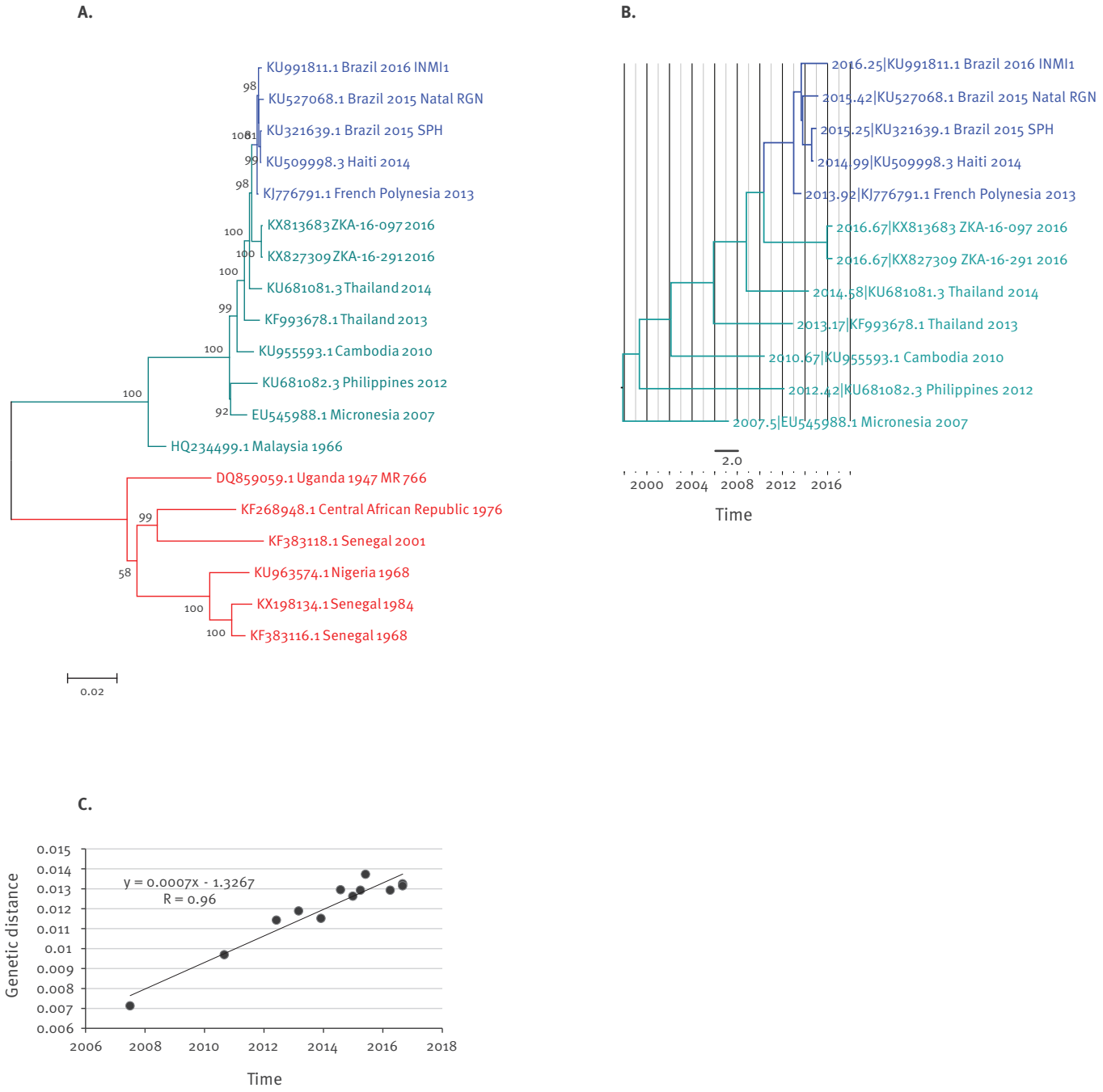
A total of 153 individuals fitting the case definition were tested for ZIKV on 27 and 28 August, of which, only 56 cases were confirmed positive by real-time PCR assay [2]. Respective samples from two cases, ZKA-16–097 and ZKA-16–291, were selected based on their high viral titre (cycle threshold (Ct) values 21.7 and 24.6). Viral RNA was extracted from urine samples using the QIAamp viral RNA kit (Qiagen) and a series of overlapping reverse transcription (RT)-PCR reactions were performed using one-step Superscript III/ Hi-Fidelity platinum Taq polymerase (Thermo Fisher). The primers were designed to target conserved regions among ZIKV whole genome sequences of both Asian and African lineages that were available on GenBank [3]. DNA bands of the predicted sizes were purified and sequenced by Sanger sequencing. Raw sequences were aligned and edited using CLC workbench. The consensus sequences for ZKA-16–097 and ZKA-16-291 were submitted to GenBank under the accession numbers KX813683 and KX827309, respectively.

Background on Zika virus

ZIKV is a mosquito-borne single-stranded positive-sense RNA virus belonging to the *Flaviviridae* family. First isolated in 1947 from a sentinel rhesus monkey in Uganda [4], ZIKV circulated enzoonotically within Africa and equatorial Asia as two distinct lineages: the African and Asian lineage [5]. Prior to 2007, only 14 sporadic human infections, confined to Africa and equatorial Asia, were documented [6]. Since then, three outbreaks of ZIKV belonging to the Asian lineage have ensued: in 2007, on Yap island within the Federated States of Micronesia, in 2013 and 2014, within the French Polynesian islands and most notably, the current large outbreak in the Americas which was first detected in Brazil in 2015. Unlike the Yap island outbreak which was characterised by cases with relatively mild dengue-like symptoms [7], the outbreaks in French Polynesia and Brazil coincided with an unusual rate of cerebral congenital anomalies, including microcephaly [8]. Since 2015, 70 countries and territories reported evidence of ZIKV transmission [9]. The magnitude of spread of the Asian lineage and its disease association prompted the World Health Organization to declare

Figure 1

Phylogenetic trees with Zika virus genomes, 1947–2016



A) Maximum likelihood phylogenetic tree of representatives from the African lineage coloured red and Asian lineage coloured blue or cyan. The tight cluster with strains from French Polynesia and the Americas is in blue and strains from south-east Asia are in cyan. The scale beneath the tree represents the number of substitutions per site. B) Time-resolved Bayesian tree of recent Asian lineage. The scale beneath the tree represents the years of divergence. C) Correlation of sampling date with phylogenetic distance for the recent Asian lineage.

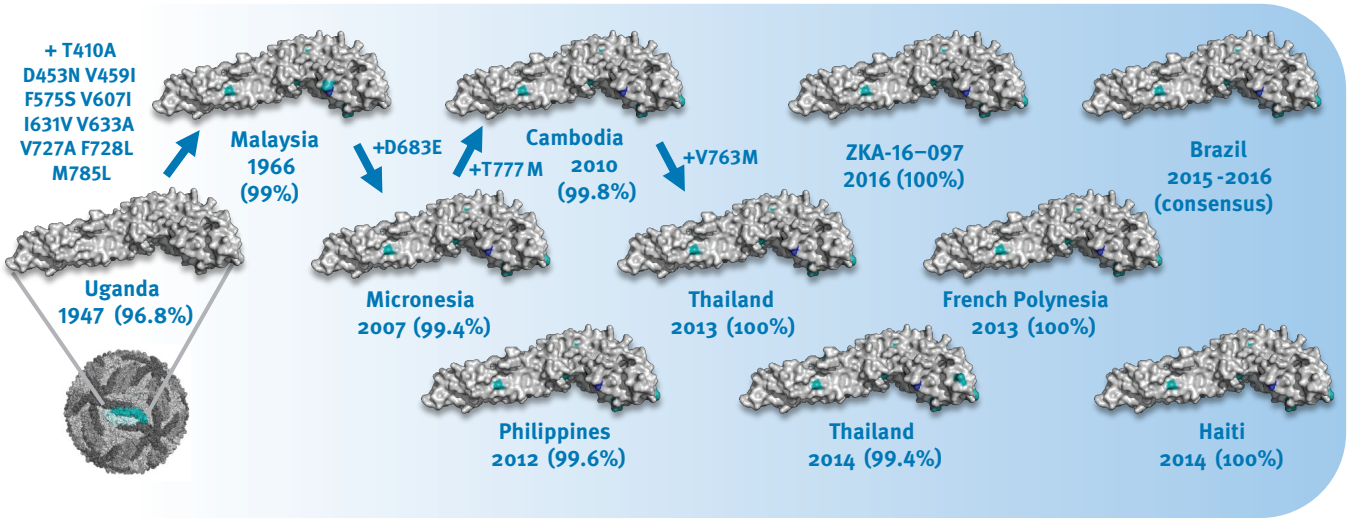
ZIKV as a Public Health Emergency of International Concern in 2016 [10].

Phylogenetic analysis

To gain further information on the ZIKV strains circulating within the current Singapore outbreak, the sequences recovered from the two cases were phylogenetically analysed. Complete ZIKV genomes available on GenBank were downloaded [3], and selected non-redundant representatives were aligned with the sequences from Singapore using Multiple Alignment using Fast Fourier Transform (MAFFT) [11]. The two Singapore sequences are 99.9% identical with only seven nucleotides different over 10,272 bases in

Figure 2

Mutations on the envelope (E) protein surface of different Zika virus strains



Mutated residues in the Asian strain of Zika virus relative to the reference strain MR766 are indicated in cyan. Amino-acid residue numbers are according to polyprotein numbering. The highly conserved glycosylation site at N444 is indicated in blue. The schematic in the bottom left illustrates how the E proteins are arranged in a set of dimeric, trimeric, and pentameric elements to form a pseudo-icosahedral structure on the viral surface. 3D homology models were created using MODELLER [15] based on PDB:5IZ7 [16] and visualised with the PyMOL Molecular Graphics System (Version 1.8 Schrödinger, LLC.). The percent identity of each depicted strain's E protein relative to the consensus of the Brazil 2015 sequences ranges from 96.8 to 100% and is indicated in parentheses.

the coding region. A maximum likelihood (ML) phylogenetic tree was created in Molecular Evolutionary Genetics Analysis (MEGA) [12] using the Tamura–Nei model with gamma distributed rate differences (5 categories, including invariant) and 1,000 bootstrap step validation. The phylogeny (Figure 1A) clearly shows the separate African and Asian lineages. Within the Asian lineage, the two sequences found in Singapore form a distinct branch, which stems from an ancestral node separating this branch from the tight cluster of strains from French Polynesia (2013), Haiti (2014) and Brazil (2015-2016) with 98% bootstrap support. The preceding shared ancestral node is with a strain circulating in Thailand in 2014 with 100% bootstrap support. Consequently, the viruses detected in Singapore evolutionarily arose in parallel to the large tight cluster of recent strains in South and Central America indicating that the two cases studied here were not infected by viruses imported from the Americas but rather by representatives of the Asian lineage already circulating in south-east Asia. The same clustering can be shown using other tree building methods and parameters (Bayesian: Figure 1B; neighbour joining, uniform rates, HKY model: data not shown).

Furthermore, in the tree, the long branch of the two new south-east Asian viruses found in this study suggests undetected evolution for several years. In order to estimate the time of divergence from the last common ancestor with the later cluster from French Polynesia and the Americas, we used a phylogenetic molecular clock approach through the Bayesian Evolutionary Analysis Sampling Trees (BEAST) package [13]. We first confirmed that the measured genetic distance within our set of the recent Asian lineage correlates well with the sample dates (Figure 1C) and created a time-resolved Bayesian tree with parameters previously established for ZIKV (strict clock model, Bayesian skyline, generalised time-reversible (GTR) [14]). The resulting tree (Figure 1B) is consistent with the clustering described in the ML tree and the time of the last common ancestor of the new south-east Asian strains and the French Polynesia/Americas cluster is estimated as 6.2 years ago (95% highest posterior density interval: 4.58–8.15 years) suggesting the observed clades diverged from each other around early 2010. Given this long unsampled evolution, future studies should establish possible reservoirs and circulation in the region.

Conclusions from sequence and structure analysis

This study shows that there are still multiple ZIKV strains in circulation globally in 2016 which raises the question on their antigenic diversity or similarity for vaccine development. Therefore, we systematically mapped the outer surface envelope (E) protein changes from 1947 onwards across the whole Asian lineage (Figure 2).

At least within the Asian lineage the antigenic protein E surface is highly conserved and homogeneous with, from 2007 onwards, typical identities from 99.4% to 100% (or 3 to zero mutations) relative to the consensus of recent strains from Brazil. Moreover all

of the E proteins in this lineage contain the typical N-glycosylation site (N444 in polyprotein numbering which is N154 in the E protein). The lack of surface mutation drift over the past 50 years (99% identity between Malaysia 1966 and consensus of Brazil 2015) also suggests that immune pressure on the E protein has not been a dominant factor in the Asian lineages' virus fitness and evolution so far. While the highly similar surface E proteins of the different strains in the Asian lineage should facilitate global vaccine development it is too early to judge if the strain linked to the Singapore outbreak would show any different disease characteristics from the one in French Polynesia and the Americas. Further studies are necessary and underway in Singapore to collect more data.

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Conflict of interest

None declared.

Authors' contributions

TMM, YKN, SPP, LC, RL contributed sample processing, primer preparation, sequencing, data analysis, interpretation and co-wrote the paper. SMS, RGH, JKM, DAH and RTCL contributed phylogenetic analysis, structural modelling and co-wrote the paper.

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Research article

Lack of virological and serological evidence for continued circulation of highly pathogenic avian influenza H5N8 virus in wild birds in the Netherlands, 14 November 2014 to 31 January 2016

MJ Poen [1,2], JH Verhagen [1,2], RJ Manvell [3], I Brown [3], TM Bestebroer [1], S van der Vliet [1], O Vuong [1], RD Scheuer [1], HP van der Jeugd [4,5], BA Nolet [4,6], E Kleyheeg [4,5], GJDM Müskens [7], FA Majoor [8], C Grund [9], RAM Fouchier [1]

1. Erasmus MC, Department of Viroscience, Rotterdam, the Netherlands
2. These authors contributed equally to this work
3. Animal and Plant Health Agency (APHA) - Weybridge, Addlestone, Surrey, United Kingdom
4. Netherlands Institute of Ecology (NIOO-KNAW), Department of Animal Ecology, Wageningen, the Netherlands
5. Vogeltrekstation – Dutch Centre for Avian Migration and Demography (NIOO-KNAW), Wageningen, the Netherlands
6. University of Amsterdam, Institute of Biodiversity and Ecosystem Dynamics, Computational Geo-Ecology, Amsterdam, the Netherlands
7. Alterra, Center for Ecosystem Studies, Wageningen University, Wageningen, the Netherlands
8. Sovon, Dutch Centre for Field Ornithology, Nijmegen, the Netherland
9. Friedrich-Loeffler-Institut, Greifswald - Insel Riems, Germany

Correspondence: Ron A M Fouchier (r.fouchier@erasmusmc.nl)

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In 2014, H5N8 clade 2.3.4.4 highly pathogenic avian influenza (HPAI) viruses of the A/Goose/Guangdong/1/1996 lineage emerged in poultry and wild birds in Asia, Europe and North America. Here, wild birds were extensively investigated in the Netherlands for HPAI H5N8 virus (real-time polymerase chain reaction targeting the matrix and H5 gene) and antibody detection (haemagglutination inhibition and virus neutralisation assays) before, during and after the first virus detection in Europe in late 2014. Between 21 February 2015 and 31 January 2016, 7,337 bird samples were tested for the virus. One HPAI H5N8 virus-infected Eurasian wigeon (*Anas penelope*) sampled on 25 February 2015 was detected. Serological assays were performed on 1,443 samples, including 149 collected between 2007 and 2013, 945 between 14 November 2014 and 13 May 2015, and 349 between 1 September and 31 December 2015. Antibodies specific for HPAI H5 clade 2.3.4.4 were absent in wild bird sera obtained before 2014 and present in sera collected during and after the HPAI H5N8 emergence in Europe, with antibody incidence declining after the 2014/15 winter. Our results indicate that the HPAI H5N8 virus has not continued to circulate extensively in wild bird populations since the 2014/15 winter and that independent maintenance of the virus in these populations appears unlikely.

Introduction

Wild birds are the natural hosts of low pathogenic avian influenza (LPAI) viruses, which generally do not cause clinical signs of disease in these host species [1]. So far, virus subtypes H1 to H16 and N1 to N9 have been detected in wild birds, of which viruses of subtypes H5 and H7 have shown the ability to evolve to highly pathogenic avian influenza (HPAI) viruses in poultry, causing severe disease with high mortality in such animals. These HPAI viruses were historically mainly detected in rapidly contained sporadic outbreaks in poultry, until H5N1 viruses of the A/Goose/Guangdong/1/1996 (GsGd) lineage emerged in Asia in 1997. Subsequently, these viruses have continuously circulated in poultry with frequent detections in wild birds [2] and with significant expansion in global range.

HPAI H5N8 viruses of the GsGd lineage of clade 2.3.4.4 emerged in poultry and wild birds on multiple continents in 2014. The ancestral influenza H5N8 virus to the strains causing outbreaks from 2014 onwards was first detected in China in 2010 in a captive-held mallard *(Anas platyrhynchos)* [3]. In early 2014, HPAI H5N8 GsGd virus of clade 2.3.4.4 occurred for the first time in poultry in South Korea, soon after causing outbreaks also in Japan [4]. From late 2014 onwards, this virus spread to other areas of the world including Europe, North America, Russia and Taiwan [5-8]. The HPAI H5N8 virus detections in Europe were limited to sporadic cases in wild birds and a relatively small

Table 1

Wild bird species sampled for virus detection during and after the emergence of highly pathogenic avian influenza H5N8 virus in Europe, the Netherlands, 21 February 2015–31 January 2016 (n = 7,337 animals)

Order	Family	Species	During outbreak: 21 Feb 2015–13 May 2015				After outbreak: 14 May 2015–31 Jan 2016			
			Birds sampled N	AIV-positive birds N	H5-positive birds N	Pathotype	Birds sampled N	AIV-positive birds N	H5-positive birds N	Pathotype
Anseriformes	Ducks	Common pochard *(Aythya ferina)*	0	0	0	NA	1	0	0	NA
		Common teal *(Anas crecca)*	8	0	0	NA	221	39	4	LPAI
		Egyptian goose *(Alopochen aegyptiaca)*	58	0	0	NA	136	0	0	NA
		Eurasian wigeon *(Anas penelope)*	175	1	1	HPAI	1,034	101	2	LPAI
		Gadwall *(Anas strepera)*	1	0	0	NA	175	15	0	NA
		Mallard *(Anas platyrhynchos)*	748	50	0	NA	2,464	354	15	LPAI
		Mandarin duck *(Aix galericulata)*	2	0	0	NA	0	0	0	NA
		Northern pintail *(Anas acuta)*	0	0	0	NA	7	3	0	NA
		Northern shoveler *(Anas clypeata)*	0	0	0	NA	17	2	0	NA
		Tufted duck *(Aythya fuligula)*	0	0	0	NA	1	0	0	NA
	Geese	Barnacle goose *(Branta leucopsis)*	96	5	4	LPAI	926	3	0	NA
		Bean goose *(Anser fabalis)*	0	0	0	NA	8	0	0	NA
		Brent goose *(Branta bernicla)*	54	0	0	NA	0	0	0	NA
		Canada goose *(Branta canadensis)*	3	0	0	NA	72	0	0	NA
		Greylag goose *(Anser anser)*	59	0	0	NA	239	0	0	NA
		Pink-footed goose *(Anser brachyrhynchus)*	0	0	0	NA	1	0	0	NA
		Greater white-fronted goose *(Anser albifrons)*	0	0	0	NA	55	0	0	NA
	Swans	Mute swan *(Cygnus olor)*	3	0	0	NA	31	1	0	NA
Charadriiformes	Gulls	Black-headed gull (Chroicocephalus ridibundus)	84	0	0	NA	392	53	0	NA
		Caspian gull *(Larus cachinnans)*	4	0	0	NA	4	0	0	NA
		Common gull *(Larus canus)*	1	0	0	NA	18	0	0	NA
		Great black-backed gull *(Larus marinus)*	1	0	0	NA	0	0	0	NA
		Herring gull *(Larus argentatus)*	15	0	0	NA	32	2	0	NA
		Lesser black-backed gull *(Larus fuscus)*	0	0	0	NA	33	2	0	NA
		Mediterranean gull *(Larus melanocephalus)*	1	0	0	NA	3	1	0	NA
		Yellow-legged gull *(Larus michahellis)*	0	0	0	NA	1	0	0	NA
	Lapwings	Northern lapwing *(Vanellus vanellus)*	6	0	0	NA	0	0	0	NA
	Terns	Black tern *(Chlidonias niger)*	0	0	0	NA	0	0	0	NA
		Common tern *(Sterna hirundo)*	0	0	0	NA	0	0	0	NA
Columbiformes	Pigeons	Common wood-pigeon *(Columba palumbus)*	1	0	0	NA	0	0	0	NA
Gruiformes	Coots	Common coot *(Fulica atra)*	46	0	0	NA	92	0	0	NA
	Rails	Little crake *(Porzana parva)*	0	0	0	NA	1	0	0	NA
		Common moorhen *(Gallinula chloropus)*	3	0	0	NA	4	0	0	NA
Total			**1,369**	**56**	**5**	**NA**	**5,968**	**576**	**21**	**NA**

AIV: avian influenza virus; HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; N: number; NA: not applicable.
Surveillance activities were intensified from 21 February to 13 May 2015 (n = 1,369) and 1 September to 31 December 2015 (n = 3,736).

Table 2

Wild bird species sampled for H5-specific antibody detection before, during and after the emergence of highly pathogenic avian influenza H5N8 virus in Europe, the Netherlands, 2007–2015 (n = 1,443)

Order	Family	Species	Number of individuals sampled		
			Before outbreak (before 2014)	During outbreak (14 Nov 2014–13 May 2015)	After outbreak (1 Sep 2015–31 Dec 2015)
Anseriformes	Ducks	Common teal *(Anas crecca)*	0	15	111
		Egyptian goose *(Alopochen aegyptiaca)*	9	62	28
		Eurasian wigeon *(Anas penelope)*	0	78	46
		Gadwall *(Anas strepera)*	1	3	1
		Mallard *(Anas platyrhynchos)*	21	93	18
		Mandarin duck *(Aix galericulata)*	1	2	0
		Northern pintail *(Anas acuta)*	0	0	1
		Northern shoveler *(Anas clypeata)*	0	2	3
		Ruddy shelduck *(Tadorna ferruginea)*	1	0	0
	Geese	Barnacle goose *(Branta leucopsis)*	20	19	0
		Bean goose *(Anser fabalis)*	5	0	0
		Brent goose *(Branta bernicla)*	0	19	0
		Greylag goose *(Anser anser)*	0	2	0
		Lesser white-fronted goose *(Anser erythropus)*	0	3	0
		Pink-footed goose *(Anser brachyrhynchus)*	0	1	0
		Greater white-fronted goose *(Anser albifrons)*	20	77	0
	Swans	Bewick's swan *(Cygnus columbianus bewickii)*	0	20	0
		Mute swan *(Cygnus olor)*	10	90	29
		Whooper swan *(Cygnus cygnus)*	0	1	0
Charadriiformes	Gulls	Black-headed gull *(Chroicocephalus ridibundus)*	20	262	31
		Caspian gull *(Larus cachinnans)*	0	6	3
		Common gull *(Larus canus)*	12	34	17
		Great black-backed gull *(Larus marinus)*	0	1	0
		Herring gull *(Larus argentatus)*	7	61	28
		Lesser black-backed gull *(Larus fuscus)*	1	3	8
		Mediterranean gull *(Ichthyaetus melanocephalus)*	2	1	0
		Yellow-legged gull *(Larus michahellis)*	0	0	1
Gruiformes	Rails	Common coot *(Fulica atra)*	19	84	24
		Moorhen *(Gallinula chloropus)*	0	6	0
Total			**149**	**945**	**349**

number of unrelated outbreaks in poultry. However in North America HPAI H5N8 viruses reassorted with co-circulating LPAI viruses, giving rise to new HPAI H5N1 and H5N2 virus subtypes that caused a large number of outbreaks in poultry with numerous detections in wild birds [9]. Despite mild clinical symptoms caused by infection with HPAI H5N8 viruses of clade 2.3.4.4 in experimentally infected mammals [10-12] and ducks [11], the widespread detection and rapid global spread of HPAI H5 clade 2.3.4.4 viruses pose a potential threat to domestic and wild animals and should be studied further.

The major challenges in understanding the epidemiology of emerging influenza viruses in wild birds are the large numbers of potential host species and the usually short period of viral shedding, combined with the difficulty of catching and sampling representative numbers per species. For instance, mallards that were experimentally infected with HPAI H5N8 virus shed infectious virus in tracheal swabs for only up to 5 days post infection [11]. These impediments result in a low probability of detecting newly emerging avian influenza viruses in wild birds through active virological surveillance and result in a delay of implementation of effective control measures. Nevertheless, to date HPAI H5N8 virus has been detected in 30 wild bird species. In addition to the host species previously described [13,14], HPAI H5N8 viruses have been detected in wild bird species belonging to the orders *Anseriformes* in Asia (*Aythya*

Table 3

Details of positive control sera titres from experimentally infected ferrets, a domestic duck, and a domestic goose with one low pathogenic (LPAI) H5 and different highly pathogenic avian influenza (HPAI) H5 clades (n = 8 antisera)

Antiserum raised against	Characteristics	Species	Haemagglutination inhibition assay						Virus neutralisation assay		
			Viruses						Viruses		
			LPAI	HPAI clade					HPAI clade		
				1[a]	2.1[b]	2.2[c]	2.3[d]	2.3.4.4[e]	2.1[b]	2.3[d]	2.3.4.4[e]
A/Mallard/Netherlands/3/1999	LPAI H5N2	Ferret	**160**	<10	<10	<10	<10	<10	ND	ND	ND
A/Viet Nam/1194/2004	HPAI H5N1 clade 1	Ferret	<10	**80**	<10	<10	<10	<10	ND	ND	ND
A/Indonesia/5/2005	HPAI H5N1 clade 2.1	Ferret	<10	<10	**120**	<10	60	<10	**80**	<10	<10
A/Turkey/Turkey/1/2005	HPAI H5N1 clade 2.2	Ferret	<10	<10	<10	**1,280**	60	<10	ND	ND	ND
A/Anhui/1/2005	HPAI H5N1 clade 2.3	Ferret	<10	<10	<10	20	**320**	<10	<10	**160**	<10
A/Chicken/Netherlands/EMC-3/2014	HPAI H5N8 clade 2.3.4.4	Ferret	<10	<10	<10	<10	<10	**160**	<10	<10	**40**
Turkey/Germany/AR2487/2014	HPAI H5N8 clade 2.3.4.4	Domestic duck	<10	<10	<10	<10	<10	160	ND	ND	ND
Turkey/Germany/AR2487/2014	HPAI H5N8 clade 2.3.4.4	Domestic goose	<10	<10	<10	<10	<10	80	ND	ND	ND

HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; ND: not determined.

Lowest serum dilution tested was 10. Titres indicating the reactivity of sera to viruses homologous to the viruses, which the sera were raised against are in bold.

[a] A/Viet Nam/1194/2004.

[b] A/Indonesia/5/2005.

[c] A/Turkey/Turkey/1/2005.

[d] A/Anhui/1/2005.

[e] A/Chicken/Netherlands/EMC-3/2014

spp.) and North America (*Branta* spp.) [6]. In Europe, HPAI H5N8 viruses have been detected in bird species of the orders *Anseriformes* (*Anas* spp. and *Cygnus* spp.) and *Charadriiformes* (*Larus* spp.) [5,6,14].

To estimate the likelihood of the involvement of live wild birds in local and long distance movement of HPAI H5 viruses, information on recent exposure of wild bird populations to HPAI H5N8 viruses using serology, in addition to virology, would add substantial power to surveillance programmes. Studies with ferret sera have shown serological tests to have substantial discriminative power between antibodies directed to HPAI H5 viruses of different clades and LPAI H5 viruses using haemagglutination inhibition (HI) assays [12,15]. Although less is known about serology in wild birds, a study on wild birds sampled in Europe and Mongolia showed that antigenic differences between the haemagglutinin (HA) of classical Eurasian LPAI H5 viruses and GsGd lineage HPAI H5 viruses can be used to define bird populations in which HPAI viruses have previously been circulating [16]. With regard to HPAI H5N8 viruses specifically, a 2014 South Korean serology study showed evidence of a rise of H5 virus antibodies occurring in long distance migratory duck species after the onset of the HPAI H5N8 virus emergence in South Korea [4].

In this study, in response to the emergence of HPAI H5N8 virus in Europe, we present data on wild bird surveillance activities in the Netherlands, including results of virological and serological assays.

Methods

Ethical statement

The capture of free-living birds was approved by the Dutch Ministry of Economic Affairs based on the Flora and Fauna Act (permit number FF/75A/2009/067 and FF/75A/2014/054). Handling and sampling of free-living birds was approved by the Animal Experiment Committee of the Erasmus Medical Centre (permit number 122–11–31). Free-living birds were released into the wild after sampling and all efforts were made to minimise animal suffering throughout the studies.

Study population

Immediately after the first detection of HPAI H5N8 virus in poultry in Europe, ongoing influenza surveillance activities in migrating and overwintering wild birds in the Netherlands were intensified (14 November 2014–13 May 2015). Hereafter, this period will be referred to as 'during the outbreak'. Surveillance activities in wild birds in the Netherlands were again intensified from the onset of the arrival of wild migrating birds a year after the initial HPAI H5N8 virus detection in Europe (1 September–31 December 2015). This period will be referred to as 'after the outbreak'. Sampled populations consisted of resident birds, partial migrants and long distance migrants. During both periods of

Table 4

Detected haemagglutination inhibition antibody titres to low pathogenic avian influenza H5 virus[a] and to highly pathogenic avian influenza H5 clade 2.3.4.4 H5N8 virus[b] in birds, before, during, and after detection of the highly pathogenic avian influenza H5N8 virus in Europe, the Netherlands, 2007–2015 (n = 1,443 birds)

Strain	Period relative to the outbreak[c]	Haemagglutination inhibition titre							High background	Total tested	Total positives
		BLD	10–40	40–80	80–160	160–320	320–640	≥ 640			
LPAI H5N2[a]	Before	121	1	0	1	0	0	0	26	149	2
	During	903	16	5	2	1	0	0	18	945	24
	After	324	2	1	0	2	0	0	20	349	5
HPAI H5N8[b]	Before	123	0	0	0	0	0	0	26	149	0
	During	897	7	20	6	4	5	1	5	945	43
	After	319	4	3	2	1	0	0	20	349	10

BLD: below limit of detection; LPAI: low pathogenic avian influenza; HPAI: highly pathogenic avian influenza.

Lowest serum dilution tested was 10.

[a] A/Mallard/Netherlands/3/1999.

[b] A/Chicken/Netherlands/EMC-3/2014.

[c] The 'outbreak' refers to the six months following the detection of the highly pathogenic avian influenza H5N8 virus in Europe and this extends from 14 November 2014 to 13 May 2015. The period before the 'outbreak' is from 2007 to 2013, while the period after the 'outbreak' is from 1 September to 31 December 2015.

intensified surveillance, blood samples were obtained in addition to samples for virus detection. A matching historical set of serum samples was compiled based on similarity in species and family, hereafter referred to as 'before the outbreak' (2007–2013).

Sample collection

Wild birds were captured using duck decoys, clap nets, cannon nets, mist nets, leg-nooses, swan hooks, or manually. Birds were sampled routinely for virus detection using cloacal and/or oropharyngeal swabs as described elsewhere [14]. In addition, faecal samples were collected from a limited number of species for virus detection. Blood samples were collected for antibody detection. Blood samples were collected from the brachial or metatarsal vein and centrifuged at 3,000 rpm for 10 min in 0.8 mL gel separation tubes (MiniCollect tubes, Roche). Serum samples were stored below -20 °C until analysis.

Virus detection, isolation and characterisation

Samples for virus detection were analysed for the presence of HPAI H5(N8) virus using matrix- and H5-specific real-time polymerase chain reaction (RT-PCR) assays followed by H5 and neuraminidase sequencing as previously described [14]. Samples testing positive in matrix specific RT-PCR were inoculated in embryonated chicken eggs as described previously [17].

Antibody detection

Serum samples were first tested for the presence of H5-specific antibodies in an HI assay according to standard procedures [18]. Briefly, serum samples were incubated for 16 hours at 37 °C with *Vibrio cholerae* filtrate containing receptor-destroying enzyme to remove non-specific inhibitors of haemagglutination activity, followed by incubation for 1 hour at 56 °C. Twofold serial dilutions of serum samples with a start dilution of 1:20 were prepared using phosphate-buffered saline (PBS) in U-bottomed 96 well microtitre plates. Serum dilutions were incubated with four haemagglutinating units (HAU) of Madin–Darby canine kidney (MDCK) (all HPAI H5 clade viruses) or egg (A/Mallard/Netherlands/3/1999) cultured virus for 30 min at 37 °C. A suspension of 1% turkey red blood cells (TRBC) was added to the serum-virus dilutions. After incubation for 1 hour at 4 °C, haemagglutination patterns were read. Negative controls, based on serum incubation without virus, were used to measure non-specific haemagglutination of each serum sample. Sera showing high background (i.e. high non-specific haemagglutination) were pre-treated with 10% TRBC for 1 hour at 4 °C and retested for the presence of H5-specific antibodies as described above. Serum samples from experimentally inoculated ferrets [12,15], a domestic duck, and a domestic goose were used as positive controls.

All serum samples were initially screened for antibodies specific for classical Eurasian LPAI H5N2 virus A/Mallard/Netherlands/3/1999 and clade 2.3.4.4 HPAI H5N8 virus A/Chicken/Netherlands/EMC-3/2014. Serum samples that tested positive for HPAI H5 clade 2.3.4.4-specific antibodies were further tested against HPAI viruses of the H5 clades 1 (A/Viet Nam/1194/2004), 2.1 (A/Indonesia/5/2005), 2.2 (A/Turkey/Turkey/1/2005), and 2.3 (A/Anhui/1/2005), and retested against the clade 2.3.4.4 virus. Samples showing more than threefold differences in titre or testing negative in the second assay after showing initial titres were tested a third time. The viruses used were recombinant viruses based on an A/PR/8/34 virus backbone, containing the HA and neuraminidase (NA) of the representative H5 strains. The sequences of the HA genes were modified to remove the multi-basic cleavage site

Table 5

Birds species with antibodies to highly pathogenic avian influenza H5 clade 2.3.4.4 H5N8 virus[a], and number of respective animals, according to their haemagglutination inhibition antibody titres to the virus, during and after detection of highly pathogenic avian influenza H5N8 virus in Europe, the Netherlands, 14 November 2014–31 December 2015 (n = 382 birds)

Species	Period relative to the outbreak[b]	HI titre to HPAI H5 clade 2.3.4.4 (H5N8) virus							High background	Total tested
		BLD	10–40	40–80	80–160	160–320	320–640	≥ 640		
Eurasian wigeon (*Anas penelope*)	During	66	6	4	2	0	0	0	0	78
Lesser white-fronted goose (*Anser erythropus*)	During	2	0	1	0	0	0	0	0	3
Mute swan (*Cygnus olor*)	During	59	1	14	4	4	5	1	2	90
Common coot (*Fulica atra*)	During	83	0	1	0	0	0	0	0	84
Eurasian wigeon (*Anas penelope*)	After	42	2	1	0	0	0	0	1	46
Egyptian goose (*Alopochen aegyptiaca*)	After	27	1	0	0	0	0	0	0	28
Mute swan (*Cygnus olor*)	After	19	1	2	2	0	0	0	5	29
Common coot (*Fulica atra*)	After	21	0	0	0	1	0	0	2	24

BLD: below limit of detection; HI: haemagglutination inhibition; HPAI: highly pathogenic avian influenza.

Lowest serum dilution tested was 10.

[a] A/Chicken/Netherlands/EMC-3/2014.

[b] The 'outbreak' refers to the six months following the detection of the highly pathogenic avian influenza H5N8 virus in Europe and this extends from 14 November 2014 to 13 May 2015. The period after the 'outbreak' is from 1 September to 31 December 2015.

to enable this study within biosafety level 2 laboratories. HPAI H5 virus of clade 0 was excluded from the analyses due to high overall reactivity with all avian positive control sera as previously described [16] and thus of limited discriminative value.

A representative selection (based on titre and serum availability) of serum samples that tested positive for HPAI H5 clade 2.3.4.4 antibodies were sent to the Animal and Plant Health Agency (APHA) (Weybridge, UK) for confirmation of HPAI H5 clade 2.3.4.4-specific antibodies using an HI assay. The HI assay procedure used by the APHA differed from the HI assay described above and was carried out in accordance to the World Organisation for Animal Health (OIE) [19]. In short, twofold serial dilutions of serum samples with a start dilution of 1:12 were made using phosphate-buffered saline (PBS) and prepared in V-bottomed microtitre plates. Serum dilutions were incubated with four HAU of egg cultured virus for 30 min at room temperature. A solution of 1% chicken red blood cells (CRBC) was added to the serum–virus dilutions. After incubation for 30 min at room temperature, haemagglutination patterns/streaming of red cells were read. Polyclonal chicken sera raised against the same clade 2.1, 2.2, 2.3, and 2.3.4.4 viruses as mentioned above were used as positive controls, supplemented with LPAI H5N3 virus A/Teal/England/7394–2805/2006 and clade 2.3.4.4 HPAI H5N8 virus A/Duck/England/36254/2014.

All samples that tested positive for HPAI H5 clade 2.3.4.4-specific antibodies in the initial HI assay were tested in a virus neutralisation (VN) assay if sufficient amounts of serum were available. The VN assay was performed as described previously [20], using titrated virus stocks of clade 2.1, 2.3, and 2.3.4.4. Briefly, serum was heat inactivated for 30 min at 56 °C and twofold serial dilutions of the sera starting at a 1:20 dilution were prepared and 100 median tissue culture infectious dose (TCID50) was added. After incubating antigen and serum for 1 hour at 37 °C with 5% CO_2, the mixtures were transferred to 96 well flat bottom plates containing MDCK cells, which were washed once with infection medium before inoculation. The plates were incubated for 1 hour at 37 °C with 5% CO2, after which the cells were washed once with 100 µL infection medium and the medium was replaced by 200 µL infection medium. Three days later, a haemagglutination assay was performed with the supernatant to determine the antibody titres.

Results

Study population

A total of 11,355 birds were sampled for virus detection during and after the first detection of HPAI H5N8 viruses in poultry and wild birds in Europe. Of those, 5,387 birds were sampled during the outbreak and 5,968 after the outbreak. This report describes the results

Table 6a

Titres of confirmatory haemagglutination inhibition and virus neutralisation assays for sera positive for highly pathogenic avian influenza H5 clade 2.3.4.4-specific antibodies in the initial screening, the Netherlands, 14 November 2014–31 December 2015 (n = 53 serum samples)

Period	Species[a]	Haemagglutination inhibition assay											Virus neutralisation assay		
		Initial						Confirmatory							
		LPAI H5	HPAI clade					LPAI H5	HPAI clade				HPAI clade		
			1	2.1	2.2	2.3	2.3.4.4[b]		2.1	2.2	2.3	2.3.4.4	2.1	2.3	2.3.4.4
During the outbreak:2014/15	Eurasian wigeon	<10	<10	<10	<10	<10	50	ND	ND	ND	ND	ND	ND	ND	ND
	Eurasian wigeon	<10	<10	<10	<10	<10	100	ND	ND	ND	ND	ND	ND	ND	ND
	Eurasian wigeon	20	<10	<10	<10	<10	15	ND	ND	ND	ND	ND	ND	ND	ND
	Eurasian wigeon	<10	<10	<10	<10	<10	60	ND	ND	ND	ND	ND	ND	ND	80
	Eurasian wigeon	<10	<10	<10	<10	<10	20	<6	<6	<6	<6	<6	ND	ND	20
	Eurasian wigeon	<10	<10	<10	<10	<10	40	<6	<6	<6	<6	<6	ND	ND	20
	Eurasian wigeon	<10	<10	<10	<10	<10	25	ND	ND	ND	ND	ND	ND	ND	40
	Eurasian wigeon	<10	<10	<10	<10	<10	15	ND	ND	ND	ND	ND	ND	ND	20
	Eurasian wigeon	<10	<10	<10	<10	<10	15	ND	ND	ND	ND	ND	ND	ND	10
	Eurasian wigeon	<10	<10	<10	<10	<10	20	ND	ND	ND	ND	ND	ND	ND	20
	Eurasian wigeon	<10	<10	<10	<10	<10	40	<6	<6	<6	<6	<6	ND	ND	40
	Eurasian wigeon	<10	<10	<10	<10	<10	120	ND	ND	ND	ND	ND	ND	ND	160
	Common coot	<10	40	<10	<10	30	40	ND	ND	ND	ND	ND	ND	ND	<10
	Lesser white-fronted goose	20	<10	<10	<10	<10	70	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<10	120	320	<30	640	40	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<10	160	160	<30	640	200	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<30	<180	320	<180	960	60	ND	ND	ND	ND	ND	ND	ND	ND
	Mute swan	<120	<120	120	<120	320	240	ND	ND	ND	ND	ND	<10	<10	80
	Mute swan	<30	30	160	40	640	480	ND	ND	ND	ND	ND	<10	<10	60
	Mute swan	<60	<60	<40	<40	60	480	ND	ND	ND	ND	ND	<10	<10	240
	Mute swan	<60	<40	240	<30	640	70	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<120	<60	160	<60	640	960	12	<6	<6	<6	192	<10	10	240
	Mute swan	<10	<40	320	<40	1,280	70	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<10	60	480	30	2,560	60	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<30	<120	240	<120	480	70	ND	ND	ND	ND	ND	ND	ND	<10
	Mute swan	<60	<120	320	<120	640	50	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<60	<120	320	<120	640	80	ND	ND	ND	ND	ND	<10	<10	20
	Mute swan	<10	<60	320	<60	960	70	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<120	160	640	30	2,560	240	<6	<6	<6	<6	<6	<10	<10	<10
	Mute swan	<60	40	320	30	1,280	120	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<30	30	160	<30	640	50	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<10	ND	ND	ND	ND	50	ND	ND	ND	ND	ND	ND	ND	20
	Mute swan	<120	<120	160	<120	640	70	ND	ND	ND	ND	ND	20	<10	<10
	Mute swan	<10	160	320	<120	1,280	70	ND	ND	ND	ND	ND	ND	ND	ND
	Mute swan	<60	<120	160	<120	640	50	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<30	40	160	<60	640	50	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<30	<30	160	<30	320	35	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<10	<180	320	<180	640	100	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	40	<60	160	<60	640	80	ND	ND	ND	ND	ND	ND	ND	ND
	Mute swan	<60	<60	<60	<60	160	240	ND	ND	ND	ND	ND	<10	<10	10
	Mute swan	<60	<240	<240	<240	<240	480	12	<6	<6	<6	96	<10	<10	60
	Mute swan	<60	<30	<30	<30	60	480	<6	<6	<6	<6	96	<10	<10	240
	Mute swan	<120	<120	<120	<120	320	480	ND	ND	ND	ND	ND	<10	<10	60

HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; ND: not determined.

Lowest serum dilution tested was 10 for the initial haemagglutination inhibition (HI) and virus neutralisation assay and 6 for the confirmatory HI assay.

[a] Species included common coot (Fulica atra), Egyptian goose (Alopochen aegyptiaca), Eurasian wigeon (Anas penelope), lesser white-fronted goose (Anser erythropus), mute swan (Cygnus olor).

[b] Mean titre of in duplo tested samples.

Table 6b

Titres of confirmatory haemagglutination inhibition and virus neutralisation assays for sera positive for highly pathogenic avian influenza H5 clade 2.3.4.4-specific antibodies in the initial screening, the Netherlands, 14 November 2014–31 December 2015 (n = 53 serum samples)

Period	Species[a]	Haemagglutination inhibition assay											Virus neutralisation assay		
		Initial						Confirmatory							
		LPAI H5	HPAI clade					LPAI H5	HPAI clade				HPAI clade		
			1	2.1	2.2	2.3	2.3.4.4[b]		2.1	2.2	2.3	2.3.4.4	2.1	2.3	2.3.4.4
After the outbreak: 2015	Eurasian wigeon	<10	<10	<10	<10	<10	20	ND	ND	ND	ND	ND	ND	ND	160
	Eurasian wigeon	<10	<10	<10	<10	<10	10	ND	ND	ND	ND	ND	ND	ND	20
	Eurasian wigeon	<10	<10	<10	<10	<10	40	ND	ND	ND	ND	ND	ND	ND	80
	Common coot	<10	80	60	60	320	160	ND	ND	ND	ND	ND	ND	ND	20
	Egyptian goose	<10	<10	<10	<10	80	25	ND	ND	ND	ND	ND	ND	ND	<10
	Mute swan	160	80	60	<30	160	120	ND	ND	ND	ND	ND	<10	<10	40
	Mute swan	40	80	80	80	320	45	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<10	<10	<10	<10	30	15	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<10	80	80	80	320	60	ND	ND	ND	ND	ND	<10	<10	<10
	Mute swan	<10	160	160	240	320	80	ND	ND	ND	ND	ND	80	20	<10

HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; ND: not determined.

Lowest serum dilution tested was 10 for the initial haemagglutination inhibition (HI) and virus neutralisation assay and 6 for the confirmatory HI assay.

[a] Species included common coot *(Fulica atra)*, Egyptian goose (*Alopochen aegyptiaca*), Eurasian wigeon (*Anas penelope*), lesser white-fronted goose (*Anser erythropus*), mute swan (*Cygnus olor*).

[b] Mean titre of in duplo tested samples.

on 7,337 samples obtained from 21 February 2015 onwards in addition to the previously reported 4,018 samples obtained until 20 February 2015 [14]. Sampled species mainly belonged to the orders *Anseriformes*, *Charadriiformes* and *Gruiformes* (Table 1).

For antibody detection, 1,443 serum samples were analysed. Among these, 945 samples from 25 avian species were obtained during the outbreak, while 349 samples from 15 species originated from after the outbreak. A total of 149 serum samples from 15 species sampled before the HPAI H5N8 virus emergence, obtained between 2007 and 2013, served as controls (Table 2). The majority of these samples were collected from birds wintering in Dutch wetlands.

Virus detection, isolation and characterisation

In addition to the two previously reported HPAI H5N8 virus-infected Eurasian wigeons detected in the Netherlands in November 2014 [14], the virus was detected in a third Eurasian wigeon faecal sample obtained on 25 February 2015 (1/1,369 birds sampled in 21 February–13 May 2015), near Ilpendam (52°28'N 4°57'E) (GenBank accession numbers: AKH14448–AKH14459). Since then, no HPAI H5N8 virus has been detected in any of the samples tested (0/5,968 birds sampled in 14 May 2015–31 January 2016) (Table 1).

Influenza A H5 virus clade-specific antibody detection

As shown previously, ferret antisera raised against prototype strains representing LPAI and HPAI H5 viruses of various clades showed almost exclusive reactivity with homologous viruses in HI assays [12] (Table 3). Importantly, a ferret antiserum raised against the clade 2.3.4.4 virus did not react with other H5 viruses, and antisera raised against other prototype H5 strains did not react with the clade 2.3.4.4 virus A/Chicken/Netherlands/EMC-3/2014. Sera obtained upon inoculation of a domestic duck and a domestic goose with the clade 2.3.4.4 virus A/Turkey/Germany/AR2487/2014 reacted similar to the ferret clade 2.3.4.4 antiserum; no cross-reactivity was seen with other prototype H5 strains (Table 3). These data indicate that the antigenic differences between clade 2.3.4.4 HA and HA of LPAI and HPAI viruses belonging to other clades were sufficiently large to allow serological discrimination by HI assay.

Influenza A virus H5-specific antibody detection in wild birds

Haemagglutination inhibition assays

Of the serum samples initially tested in the HI assay with LPAI H5N2 (A/Mallard/Netherlands/3/1999) and HPAI H5 clade 2.3.4.4 H5N8 (A/Chicken/Netherlands/EMC-3/2014) virus, LPAI H5-specific antibodies were detected in 31 of 1,443 serum samples and HPAI H5 clade 2.3.4.4-specific antibodies in 53 of 1,443 serum samples (Table 4). Among these, seven samples tested positive for both LPAI H5- and HPAI H5 clade 2.3.4.4-specific antibodies. The incidence of LPAI H5-specific antibodies was similar before, during and after the HPAI H5N8 virus emergence in Europe (Fisher exact test,

p = 0.76 before vs during the outbreak; p = 0.39 during vs after the outbreak), while HPAI H5 clade 2.3.4.4-specific antibodies were detected exclusively in sera from five bird species, obtained during and after the HPAI H5N8 virus emergence in Europe (Table 4, Table 5). The incidence of HPAI H5 clade 2.3.4.4-specific antibodies a year after the outbreak (10/329 (20 samples with high background excluded), 3.0%) was lower than during the outbreak (43/940 (5 samples with high background excluded), 4.6%) (Fisher exact test, p = 0.27).

Serum samples obtained during (43/940 (5 samples with high background excluded), 4.6%) and after (10/329 (20 samples with high background excluded), 3.0%) the outbreak that tested positive for HPAI H5 clade 2.3.4.4-specific antibodies were subsequently tested in an HI assay against prototype viruses of clades 1, 2.1, 2.2, 2.3, and 2.3.4.4. Of the sera collected during the outbreak, 29/90 mute swans (*Cygnus olor*), 12/78 Eurasian wigeons, 1/3 lesser white-fronted geese (*Anser erythropus*) and 1/84 common coots (*Fulica atra*) tested positive for HPAI H5 clade 2.3.4.4-specific antibodies (Table 5). In these HPAI H5 clade 2.3.4.4-specific antibody positive sera, no cross-reactivity was observed in sera of Eurasian wigeons (12/12) and the lesser white-fronted goose (1/1). In contrast, the common coot (1/1) serum showed an additional titre to the clade 2.3 virus and sera of mute swans showed cross-reactivity to clade 2.3 (27/29), 2.1 (23/29), 1 (9/29) and 2.2 (4/29) viruses. In the majority of samples (22/29), titres to clade 2.1 and 2.3 exceeded those detected to clade 2.3.4.4 (Table 6).

Of the sera collected after the outbreak, 5/29 mute swans, 3/46 Eurasian wigeons, 1/28 Egyptian geese (*Alopochen aegyptiaca*) and 1/24 common coots tested positive for HPAI H5 clade 2.3.4.4-specific antibodies (Table 5). The sera of the Eurasian wigeons reacted with HPAI H5N8 virus exclusively. However, the common coot as well as 1/5 mute swans showed HI titres to all five H5 clades. The other 3/5 mute swans showed HI titres to multiple but not all H5 clades, while 1/5 mute swans and 1/1 Egyptian goose only showed an additional titre to clade 2.3 (Table 6).

Seven of the HPAI H5 clade 2.3.4.4-seropositive bird sera obtained during the outbreak, from four mute swans and three Eurasian wigeons, were retested in an HI assay at the APHA. Here, 3/4 mute swan samples with high initial HI antibody titres against HPAI H5 clade 2.3.4.4 (H5N8) virus were confirmed. However, 1/4 mute swan sera could not be confirmed, and HPAI H5 clade 2.3.4.4-specific antibodies were also not detected in 3/3 sera of the Eurasian wigeons that had low antibody titres in the initial tests (Table 6).

Virus neutralisation assays

For 37/43 HPAI H5 clade 2.3.4.4-positive sera collected during and 10/10 sera collected after the outbreak, sufficient serum volumes were available for retesting in a VN assay. In this assay, HPAI H5 clade 2.3.4.4-specific antibodies were detected in sera of 9/9 Eurasian wigeons and of 10/26 mute swans obtained during the outbreak. Sera of the mute swans did not react with viruses of other H5 clades. HPAI H5 clade 2.3.4.4-specific antibodies were not detected in the sera of the common coot and the lesser white-fronted goose by VN assay. HPAI H5 clade 2.3.4.4-specific antibodies were confirmed by VN assay in sera from 3/3 Eurasian wigeons, 1/5 mute swans, 1/1 common coot and 0/1 Egyptian goose collected after the outbreak (Table 6).

Discussion

In this report surveillance data for HPAI H5N8 in birds in the Netherlands are presented. In addition to bird samples previously investigated for the virus from 14 November 2014 to 20 February 2015, a new set of 7,337 samples obtained between 21 February 2015 and 31 January 2016 is analysed. One faecal sample obtained from a Eurasian wigeon (*Anas penelope*) on 25 February 2015 tested positive for the HPAI H5N8 virus, adding to the previous finding of the virus in two Eurasian wigeons in the country in late 2014 [14]. Virological surveillance moreover suggests that only very limited numbers of wild bird species were identified as potential hosts in Europe. Importantly, to the best of our knowledge, there are no reports of additional findings of HPAI H5N8 viruses in wild birds and poultry in Europe, since the last detection of the virus in February 2015 in the Netherlands.

Given the difficulty of detecting newly emerging HPAI virus strains in wild birds however, the application of a more sensitive and cost-effective method to detect potential host species is warranted. For this purpose, we performed serological assays specifically aimed to detect antibodies specific to HPAI H5 clade 2.3.4.4 viruses in a substantial number of sera obtained before, during, and after HPAI H5N8 emergence in the Netherlands. Three potential HPAI H5N8 host species were identified by HI assays and confirmed by VN assays; Eurasian wigeons, mute swans and common coots. Considering the results of virological studies performed worldwide since the onset of the HPAI H5N8 virus emergence in early 2014, the detection of HPAI H5 clade 2.3.4.4-specific antibodies in these species is not surprising. HPAI H5N8 virus was isolated from Eurasian wigeons in Russia [8] and the Netherlands [14], from mute swans in Sweden [6], and from a common coot in South Korea [21].

The serological results reported here were not entirely consistent between HI and VN assays and between HI assays performed in two different laboratories. Although low HI titres (e.g. in Eurasian wigeons) were reproducible within a laboratory with the same HI assay and a VN assay, they were not detected by HI assay in a second laboratory, potentially due to differences in the methods used and hence differences in sensitivity and specificity. High antibody titres in mute swan sera were reproduced by HI assay in a second laboratory and by VN assay, but low antibody titres in mute swans

were not always reproduced. While it is thus clear that individual HI titres in avian sera obtained from a single test cannot be used reliably for diagnosis, use of serum panels from cohorts of birds, use of multiple tests to cross-validate results, a panel of relevant viruses and use of collections of control antisera may still enable the use of serological tests in support of HPAI H5 surveillance studies.

Previously, HI assays were shown to be discriminative enough to detect antibodies in serum samples collected from free-living wild birds in Europe and Asia to be directed to either HPAI or LPAI H5 viruses. However, widely varying results were obtained as far as HPAI H5 clade-specific antibodies were concerned [16]. In this study, most birds that tested positive for HPAI H5 clade 2.3.4.4-specific serum antibodies showed relative low HI titres. This is in accordance with findings based on experimental HPAI H5N8 virus infections of ferrets [10-12], possibly indicating low immunogenicity upon infection. In addition, there is limited knowledge about the longevity of avian antibodies after naturally occurring infection with avian influenza viruses. Antibodies specific to LPAI viruses were detected up to several months after experimental or natural infection [22-24], whereas little is known about the duration of detection of antibodies specific to HPAI viruses with a reported maximum of detection of 28 days after experimental infection in domestic ducks [25]. To date, there is no knowledge on the effect of a prior exposure to an unrelated subtype or on the phenomena of antigenic sin in avian species. Hypothetically, low immunogenicity in combination with decreasing titres in time could be an explanation for the low incidence and relative low titres of antibodies detected in wild bird sera in this study.

In conclusion, our results provide evidence that clinically unaffected long distance migratory and local wild birds sampled in the Netherlands during the H5N8 outbreak late 2014 and early 2015, and again late 2015, have been exposed to HPAI H5N8 or closely related HPAI H5 clade 2.3.4.4 viruses and seroconverted upon exposure. Since HPAI H5N8 virus has not been detected in Europe since early 2015 and because HPAI H5 clade 2.3.4.4-specific antibody incidence decreased in time, we conclude that the virus has not circulated extensively at the breeding grounds in summer and upon the return of the birds to their wintering areas in the 2015/16 winter. As a consequence, the newly emerging HPAI H5N8 clade 2.3.4.4 virus subtype appears to have already disappeared from European wild birds indicating that sustained transmission and independent maintenance may be less likely. This is an important consideration in the ongoing evolution and ecology of these viruses in wild birds and the potential risks they pose for introduction to poultry and the pathways through which they might spread. Finally we recommend that serological tools be further optimised, harmonised, and validated for avian influenza surveillance studies in wild birds.

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Conflict of interest

None declared.

Authors' contributions

MP and JV: performing serological assays, compiling the data and drafting the manuscript; RM and IB: confirmatory serological assays; TB and SV: excellent technical assistance in preparing, performing and interpreting serological assays; OV and RS: analysing samples for virus detection; HJ, BN and EK: initiation of the study, providing field data; FM and GM: collecting field data; CG; providing positive control sera; RF: initiation of study, critically revised the manuscript.

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Research article

Epidemiological and molecular investigation of a rubella outbreak, Romania, 2011 to 2012

M Lazar [1,7], E Abernathy [2], M Chen [2], J Icenogle [2], D Janta [3], A Stanescu [3], A Pistol [3], S Santibanez [4], A Mankertz [4], JM Hübschen [5], G Mihaescu [7], G Necula [1,6], E Lupulescu [1]

1. National Institute of Research-Development for Microbiology and Immunology "Cantacuzino", Bucharest, Romania
2. National Center for Immunizations and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, United States
3. National Centre for Communicable Diseases Surveillance and Control, National Institute of Public Health, Bucharest, Romania
4. WHO-EURO, Regional Reference Laboratory for Measles and Rubella, Robert Koch Institute, Berlin, Germany
5. Department of Infection and Immunity, WHO-EURO Regional Reference Laboratory for Measles and Rubella, Luxembourg Institute of Health Esch-sur-Alzette, Grand-Duchy of Luxembourg
6. Horia Hulubei National Institute for R&D in Physics and Nuclear Engineering, Bucharest, Romania
7. University of Bucharest, Faculty of Biology, Department of Virology, Bucharest, Romania

Correspondence: Mihaela Lazar (mlazar@cantacuzino.ro)

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We describe a rubella outbreak that occurred in Romania between September 2011 and December 2012. During this period 24,627 rubella cases, 41.1% (n=10,134) of which female, were notified based on clinical criteria, and a total of 6,182 individuals were found serologically positive for IgM-specific rubella antibody. The median age of notified cases was 18 years (range: <1–65) and the most affected age group 15 to 19 years (n=16,245 cases). Of all notified cases, 24,067 cases (97.7%) reported no history of vaccination. Phylogenetic analysis of 19 sequences (739 nucleotides each), from 10 districts of the country revealed that the outbreak was caused by two distinct rubella virus strains of genotype 2B, which co-circulated with both temporal and geographical overlap. In addition to the 6,182 IgM-positive rubella cases, 28 cases of congenital rubella syndrome (CRS) were identified, including 11 neonatal deaths and one stillbirth. The outbreak underscores the need to encourage higher vaccination uptake in the population, particularly in women of reproductive age, and to strengthen epidemiological and laboratory investigations of suspected rubella cases. Genetic characterisation of wild-type rubella virus is an essential component to enhance surveillance and here we report rubella virus sequences from Romania.

Introduction

Rubella virus (RuV), the sole member of the Rubivirus genus in the Togaviridae family, is a positive strand RNA virus with a non-segmented genome of ca 9,762 nucleotides (nt). The genome encodes two non-structural (P90 and P150) and three structural (virion) proteins (the capsid and 2 envelope glycoproteins, E2 and E1). A 739-nt region between nt 8,731 and 9,469 within the E1 glycoprotein is the standard genotyping window for RuV [1,2]. Based on phylogenetic analysis of sequences of the structural protein coding region, two virus clades including a total of 13 genotypes, have been identified.

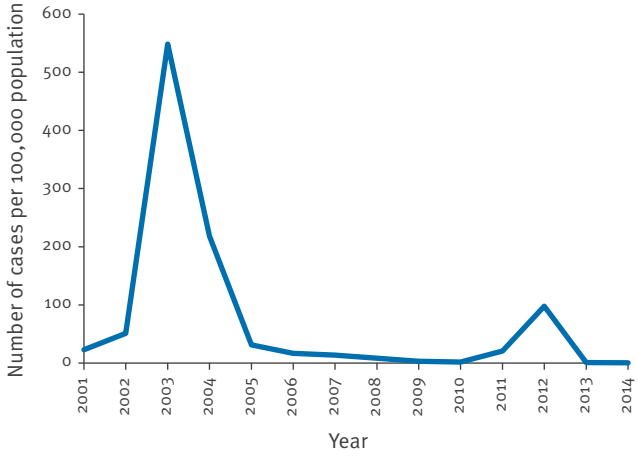
Infection with RuV generally leads to mild disease with symptoms that can include rash and low fever (<39°C) [3]. In pregnancy, however, RuV infection can cause miscarriages and serious birth defects including hearing, vision, mental, and heart impairment, which are collectively known as congenital rubella syndrome (CRS). CRS occurs in up to 85% of children born to women with RuV infection during the first trimester of pregnancy [4]. In addition, CRS can lead to neonatal deaths in up to 30% of cases [5].

Laboratory investigation plays an important role in both diagnosis and surveillance of rubella and CRS, since clinical diagnosis is unreliable and up to 50% of infections are estimated to be subclinical [6]. Typically, rubella is diagnosed by RuV specific IgM, but in pregnancy additional testing such as IgG avidity may be necessary.

False-negative rubella IgM can occur when specimens are taken within the first three days post-rash onset while false-positive IgM can result from cross reactions with rheumatoid factor or other viruses (such as parvovirus B19) [7,8]. In addition to serology, detection of viral RNA from nasopharyngeal swabs or oral fluid has been widely employed to confirm RuV infection. Moreover, polymerase chain reaction (PCR) can be

Figure 1

Rubella incidence in Romania, 2000–2014



used to obtain genetic information about circulating wild-type viruses to investigate transmission events [9,10].

When the European Region of the World Health Organization (WHO) adopted the goal of eliminating endemic rubella and measles by the end of 2015, the two key strategies were to achieve and sustain high vaccination coverage (≥95%) with two doses of measles, mumps, and rubella (MMR) vaccine and to strengthen surveillance systems through rigorous investigation and laboratory confirmation of outbreak-related and sporadic cases [11]. Because phylogenetic analysis of RuV genotypes can help determine whether circulating RuV strains result from endemic transmission or importations, laboratory surveillance for rubella also included the molecular characterisation of viruses.

In Romania, selective vaccination for rubella and measles was offered to adolescent girlsagedbetween 15 and 18 years (birth cohorts 1980 – 1983) as part of a mass vaccination campaign following a nation-wide measles outbreak in 1998 [12]. In 2004, MMR vaccination was introduced into the national immunisation programme with the first dose administered at 12 to 15 months of age and the second dose at seven years-old, and a rubella-containing vaccine was offered to girls aged between 13 and 14 years until 2008 (birth cohort 1994) [13]. Based on recent assessments of 18 month-old children however, the estimated MMR vaccine (one dose) coverage has decreased from 96.5% in 2010 to 89.3% in 2014 [14].

Rubella epidemics follow a 6 to 9 year cycle in the country. Between 2002 and 2003, Romania experienced a large rubella outbreak with more than 115,000 reported cases nationwide corresponding to an incidence of 549 cases per 100,000 population, the highest incidence ever observed in the 24 prior years [12]. In 2011 and 2012, another rubella outbreak occurred, with an incidence of 20.6 cases per 100,000 population in 2011 and 97.5 per 100,000 in 2012 [15]. This outbreak coincided with a measles outbreak, which took place between 2010 and 2013 and included 8,170 notified cases [16]. Here we provide an overview on the 2011 to 2012 rubella outbreak in Romania in terms of time, place and person, with a focus on laboratory and molecular analysis

Methods

Description of the surveillance systems

Since 1978 measles and rubella are statutorily notifiable diseases in Romania. Medical practitioners must report all possible measles or rubella cases to the regional public health authorities. The definition of a possible case in Romania concurs with the European Union (EU) case definition for possible cases and comprises any person with sudden onset of generalised maculopapular rash and at least one of the following five manifestations: cervical adenopathy, suboccipital adenopathy, post-auricular adenopathy, arthralgia, or arthritis [17,18].

A rubella surveillance system with case-based reporting with mandatory laboratory confirmation started in 2010. IgM antibody detection by enzyme-linked immunosorbent assay (ELISA) is the standard test for routine rubella surveillance recommended in the country [18]. In case of clusters/outbreaks, only five to ten sera from rubella possible cases are collected for testing [11,19].

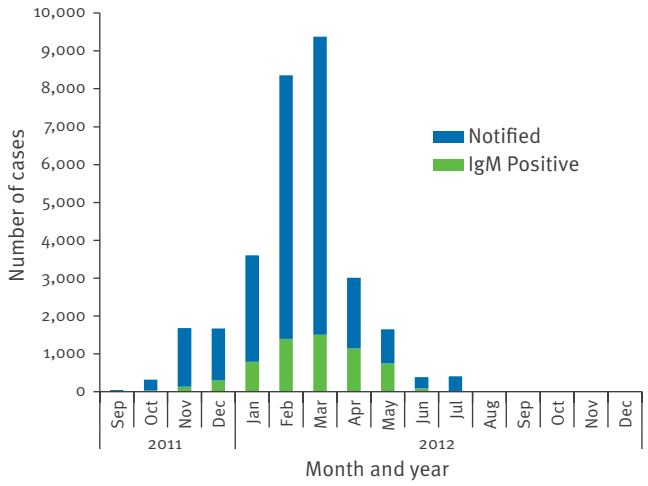
Laboratory confirmation of cases in Romania is conducted according to a national methodology. Except for pregnant women, cases in Romania are either laboratory-confirmed by detecting rubella IgM antibodies in serum samples, or a significant rise in rubella IgG antibody levels, or PCR detection of RuV genetic material in nasopharyngeal swabs. In pregnancy, a rubella-specific IgG avidity test is additionally used to confirm rubella infection in rubella IgM-positive patients. Moreover, pregnant women, who are known to have been exposed to rubella, are assessed for rubella specific IgM and IgG antibodies and for those found to be negative another sample of serum is requested after 14 days to monitor IgM and/or IgG seroconversion [18]. As for measles, rubella surveillance is carried out among the general population, nationwide and all year round. The objectives of the surveillance are to facilitate the detection and laboratory confirmation of all possible sporadic cases, to identify chains of transmission and to investigate outbreaks.

National surveillance for CRS, which is notifiable, was initiated in the year 2000 according to Romanian methodology. The clinical criteria for CRS apply to any infant ‹ 1 year of age or any stillborn and include at least two of the following conditions: cataract(s), congenital glaucoma, congenital heart disease, loss of hearing, pigmentary retinopathy, or one of the above and either one of the subsequent manifestations: purpura,

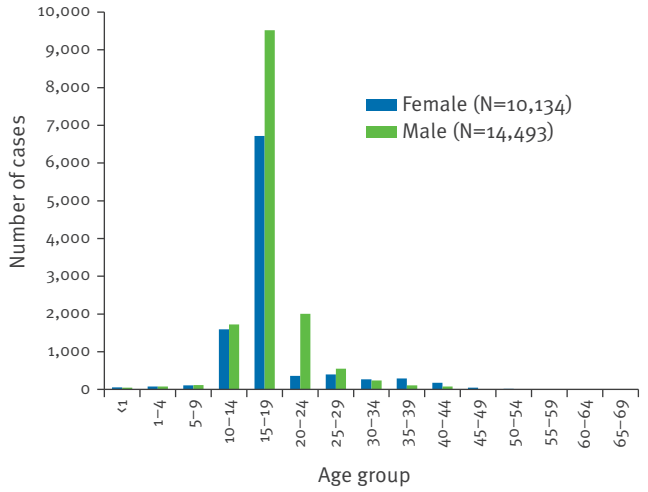
Figure 2

Distributions of notified rubella outbreak cases, Romania, September 2011–December 2012 (n = 24,627)

A. Monthly distribution of notified (N=24,627) and serologically-confirmed[a] (N=6,182) rubella cases



B. Rubella outbreak notified cases by age group and sex (N=24,627)



[a] IgM positive.

splenomegaly, microcephaly, developmental delay, meningo-encephalitis, radiolucent bone disease, or jaundice that begins within 24 hours after birth [20].

Infants who meet the CRS clinical criteria are usually investigated for rubella-specific IgM and IgG antibodies: a serum sample is collected as soon after birth as possible; for infants with IgM negative and IgG positive results, a second serum sample is required, according with the EU case definition [20].

Collection and processing of samples

From September 2011 to December 2012, within the routine surveillance system, the Romanian Public Health Districts collected 9,627 serum samples from possible rubella cases for laboratory confirmation. These 9,627 samples corresponded to 9,615 possible rubella cases, including 314 pregnant women (whereby two serum samples were respectively received from 12 pregnant women).

During this time period 832 measles IgM-negative serum samples were also tested for rubella IgM.

In accordance with the national surveillance for CRS, during the epidemic and post epidemic period (2012–2013) 178 serum samples were collected from 137 infants who met the clinical definition.

From May 2011 to December 2012 (i.e. before and during the outbreak), 68 nasopharyngeal (NP) swabs from sporadic and outbreak-related cases were collected in different districts. Necropsy samples were obtained from one CRS case.

Sera, swabs and necropsy samples were transferred for testing to the Cantacuzino Institute laboratory. Sera were maintained at 2–8 °C until testing (maximum of 6 days), then stored at –20 °C. The RNA extraction from swabs and the necropsy samples was done on the same day than the samples were received, followed by reverse transcription-(RT)-PCR detection, and in case of positive results by genotyping. The remaining swab samples and the extracted RNA were maintained at –70 °C.

Serological assays

Detection of RuV specific IgM antibodies was performed using the Enzygnost Anti-Rubella Virus/IgM antibody enzyme immunoassay (EIA; Siemens, Marburg, Germany) or the Rubella virus IgM micro-capture EIA (IBL International). The Euroimmun Anti-Rubella Virus IgG and Avidity ELISA kit (Lubeck, Germany) was used for IgG and avidity testing. According to the manufacturer, relative avidity indexes are interpreted as follows: ‹40% indicates low avidity antibodies and ›60% indicates high avidity antibodies, with 40–60% considered as intermediate (high avidity excludes rubella infection within the last 4 to 6 weeks before sample collection).

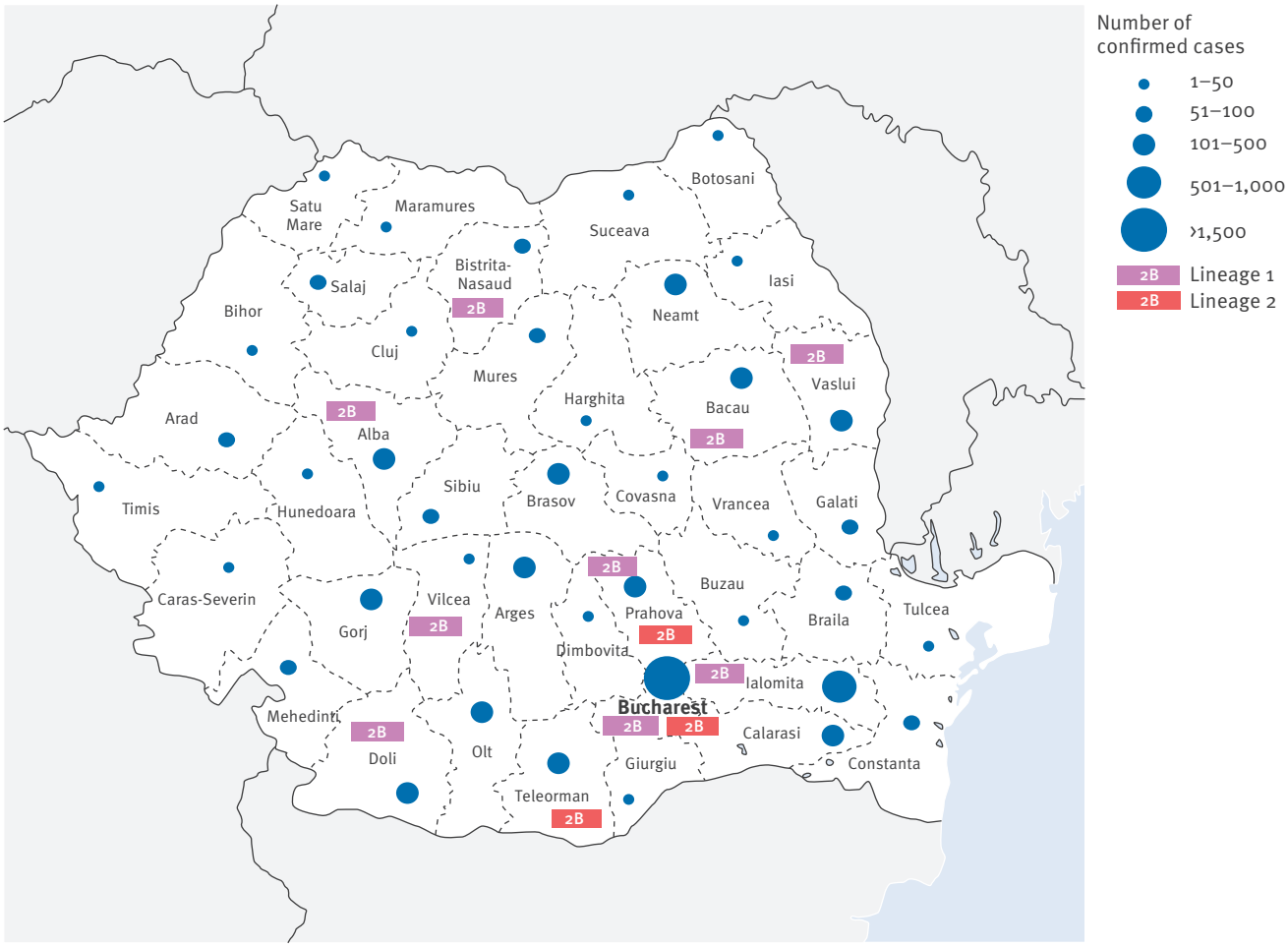
RNA extraction from clinical specimens

In Romania, detection of RuV RNA or RNA extraction and subsequent genotyping were conducted only on NP swab samples and from necropsy samples (one case).

As the number of swab samples collected during the outbreak was low (n=61), it was tested whether RNA could be obtained from IgM-positive serum samples that had been collected within three days after rash with a protocol used at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia. Therefore in July 2014, 93 aliquots from such selected serum specimens were transported to the CDC for detection of RuV RNA and genotyping.

Figure 3

Geographical distribution of rubella serologically confirmed cases (n = 6,182)[a] and virus genotype, Romania, September 2011–December 2012



[a] Confirmed cases included 88 pregnant women and 5,820 other patients with rubella symptoms, as well as 274 patients suspected of measles but who, after being found negative for measles IgM, were found positive for rubella IgM.

Total RNA was extracted from NP swabs with the Nucleospin Viral RNA kit (Macherey, Germany) according to the manufacturer's instructions, except that 20 µL of proteinase K (20 mg/mL) was added in the lysis step and the RNA was eluted in 30 µL RNase-free H2O. RNA was also isolated from tissues (lung, kidney, spleen, lens, liver, brain, and thymus) from a deceased infant with CRS using TRIzol (Invitrogen, US). Extracted RNAs were stored at –70 °C.

For RNA extraction from sera shipped to CDC, the Qiagen ViralAmp RNA Mini kit (Qiagen, Valencia, CA) was used according to the manufacturer's instructions.

Detection of rubella virus RNA

In Romania, two detection methods were used to detect rubella RNA in the clinical samples. Prior to 2012 a nested RT-PCR assay [21], which amplified a 143-nt region in the E1 coding region, was performed using GoScript Reverse Transcriptase and GoTaq Flexi DNA Polymerase (Promega,Madison, WI, US) according to the manufacturer's instructions, followed by gel electrophoresis. In 2012 a real-time RT-PCR assay for RuV RNA detection using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, US) [22] was implemented.

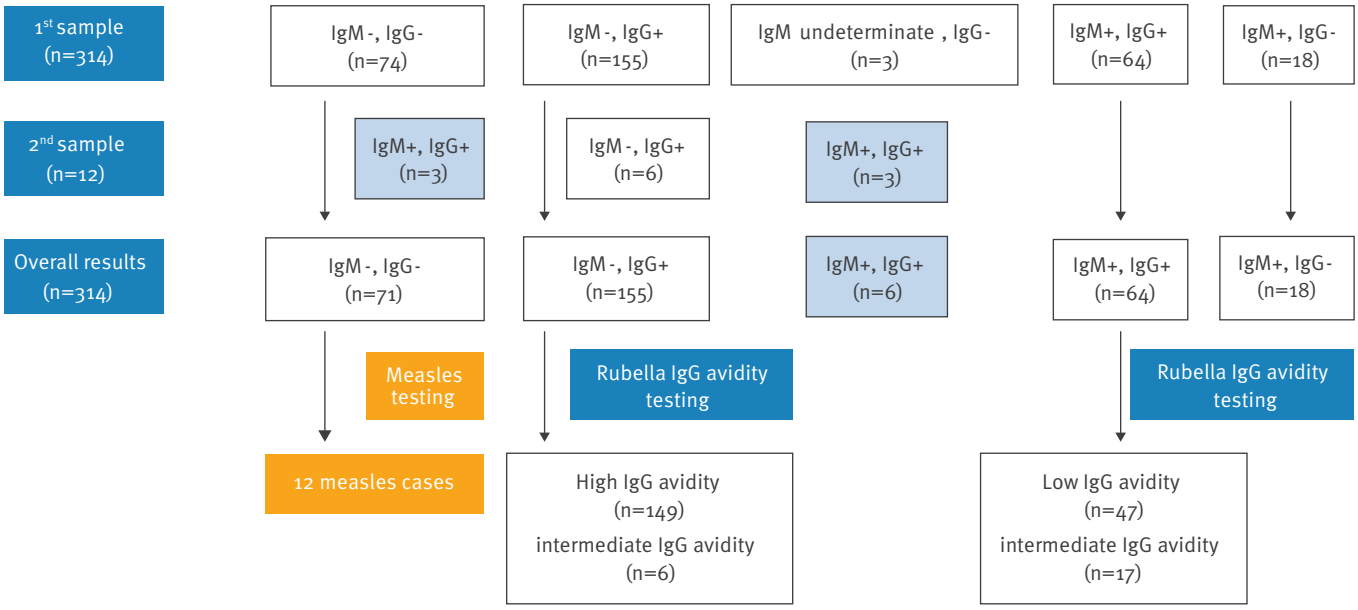
At CDC, a TaqMan real-time PCR assay targeting a 154 nt region near the 5' end of the rubella genome and the same SuperScript kit was used (data not shown).

Genotype determination

All genotyping assays were targeted to the RuV E1 coding region which contains the 739-nt region recommended by WHO for RuV genotyping. Generation of genotyping templates using RNAs from NP swab and tissue samples was performed by conventional RT-PCR reactions with the Qiagen OneStep RT-PCR Kit (Hilden, Germany) as described in Namuwulya et al. [11], except that the primers for the 5' fragment were replaced by

Figure 4

Serological testing of pregnant women with clinical symptoms of rubella or exposed to rubella, Romania, September 2011–December 2012 (n=314)



IgG-: IgG negative; IgG+; IgG positive; IgM-: IgM negative; IgM+: IgG positive.

The boxes shaded in light blue, in the respective 2nd sample and in overall result rows, highlight samples with evidence of seroconversion.

primers 8656F (5'-CCCCACCGACACCGTGATGAG-3') and 9182R (5'-CGTGGATCCACTCGGGGATTT-3'). RNAs from sera which were positive by real-time RT-PCR were used as templates in one or more of three nested RT-PCR assays using specific primers pairs (Table). The nested assay 1 was used initially; samples that tested negative in this assay were subsequently tested using the assays 2 and 3.

Sequences derived from assays 2 and 3 were combined to obtain the 739-nt sequence. All genotyping nested RT-PCR assays were performed with the Superscript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA polymerase kit (Invitrogen) modified by the addition of betaine (Sigma, St. Louis, MO) to a final concentration of 1M. Cycling conditions for the first round consisted of one cycle of 30 min at 55 °C, 2 min at 94 °C, and 40 cycles of 10 s at 94 °C, 15 s at 55 °C, and 1 min at 68 °C. For the second round, 1 µL of the first round PCR was transferred and the 30 min at 55 °C RT step was eliminated. Negative and positive controls were carried through both rounds and master mix preparation and template addition were strictly separated.

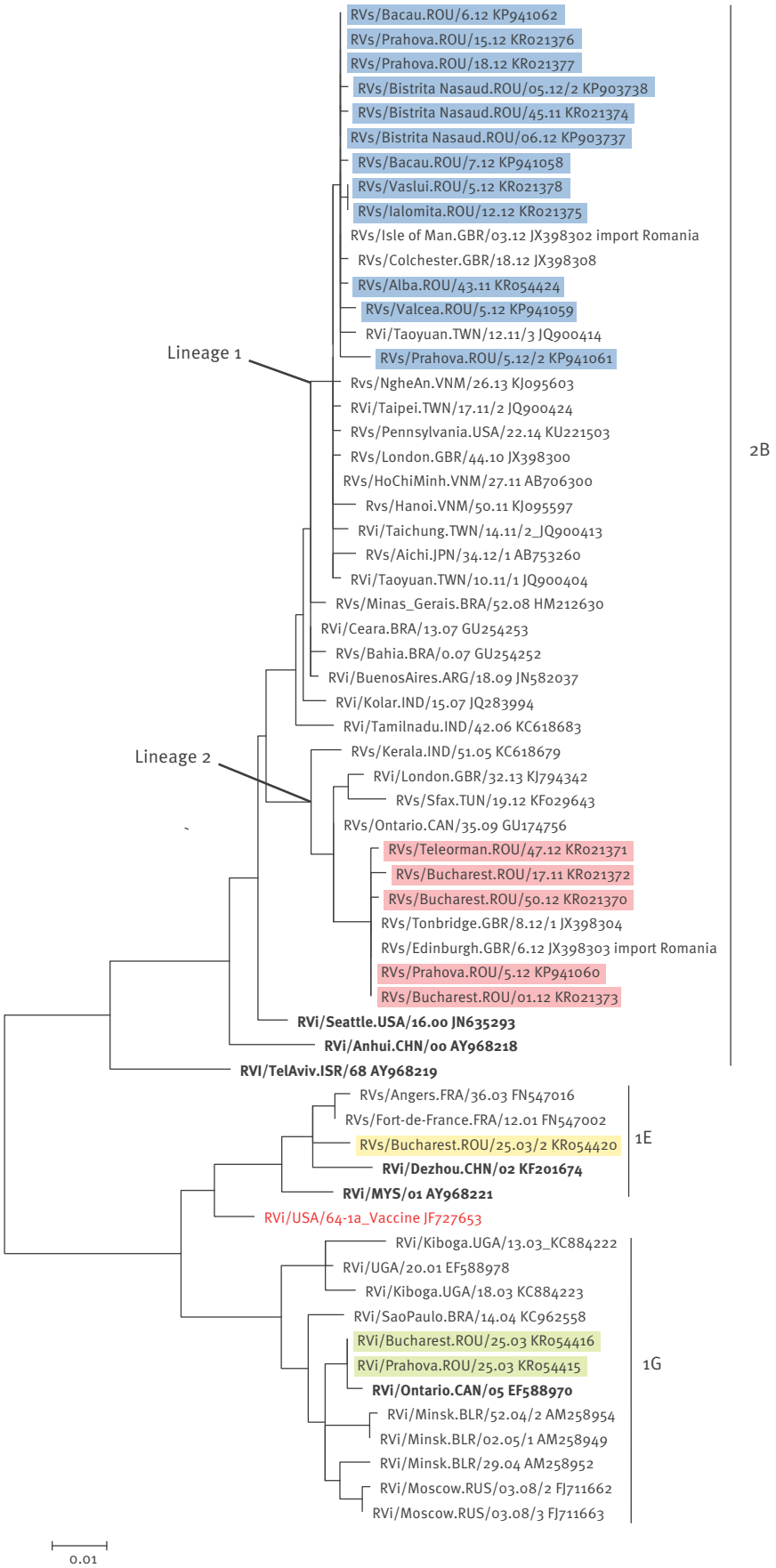
To sequence the DNA templates, the PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, California) was used on a PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis

The Romanian sequences were genotyped using the method recommended by the WHO [1]. GenBank accession numbers for the Romanian sequences are KP903737, KP903738, KP941058–62, KR021370–9 and KR054415–24. For phylogenetic analysis, an alignment was created and comprised 19 sequences from the 2011–2012 outbreak, three genotypes 1E and 1G sequences from the 2003–2004 outbreak, the 1E, 1G, and 2B WHO reference virus sequences and selected sequences from different parts of the world (26 2B sequences (2005–2014), seven 1G sequences (2003–2008) and two 1E sequences (2001–2003)). Searches to select the representative global strains were performed with basic local alignment search tool (BLAST) [23] and the selection was based on the degree of nt sequence homology with data from the Romanian outbreaks (≥ 99% identity), geographical distribution and collection date. Phylogenetic analysis was performed with the programme RAxML v8.00 [24] and the resulting tree was edited with the FigTree v1.4.2 programme [25] and the Inskape [26] programme for scalable vector graphics editing. The genetic distances were computed using the maximum-likelihood inference with generalised time-reversible (GTR) model of nt substitution and gamma model rate heterogeneity.

Figure 5

Phylogenetic analysis of sequences from rubella viral strains retrieved in Romania in 2003 and 2011–2012



Romanian sequences from 2003 are highlighted in yellow (1E) and green (1G) while those from 2011–12, are highlighted in blue (2B lineage 1), and red (2B lineage 2). The sequences are according to the World Health Organization recommended standard 739-nt window in the envelope (E)1 coding region of the rubella virus genome, and are compared with WHO reference strains (in bold) and representative sequences from RuV genotypes 1E, 1G, and 2B.

Results

Rubella incidence in Romania

After the year 2000, the incidence of rubella in Romania decreased following the 2003–2004 epidemic, from 218.5 cases per 100,000 population in 2004 to 1.6 in 2010 (Figure 1). In April and May of 2011, sporadic cases were notified in the south and south-east of the country. Subsequently in September, the outbreak started in the north-west, and spread further so that by the end of 2011 the total number of notified rubella cases amounted to 3,815 cases (18.2/100,000 population). In 2012, the whole country had become affected with 20,812 cases notified in that year (97.5/100,000).

Description of the rubella outbreak

From September 2011 to December 2012, a total of 24,627 cases were notified, 6,182 were confirmed (based on detection of IgM antibody), 18,442 were probable (based on an epidemiological link to a laboratory-confirmed case) and three were possible. Overall, 41.1% (n=10,134) of cases were of female sex and the median age was 18 years (range: ‹1–65), with the majority of cases (n=16,245) in the 15 to 19 year-old age group (Figure 2 A and B). Of all notified cases, 24,067 cases (97.7%) reported no history of vaccination, 528 cases (2.1%) reported one dose of MMR vaccine, and 23 cases (0.1%) reported two doses (vaccination histories were self-reported). For case-patients reporting vaccination, 114 (19.5%) were laboratory confirmed and 437 (80.5%) were considered probable by epidemiological link to laboratory-confirmed cases [15].

Serological analysis

Between September 2011 and December 2012, aside from sera obtained from 314 pregnant women (which are further described below), 9,301 serum samples were collected from possible cases of rubella and tested for the presence of rubella-specific IgM antibodies. Of these, 5,820 cases were positive for rubella IgM-specific antibody. Cases were from all parts of the country (Figure 3). Of the 3,481 IgM-negative serum samples, 1,726 (49.6%) were collected within 3-days post-rash onset.

Serum samples received via the national measles surveillance programme, which were negative for measles specific IgM were also tested for rubella IgM. Between September 2011 and December 2012, 274 (30.3%) of the 832 measles IgM-negative serum samples, were positive for rubella-specific IgM.

Rubella in pregnant women and congenital rubella syndrome cases

Sera from 314 pregnant women with clinical symptoms of rubella or known to have been exposed to rubella were tested for rubella-specific IgM and IgG antibodies.

In a first respective serum sample, 232 pregnant women tested negative or indeterminate for IgM and 82 tested IgM positive. The 232 IgM-negative or indeterminate women consisted of 74 women negative for both IgM and IgG, 155 IgM-negative IgG-positive women, and three IgM-indeterminate IgG-negative women. The 82 IgM-positive pregnant women comprised 18 women testing IgM positive IgG negative and 64 testing IgM positive IgG positive (Figure 4).

Follow-up samples for further laboratory confirmation could not be obtained for all pregnant women, however 12 women with negative or indeterminate IgM results were retested on a second sample received 14 days after the first. Six of these 12 women were initially among the 155 IgM negative IgG positive women, three were initially IgM indeterminate IgG negative, and three were initially part of the 74 women negative for both IgM and IgG. The first six women's tests remained unchanged in the second sample (i.e. still IgM negative, IgG positive, and with an intermediate IgG avidity), while for the latter six there was evidence of seroconversion, as they tested positive for both IgM and IgG in the second sample.

In total, 88 pregnant women were found to have rubella specific IgM-antibodies. The remaining rubella IgM-negative sera were subsequently tested for measles-specific IgM antibodies and 12 pregnant women were determined to be measles cases.

Overall, the number of women who tested positive for rubella-specific IgG only (i.e. IgM negative, IgG positive) amounted to 155. All had IgGs tested for avidity, and 149 were found with high avidity IgG antibodies, while six had intermediate avidity IgGs.

Of the total 88 IgM positive pregnant women, six could be confirmed as rubella cases by evidence of seroconversion in the second serum sample. For the 64 IgM-positive women who were IgG positive in the first sample, IgG avidity testing was conducted, whereby 47 had low and 17 intermediate avidity IgG, confirming primary rubella infection. Because a second serum sample could not be obtained from 18 women with initial IgM positive IgG negative results, IgG avidity testing was not possible for these persons. Taking into account their symptoms and the epidemiological context however, they were nevertheless included as outbreak cases.

When available, IgG avidity was used as a complementary test to the IgM antibody results, to determine the possible timing of contracting rubella. Based on this approach, it was estimated that 25 pregnant women (28.4 %; 25/88) were likely infected during the first trimester. A total of 14 pregnancies were terminated.

Serum samples from 137 infants suspected of having been exposed to RuV during fetal development were collected, 38 were IgM positive. In addition, RNA was

Table

Primer sequences for three nested reverse transcription-polymerase chain reaction genotyping assays

Nested assay set number	Primer name	Primer sequence (5'–3')	PCR product size	Nucleotides targeted
1	RV8633F	AGCGACGCGGCCTGCTGGGG	945	8,731–9,469
	RV9577R	CGCCCAGGTCTGCCGGGTCTC		
	RV8669F	GTGATGAGCGTGTTCGCCCTT	873	
	RV9541R	GTGTGTGCCATACACCACGCC		
2	RV8812F	CAACACGCCGCACGGACAAC	766	8,869–9,469
	RV9577R	CGCCCAGGTCTGCCGGGTCTC		
	RV8823F	ACGGACAACTCGAGGTCC	727	
	RV9541R	GTGTGTGCCATACACCACGCC		
3	RV8669F-2B	GTGATGAGCGTGTTCGCCCT	328	8,731–8,869
	RV8996R	CCACGAGCCGCGAACAGTCG		
	RV8691F-2B	CTAGCTACGTCCAGCACCC	271	
	RV8961R	CAAACCGGGGAGGCCCA		

PCR: polymerase chain reaction.

also collected from a stillborn infant. Combined with clinical criteria, 27 infants were laboratory-confirmed to be CRS by IgM and IgG testing, while the stillborn was confirmed to have been infected by RuV using PCR. The other 11 infants were identified to have congenital rubella infection by an IgM-positive test at birth, and an epidemiological link (the mother was confirmed with rubella infection during pregnancy) but without observable defects. Such children are not followed-up. Of 28 infants with CRS, one was a stillbirth and 11 died after birth.

Formalin-fixed, paraffin-embedded (FFPE) tissue specimens obtained from one confirmed child with fatal congenital rubella autopsy were submitted to the CDC for additional studies (histopathological and immunohistopathological evaluation) [27].

Reverse transcription-polymerase chain reaction and genotyping

In May of 2011, two sporadic rubella cases in Bucharest were confirmed. Both cases occurred three months before the outbreak was recognised and had no recent history of travel. A virus sequence from one of these sporadic cases (RVs/Bucharest.ROU/18.11) was determined to be genotype 2B. Between May 2011 and December 2012, 68 NP swabs were collected from cases occurring in 21 of 42 districts. Thirty-three (48.5%) swabs were positive for RuV RNA by either the nested or real-time RT-PCR assay. Of these, PCR templates for genotyping were generated from 11 swab samples (36.4%).

In addition, RNAs from necropsy tissues (lung, kidney, spleen, liver, brain, thymus and lens) from one case were positive for rubella by real-time RT-PCR and RNA from the kidney was used to genotype the virus.

RNA was also extracted from 93 IgM-positive sera which were collected three days after rash onset or earlier. Of these, rubella RNA was detected by real-time RT-PCR in 20 sera (21.5%); the average cycle threshold value was 37 of 40 cycles (range: 35.7–39). Genotypes were determined from seven sera (7.5%). Three of the RNAs derived from serum were amplified by nested primers set 1 and four required the amplification of both the nested primer sets 2 and 3 to obtain the 739-nt sequence.

In total, 19 sequences were obtained from rubella cases between May and December 2012, representing samples from 10 distinct districts (Figure 3). The genotype of all the sequences was determined to be 2B by comparison to the WHO reference sequences (data not shown).

In order to compare the sequences from the 2011–2012 outbreak to earlier sequences from Romania and sequences of the same genotypes retrieved worldwide, a phylogenetic tree of genotypes 1E, 1G, and 2B is shown (Figure 5). The 2003 1E and 1G sequences (in yellow and green in Figure 5; Robert Koch Institute, Berlin) from two Romanian cities, Bucharest and Prahova, were found in the same clusters as viruses from other European countries from the same time period (e.g. for 1E, RVs/Angers.FRA/36.03; for 1G, RVi/Minsk.BLR/52.04/2). The 2B sequences from the 2011–2012 Romanian outbreak assort into two lineages with 3.11–3.92% nt (23–29 nt) difference between the two lineages. Lineage 2 appeared to have had a smaller geographical range, being found in Bucharest and two other districts, while lineage 1 was found in Bucharest and seven additional districts (Figure 3). Lineage 1 contains 12 of the Romanian sequences from 2011 and 2012 as well as 2010–2014 sequences from different areas of Asia including Japan, Taiwan and Vietnam. One of three sequences from Great Britain in this cluster

(RVs/Isle of Man.GBR/03.12) was epidemiologically linked to an importation from Romania (Kevin Brown, personal communication, 25 January 2016). This lineage descends from sequences from South America and India detected from 2006 to 2009. Lineage 2 contains seven of the Romania sequences, including a sequence from a sporadic case early in 2011 (RVs/Bucharest.ROU/18.11) and six from 2012. Lineage 2 also contains another import into Great Britain from Romania (RVs/Edinburgh.GBR/06.12) (Kevin Brown, personal communication, 25 January 2016). Other sequences from the same time period as the Romania outbreak in this lineage are from Tunisia, and Great Britain, with two older sequences from Canada (2009) and India (2005).

Discussion

Rubella is usually a mild benign disease, but due to its devastating effects in pregnancy, control and elimination programmes have been instituted in many countries; the disease has been eliminated by immunisation programmes in several countries, including those in the WHO Region of the Americas [28-31]. Universal rubella vaccination of one year-old infants was implemented in Romania in 2004; however, outbreaks continue to occur following a typical 6 to 9 year epidemic cycle. The total number of cases notified in Europe since 2007 varied from 26,827 in 2007 to 4,767 in 2010 then increased to 8,318 in 2011 and to 26,014 in 2012 [30,32]. In 2011, 97% of the rubella cases in Europe were reported from Poland and Romania [33], although it has to be taken into account that rubella surveillance has not been implemented in all European countries. During the 2011–2012 outbreak in Romania, cases occurred in all the districts of the country, amounting to 24,627 notified cases, most of which were unvaccinated (97.7%). The majority of cases were 15 to 19 year-olds who were missed by the current vaccination strategy (the MMR coverage among adolescents is not routinely monitored in Romania). The 2011–2012 outbreak resulted in the birth of 28 children with CRS, including 11 deaths and one stillbirth.

In Romania, rubella surveillance requires laboratory detection of IgM-specific antibody in serum collected from each sporadic case and the first cases from rubella outbreaks [11]. A limitation of rubella IgM tests, however, is that the IgM response may not have developed for a serum collected within the first 72 hours after rash onset, resulting in a false-negative result [22,34]. In the 2011–2012 outbreak 3,481 serum samples were negative for specific rubella IgM, but 49.6% of the negative sera were collected too close in time to the onset of rash; therefore, the total number of serologically-confirmed cases (n = 6,182) in the outbreak was likely underestimated.

In addition to serological testing, molecular detection of RuV RNA is useful for the further confirmation of rubella infection, especially in the five days after rash onset [22]. Moreover, sequence information can be obtained and used to differentiate between vaccine and wild-type infections and, in combination with well-established baseline genetic and epidemiological data, to identify indigenous or imported viruses. The ideal samples for rubella isolation and detection are NP specimens, collected as soon as possible after the onset of symptoms (‹ 5 days after rash onset), but collection of samples for virological surveillance can be challenging due to the logistics of storage and transport. Nevertheless, we were able to obtain 68 swabs and genotype 11 samples. In addition, although sera are not optimal specimens for viral detection due to the low amounts of viral RNA present, RuV RNA was detected in 21.5% of IgM positive serum samples collected close to symptom onset, while genotypes were obtained from 7.5%. These numbers are in good agreement with a previous study of RuV RNA in sera in which 26% of sera samples were real-time PCR positive and 12% yielded genotypes [35].

Genetic information obtained from the 2011–2012 outbreak in Romania revealed that it was driven by two 2B lineages with an average of 3.5% nt difference, which overlapped both temporally and geographically. Data from other countries have shown that co-circulation of multiple RuV lineages of one genotype within a country is quite common [36,37]. Lineage 1 Romanian outbreak sequences, which were most similar to those of viruses from south-east Asia, were detected from late 2011 through the spring of 2012 while lineage 2 sequences, which were most similar to a viral strain from northern Africa, were detected in May 2011 from one of the sporadic cases and then from early 2012 through December of 2012. In addition, analysis of sequence data confirmed that viruses which were identified by epidemiological data as exportations to Great Britain were identical to viruses from Romania.

The viruses of genotypes 1E and 1G detected in Romania in the previous outbreak in 2003 were not detected in the 2011–2012 outbreak and no sequences from Romania are available from the intervening time period; therefore, it is not possible to know when the genotype 2B viruses entered the country. The viruses in the 2011–2012 outbreak may have been circulating in Romania before the outbreak. However, they may have been introduced by recent importation events, as would be suggested by the high degree of sequence similarity with viruses from approximately the same time period (2011 to 2013) detected in other parts of the world such as Japan, Vietnam and Taiwan (Figure 5). The very low incidence of rubella in the two to three years before the outbreak is consistent with this hypothesis. In addition, the approximate nine month gap that elapsed between the first and second detections of lineage 2 suggests that there may have been two separate introductions of this lineage. Gaps in viral surveillance for rubella both regionally and globally limit the ability to use genetic data for identifying the source of a particular lineage. It is clear, however, that a shift occurred in the RuV genotypes over time from genotypes that were common in other European

countries (1E and 1G) to the 2B genotype that was previously found primarily in other parts of the eastern hemisphere [1]. Such genotype shifts have been documented in other countries such as China [35] and Brazil [38].

This is the first report of RuV sequences from Romania. Documenting virus genotypes is one of the essential criteria for tracking the progress of elimination of rubella in the WHO European region [39]. Thus, determining the endemic RuV genotype baseline is necessary. However, as shown here circulating genotypes can change over time and ongoing surveillance is necessary to provide up-to-date information.

In order to reach the goal of endemic rubella elimination and, thus, prevent CRS cases such as those that resulted from the 2011–2012 outbreak, it is necessary to achieve and sustain high vaccination coverage›95% (the women in reproductive age who were born before 2004 should be better informed on the risks of rubella, encouraged to get vaccinated and women in childbearing age checked for immunity to rubella prior to pregnancy) supported by high-quality surveillance including epidemiological, serological and molecular studies. This process will include the development of case-based epidemiology investigations to identify importations and prevent secondary transmissions especially in countries such as Romania where rubella virological surveillance is not yet well established.

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Conflict of interest

None declared

Authors' contributions

Mihaela Lazar - writing of the manuscript, molecular epidemiology data analysis and interpretation of the study. Emily Abernathy and Min-hsin Chen - obtained rubella sequences from serum samples in CDC, writing of the manuscript and interpretation of the study. Joseph Icenogle - analysis and interpretation of the study. Denisa Janta and Aurora Stanescu - epidemiological data analysis and contributed to the revision of the draft manuscript. Adriana Pistol - epidemiological data analysis and interpretation of the study. Sabine Santibanez and Annette Mankertz obtained the first rubella sequences from Romania, contributed to the interpretation of the study and the revision of the draft manuscript. Judith M.Hübschen - submitted part of the rubella sequences in RubeNS and contributed to the revision of the draft manuscript. Grigore Mihaescu - analysis and interpretation of the study. Gheorghe Necula - molecular epidemiology data analysis and interpretation of the study. Emilia Lupulescu - writing of the manuscript and interpretation of the study.

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Effectiveness of the live attenuated and the inactivated influenza vaccine in two-year-olds – a nationwide cohort study Finland, influenza season 2015/16

H Nohynek [1], U Baum [2], R Syrjänen [2], N Ikonen [3], J Sundman [2], J Jokinen [2]
1. Vaccine Programme Unit, Department of Health Protection, National Institute for Health and Welfare, Finland
2. Impact Assessment Unit, Department of Health Protection, National Institute for Health and Welfare, Finland
3. Viral Infections Unit, Department of Infectious Diseases, National Institute for Health and Welfare, Finland

Correspondence: Hanna Nohynek (hanna.nohynek@thl.fi)

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Although widely recommended, influenza vaccination of children is part of the national vaccination programme only in few countries. In addition to Canada and the United States (US), in Europe Finland and the United Kingdom have introduced live attenuated influenza vaccine (LAIV) for healthy children in their programmes. On 22 June 2016, the US Advisory Committee on Immunizations Practices, voted against further use of LAIV due to no observed vaccine effectiveness (VE) over three consecutive influenza seasons (2013/14 to 2015/16). We summarise the results of a nationwide, register-based cohort study (N=55,258 of whom 8,086 received LAIV and 4,297 TIV); all outcome (laboratory-confirmed influenza), exposure (vaccination) and confounding variable data were retrieved from four computerised national health registers, which were linked via a unique personal identity code assigned to all permanent Finnish residents regardless of nationality. Our study provides evidence of moderate effectiveness against any laboratory-confirmed influenza of the quadrivalent LAIV vaccine (VE: 51%; 95% confidence interval (CI): 28–66%) as well as the inactivated trivalent vaccine (VE: 61%; 95% CI: 31–78%) among two-year-olds during the influenza season 2015/16 in Finland. Based on these data, Finland will continue using LAIV for young children in its National Immunisation Programme this coming influenza season.

Introduction

Influenza causes mild to severe symptoms among one in three young children. Vaccination is considered the best available intervention to prevent influenza in children and its spread from children to other age groups reducing the disease burden in the entire population [1]. Many European countries recommend to vaccinate the elderly, medical risk groups and healthcare workers but only nine countries recommend vaccination of healthy children, i.e. Austria, Estonia, Finland, Latvia, Malta, Poland, Slovakia, Slovenia, and the United Kingdom (UK) [2].

Since 2007, influenza vaccine has been given free of charge to all children aged 6 to 35 months as part of the National Vaccination Programme of Finland (NVP) [3], following a formal cost effectiveness analysis [4] requested by the National Immunization Technical Advisory Group and favourable decision by the government. For young healthy children and those above three but under nine years of age with medical risk conditions, the recommended schedule has included two doses for those vaccinated for the first time ever and one dose if they were already vaccinated during previous seasons.

Different types of influenza vaccines have been available for large scale use since early 1970s. Inactivated influenza vaccines have been commonly used. The live attenuated influenza vaccine (LAIV) was developed already in the 1960s but it has been available for large scale use in the United States (US) since 2003 (FluMist) and in Europe since 2011 (Fluenz). Prior to season 2015/2016, in Europe, only the UK had introduced LAIV for healthy children in their programme.

During the influenza season 2015/16, for the first time in Finland, two-year-olds (i.e. children aged 24 to 35 months) were offered either one or two doses of trivalent inactivated influenza vaccine (TIV; Vaxigrip) or one dose of LAIV (FluenzTetra). No preference for either was made in the national recommendation. Both vaccines were scheduled to be given in November and December 2015, although TIV could also be used from 6 January 2016 onwards after LAIV doses available in NVP had expired.

On 22 June 2016, the US Advisory Committee of Immunization Practices (ACIP) discussed the effectiveness of LAIV given to children from 2 to 17 years of age over three consecutive seasons in the US. Due to no observed vaccine effectiveness using the test negative design methodology, the ACIP voted against the use of LAIV in children during the coming season 2016/17 [5]. However, mid-season data from both Finland and the UK made available to the ACIP via CDC demonstrated reasonable effectiveness of the LAIV vaccine produced in the same plant [6,7].

As part of its statutory tasks, the Finnish National Institute for Health and Welfare (THL) is obliged to monitor the effectiveness and safety of vaccines used, in order to measure the impact of the NVP, and to give evidence-based vaccination recommendations [3]. Finland recently established a nationwide, computerised, real-time vaccination register (NVR) [8]. Linking NVR with disease register data in real time allows comprehensive effectiveness studies in timely manner. We present the end-of-season estimate of the influenza vaccine effectiveness (VE) among all two-year-old children residing permanently in Finland during the influenza season 2015/16 using national register data.

Methods

Study design and follow-up period

This nationwide register-based cohort study retrospectively assessed influenza VE in two-year-old children, i.e. the birth cohort of 2013, during the influenza season 2015/16, defined as lasting from week 40 (28 September 2015) to week 20 (22 May 2016). All outcome, exposure and confounding variable data were retrieved from four computerised national health registers maintained by THL, which were linked via a unique personal identity code assigned to all permanent Finnish residents regardless of nationality.

Study population

The study population, i.e. the birth cohort of 2013, was defined based on the Finnish Population Register, which contains an up-to-date information of all permanent residents in Finland.

Exposure

Vaccination status was defined by the NVR, which contains individual-level vaccination records comprising the vaccinee's personal identity code, the administered vaccine (including brand name) and the date of vaccination. The NVR covers records of vaccinations given from 2009 onwards in public primary healthcare, which is responsible for the delivery of the NVP. However, small regional and temporal information gaps are assumed, mainly due to data dispatch problems [8]. Every individual within the study population and with at least one recorded influenza vaccination in the NVR in 2015/16 was considered vaccinated since the day of vaccination. For purposes of sensitivity analysis, children were also considered vaccinated only after a two-week-period following vaccination allowing them to develop a sufficiently protective immunity. Consecutive vaccinations within the same season are rare among two-year-olds, and observed in less than 1% of those vaccinated. They were not considered in the analysis.

Outcome

The outcome of interest was any laboratory-confirmed influenza (LCI) registered in the National Infectious Disease Register (NIDR). The NIDR covers nationwide data about LCI cases, diagnosed in both public and private primary and secondary care. No universal recommendation exists when a suspected case should be tested for influenza. In Finland, influenza suspected patients are tested for influenza by RT-PCR, multiplex RT-PCR, culture and/or antigen detection and all influenza-positive cases from all laboratories are reported to the NIDR, where the patient's personal identity code, the influenza type, and the date of laboratory confirmation is recorded. In this report, LCI was defined as influenza finding in RT-PCR, multiplex RT-PCR, culture and/or antigen detection test, and further stratified to LCI type A and LCI type B.

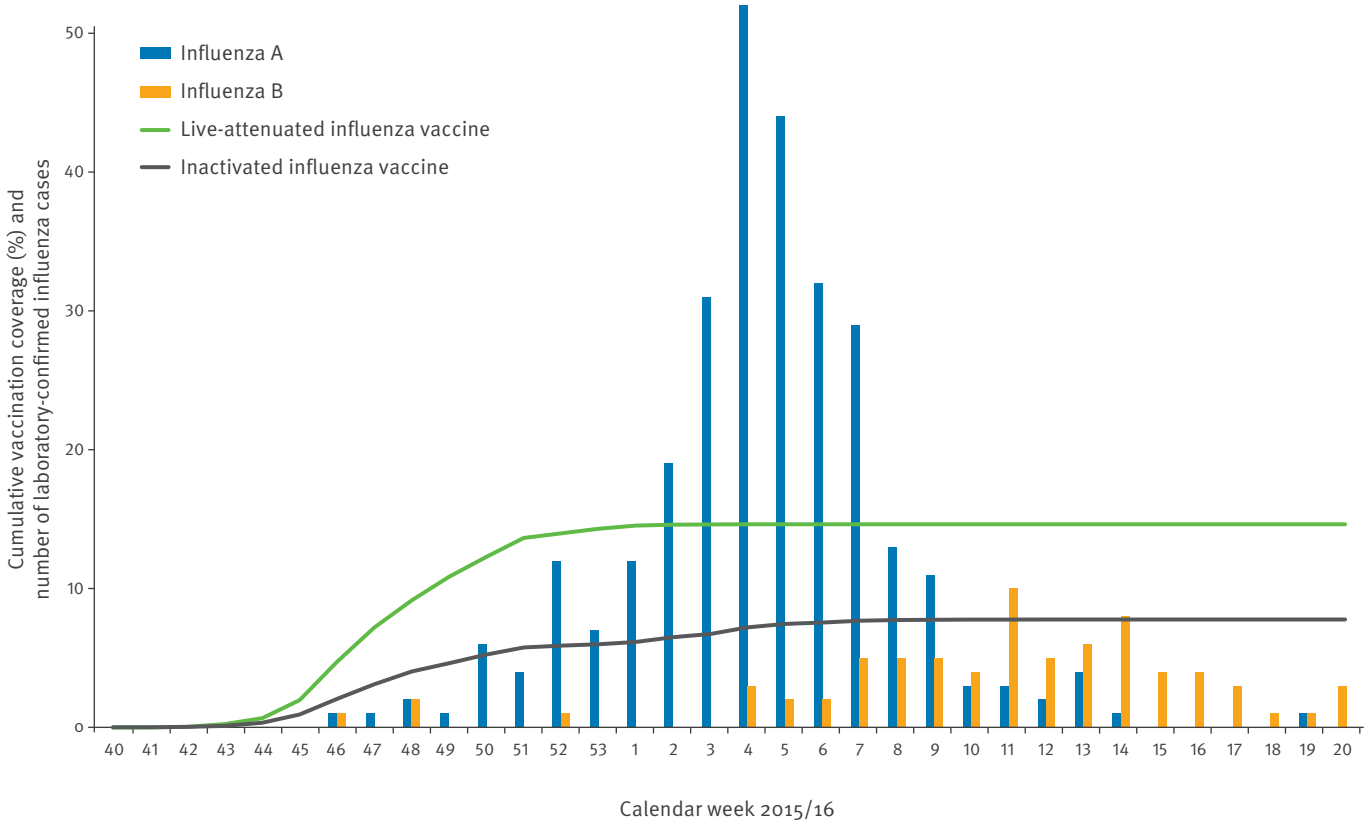
Confounders

In order to control for potential confounders, several variables describing the characteristics of the study population were included in the analysis. Background information was collected from the Finnish National Medical Birth Register (NMBR), which contains data about the status of the child and the mother at the time of child's birth [9]. The following 12 categorical variables (levels given in Table 1) were considered in the analysis: mother's age at birth in years (<20, 20–24, 25–29, 30–34, 35–39, ≥40), socio-economic status (based on mother´s profession), marital status and smoking behaviour, as well as child's birth weight in grams (<1,500, 1,500-2,499, ≥2,500), gestational age at birth in weeks, number of siblings at birth, month of birth (January–June, July–December) as indicator for the eligibility to previous seasonal influenza vaccinations, sex, nationality, place of residence, and BCG (Bacillus Calmette–Guérin) vaccination status.

Acute and chronic diagnoses made in hospitals were extracted from the National Register of Health Care (NRHC), which covers diagnosis information of all outpatient and inpatient healthcare provided in Finnish hospitals [10]. The following three acute diseases diagnosed within 6 months before the vaccination campaign (weeks 14–39 in 2015) and 13 chronic disease entities from birth until the end of 2015 were selected based on their International Statistical Classification of Diseases and Related Health Problems tenth Revision (ICD-10) codes [11]: acute bacterial and viral infections (A30–A49, A85–A89), acute diseases of the middle ear (H65–H75, H92), acute respiratory infections (J00–J06, J10–J22), HIV (B20–B24), malignant neoplasms (C69–C97), diseases of the blood and blood forming organs (D55–D89), diabetes mellitus and obesity (E10–E14,

Figure

Cumulative seasonal influenza vaccination coverage and number of laboratory-confirmed influenza in two-year-old children by calendar week, Finland, influenza season 2015/16 (n=55,258)



E65–E68), mental retardation (F71–F73, F79.1), diseases of the nervous system (G31, G40–G41, G70–G73, G80–G83), heart diseases (I34–I37, I42, I50), diseases of the respiratory system (J35, J40–J47), atopic dermatitis (L20), diseases of the musculoskeletal system and connective tissue (M02–M07, M13, M30–M36), diseases of the kidney (N00–N19), congenital malformations of the circulatory and respiratory system and Down syndrome (Q20–Q39, Q90) and undergone organ transplantations (Z94.0–Z94.6).

In contrast to the NVR and the NIDR, the NRHC does not accumulate in real time and is currently updated once a year. At the time this study was conducted, the NRHC covered patient encounters until the end of 2015, with preliminary data for 2015.

Statistical analysis

VE was defined as one minus the hazard rate ratio, estimated using Cox regression [12] with the time since the first day of week 40 as underlying time scale. Influenza vaccination was treated as time-dependent variable. VE was estimated for LAIV and TIV separately, using the unvaccinated cohort as a reference for both. Each individual of the study population was followed till the date of LCI, the date of receiving either (i) TIV (when analysing LAIV effectiveness) or (ii) LAIV (when analysing TIV effectiveness), the last day of week 20 or death, whatever occurred first. The validity of the proportional hazards assumption was evaluated using Schoenfeld residuals, and no notable deviation from proportionality was found.

The propensity score method [13] was used to account for potential confounders. In order to include also children with partially missing confounder information, missing values observed in five NMBR variables (Table 1 footnotes d and e; socio-economic status based on mother's occupation, mother's marital status, mother's smoking behaviour, birth weight, gestational age at birth) were imputed using hot deck imputation [14]. Altogether 29 variables, 12 categorical variables derived from NMBR plus one categorical (i.e. number of hospitalisations in 2015, irrespective of the ICD-10 code) and 16 binary variables derived from NRHC, were included into two separate propensity score models estimating each child's probability of being vaccinated (i) with LAIV and (ii) with TIV conditional on the covariates by applying logistic regression.

The VE estimates were adjusted for (i) LAIV propensity score quintiles in LAIV analysis and (ii) TIV propensity score quintiles in TIV analysis. In addition, further population and outcome subgroup-stratified analyses were conducted according to the child's seasonal influenza vaccination status in 2013/14 and 2014/15, as well as according to LCI type A and LCI type B.

Table 1

Baseline characteristics of two-year-old children by seasonal influenza vaccination status, Finland, influenza season 2015/16 (n= 55,258)

	Not vaccinated (N=42,875)	LAIV vaccinated (N=8,086)	TIV vaccinated (N=4,297)	p-value[a]
Mother's age at birth[b]				
Years	30 (5.3)	31 (5.0)	31 (5.0)	<0.001
Socio-economic status based on mother's occupation[c,e]				
Higher white-collar workers	8,596 (20.0)	2,158 (26.7)	1,145 (26.6)	<0.001
Lower white-collar workers	18,375 (42.9)	3,329 (41.2)	1,760 (41.0)	
Blue-collar workers	7,069 (16.5)	934 (11.6)	516 (12.0)	
Others	8,835 (20.6)	1,665 (20.6)	876 (20.4)	
Mother's marital status[d]				
Single or divorced	4,202 (9.8)	620 (7.7)	334 (7.8)	<0.001
Cohabiting	14,830 (34.6)	2,408 (29.8)	1,210 (28.2)	
Married	23,843 (55.6)	5,058 (62.6)	2,753 (64.1)	
Mother's smoking behaviour[d]				
No	35,303 (82.3)	7,284 (90.1)	3,867 (90.0)	<0.001
Quitted during first trimester	3,232 (7.5)	427 (5.3)	210 (4.9)	
Continued after first trimester	4,340 (10.1)	375 (4.6)	220 (5.1)	
Birth weight[b,d]				
Grams	3,514 (541.8)	3,470 (579.7)	3,459 (595.1)	<0.001
Gestational age at birth[d]				
<28 weeks	68 (0.2)	35 (0.4)	30 (0.7)	<0.001
≥28 and <37 weeks	4,173 (9.7)	903 (11.2)	504 (11.7)	
≥37 weeks	38,634 (90.1)	7,148 (88.4)	3,763 (87.6)	
Number of siblings at birth[c]				
0	16,156 (37.7)	4,057 (50.2)	1,830 (42.6)	<0.001
1	15,116 (35.3)	2,465 (30.5)	1,509 (35.1)	
>1	11,603 (27.1)	1,564 (19.3)	958 (22.3)	
Month of birth[c]				
January–June	22,169 (51.7)	3,424 (42.3)	1,967 (45.8)	<0.001
July–December	20,706 (48.3)	4,662 (57.7)	2,330 (54.2)	
Sex[c]				
Male	21,870 (51.0)	4,225 (52.3)	2,302 (53.6)	0.001
Female	21,005 (49.0)	3,861 (47.7)	1,995 (46.4)	
Nationality[c]				
Finnish	39,483 (92.1)	7,682 (95.0)	4,013 (93.4)	<0.001
Non-Finnish	3,392 (7.9)	404 (5.0)	284 (6.6)	
Place of residence[c]				
Urban	29,709 (69.3)	6,220 (76.9)	3,368 (78.4)	<0.001
Semi-urban	7,713 (18.0)	1,125 (13.9)	517 (12.0)	
Rural	5,453 (12.7)	741 (9.2)	412 (9.6)	
BCG vaccination status[c]				
Not vaccinated	39,403 (91.9)	7,618 (94.2)	3,988 (92.8)	<0.001
Vaccinated	3,472 (8.1)	468 (5.8)	309 (7.2)	
Presence of underlying chronic conditions[c]				
No	37,734 (88.0)	7,032 (87.0)	3,510 (81.7)	<0.001
Yes	5,141 (12.0)	1,054 (13.0)	787 (18.3)	
Presence of an acute disease between week 14–39, 2015[c]				
No	39,766 (92.7)	7,354 (90.9)	3,791 (88.2)	<0.001
Yes	3,109 (7.3)	732 (9.1)	506 (11.8)	
SIV vaccination status in2013/14 and 2014/15[c,f]				
Not vaccinated	38,288 (89.3)	3,470 (42.9)	1,386 (32.3)	<0.001
Vaccinated	4,587 (10.7)	4,616 (57.1)	2,911 (67.7)	

BCG: Bacillus Calmette–Guérin vaccine: LAIV: live attenuated influenza vaccine; TIV: trivalent inactivated influenza vaccine; SIV: seasonal influenza vaccine.

[a] One-way analysis of variance for continuous and chi-squared test of independence for categorical variables.

[b] Mean (standard deviation).

[c] Absolute frequency (relative frequency in %). Because of rounding, percentages may not total 100.

[d] Proportion of data imputed by hot deck imputation: <0.2%.

[e] Proportion of data imputed by hot deck imputation: 31.5%.

[f] Vaccinated group contains those vaccinated either in the 2013/14, 2014/15 season or both.

Table 2

Influenza vaccine effectiveness against laboratory-confirmed influenza in two-year-old children, stratified by influenza type, Finland, influenza season 2015/16 (n=55,258)[a]

Laboratory-confirmed influenza	Cases			Person-years			Crude effectiveness (95% confidence intervals)		Adjusted effectiveness (95% confidence intervals)	
Type	Not vaccinated	LAIV	TIV	Not vaccinated	LAIV	TIV	LAIV	TIV	LAIV	TIV
A and B	317	31	12	29,984	3,965	1,954	46.5% (22.7%–63.0%)	58.2% (25.6%–76.5%)	50.7% (28.4%–66.1%)	61.2% (30.7%–78.3%)
A	260	26	5	29,994	3,967	1,955	45.4% (18.2%–63.5%)	78.2% (47.3%–91.0%)	47.9% (21.6%–65.4%)	79.5% (50.3%–91.6%)
B	62	6	7	30,063	3,972	1,957	47.1% (-22.5%–77.1%)	-14.1% (-149.3%–47.8%)	57.2% (-0.0%–81.7%)	-1.0% (-122.8%–54.2%)

LAIV: live attenuated influenza vaccine; TIV: trivalent inactivated influenza vaccine.

[a] Crude and adjusted for propensity score quintiles.

When stratified by previous exposure to influenza vaccinations, there was a tendency towards higher effectiveness among those previously vaccinated (Table 3), although due to a small number of cases in each stratum, these differences were not statistically significant.

Results

Epidemiology of the 2015/16 influenza season in Finland

The Finnish sentinel surveillance [15] covering a representative sample of all age groups, demonstrated that the influenza season started earlier than usual (in week 47) and spread almost simultaneously all over the country. During the first wave of the season, influenza A(H1N1)pdm09 viruses predominated and all characterized A(H1N1)pdm09 viruses represented the new genetic subclade 6B.1. The second wave was caused by influenza B/Victoria viruses that genetically fell into the B/Brisbane/60/2008 clade. Influenza A(H3N2) viruses belonging to clades 3C.2a and 3C.3a were detected only sporadically. No B/Yamagata viruses were detected in 462 samples tested in the frame of the sentinel surveillance.

Influenza vaccine effectiveness in two-year-olds

The study population for the VE estimation comprised all permanent residents of Finland eligible for both LAIV and TIV vaccination, i.e. the birth cohort of 2013. Due to small regional and temporal information gaps in the NVR, 5% of the birth cohort 2013 were excluded because of presumably incomplete vaccination records. In addition, 2% that were not found in the NMBR were excluded, leaving 93% of the birth cohort for analysis. The final study population thus comprised 55,258 two-year-old children. The total influenza vaccination coverage was 22%; about two thirds were vaccinated with LAIV and one third with TIV. The characteristics of those included in the analyses are described in Table 1. Among the 55,258 children, a total 360 LCI were registered in the NIDR. Influenza A cases peaked in week 4 and caused 291 laboratory-confirmed infections. Influenza B mainly circulated between weeks 11 and 14 and caused 69 LCI cases in the study population (Figure). The majority of vaccinations was given before the epidemic (Figure).

The combined influenza A and B effectiveness estimates adjusted for potential confounders were similar among the LAIV and TIV recipients (51% and 61%, respectively) with widely overlapping confidence intervals (95%CI 28–66 vs. 31–78, respectively), as described in Table 2. The highest effectiveness (80%, 95%CI 50–92) was observed against influenza A among those vaccinated with TIV. Due to small numbers, the influenza B analysis yielded statistically borderline non-significant point estimates (Table 2). The results were practically the same when children were considered vaccinated only after a two-week-period following vaccination (data not shown).

When stratified by previous exposure to influenza vaccinations, there was a tendency towards higher effectiveness among those previously vaccinated (Table 3), although due to a small number of cases in each stratum, these differences were not statistically significant.

Discussion

In Finland, the overall influenza vaccine uptake during the influenza season 2015/16 among two-year-old children was low (22%) but sufficient for a meaningful effectiveness analysis using a nationwide cohort approach. The end-of-season effectiveness estimates were moderately good for both LAIV and TIV with generally slightly higher point estimates for TIV, although the confidence intervals were wide and overlapping. This is in contrast to the findings reported from the US where unlike TIV, LAIV yielded no effectiveness already for the third consecutive season [5]. The LAIV, however, was produced in the same plant for both North American and European markets. The results from the US were based on a test-negative case–control design (TND), and covered children aged 2 to 17 years, in contrast to

Table 3

Influenza vaccine effectiveness against laboratory-confirmed influenza in two-year-old children, stratified by influenza type and seasonal influenza vaccination status in the 2013/14 and 2014/15 seasons, Finland, influenza season 2015/16 (n=55,258)

Laboratory-confirmed influenza		Cases			Person-years			Crude effectiveness (95% confidence intervals)		Adjusted effectiveness (95% confidence intervals)	
Type		Not vaccinated	LAIV	TIV	Not vaccinated	LAIV	TIV	LAIV	TIV	LAIV	TIV
A and B	NPV	272	17	5	25,750	1,691	588	29.3% (-15.4%–56.7%)	40.1% (-45.1%–75.3%)	34.0% (-8.1%–59.7%)	44.1% (-35.7%–76.9%)
	PV	45	14	7	4,234	2,274	1,366	66.2% (38.4%–81.5%)	73.1% (40.4%–87.9%)	69.7% (44.0%–83.6%)	73.3% (40.4%–88.1%)
A	NPV	221	15	2	25,759	1,691	589	23.1% (-29.8%–54.4%)	69.3% (-23.4%–92.4%)	24.6% (-27.8%–55.5%)	70.6% (-18.6%–92.7%)
	PV	39	11	3	4,235	2,275	1,367	70.1% (41.6%–84.7%)	86.4% (56.0%–95.8%)	74.0% (48.5%–86.9%)	87.1% (57.9%–96.0%)
B	NPV	56	2	3	25,817	1,695	590	60.1% (-63.4%–90.3%)	-51.4% (-383.7%–52.6%)	68.5% (-29.8%–92.4%)	-29.3% (-315.5%–59.8%)
	PV	6	4	4	4,246	2,277	1,367	15.3% (-211.9%–77.0%)	-5.5% (-273.9%–70.2%)	16.7% (-213.7%–77.9%)	-25.1% (-352.0%–65.4%)

LAIV: live attenuated influenza vaccine; NPV: not previously vaccinated; PV: previously vaccinated; TIV: trivalent inactivated influenza vaccine.
[a] Crude and adjusted for propensity score quintiles.

this study's cohort design, focusing only on two-year-olds. Our findings are in agreement with those from the UK, where VE in the 2015/16 season was also moderate for influenza A and even good for influenza B [6,16,17] in children and adolescents younger than 18 years and based on a TND.

The particular strength of our study is that by utilising population-based registers, we were able to cover the whole population eligible for LAIV and TIV vaccination; monitoring VE by using routine health registers is particularly suitable for measuring the public health impact of vaccination programmes. Furthermore, the non-preferential national recommendation of influenza vaccinations for two-year-olds for the season 2015/16 allowed us to investigate the effectiveness of LAIV and TIV in parallel within the same cohort.

When using routine registers for defining the exposure, data completeness is a special concern. Therefore the quality and completeness of the NVR is constantly monitored [8] and geographic areas not fulfilling quality criteria are omitted from any cohort analysis. Based on a recent validation study [8] on childhood vaccinations – using MMR vaccination at the age of 12 months as a proxy – the register covers 96% of influenza vaccination records, translating to misclassification of approximately 500 vaccinated in our study cohort. Some LAIV doses may also have been given in the private primary care, which is not currently covered by NVR. However, since all NVP vaccinations are given in public primary care and free of charge, it is anticipated that private primary care uptake in our study cohort was negligible. This is supported by the national pharmaceutical distribution figures in 2015 of 2,120 LAIV doses distributed for the whole eligible age group of 2–17-year-olds. Finally, since lack of data completeness leads to misclassifying a subgroup of those vaccinated to the group of unvaccinated, our VE estimates can be considered conservative, i.e. an underestimation of the real VE.

As with any observational study, the VE estimates may be biased due to unobserved confounders or other types of unknown selection processes in the uptake of vaccinations or care seeking or access to care captured by routine register data. In order to account for potential biases, we adjusted our estimates with several background variables at birth and data of hospital visits prior to the 2015/16 seasonal influenza vaccination campaign. Information on baseline characteristics helps to understand the possible sources of bias in the analysis. The statistically significant differences observed between the three groups, i.e. not vaccinated, LAIV and TIV vaccinated, may not necessarily have clinical significance but underscore the need to perform adjusted analyses. Many of the characteristics thought to increase infection risk, such as siblings, non-Finnish nationality, non-urban residence, low socio-economic status, single mothers and smoking mothers, were more common among the non-vaccinated. Therefore it is somewhat surprising that the adjusted estimates are generally higher than the crude

estimates. This may be explained by healthcare-seeking behavior so that parents who get their children vaccinated are possibly also more likely to seek healthcare e.g. for acute respiratory infections like influenza. This is supported by the observation that diagnoses of both chronic and acute diseases prior to the vaccination campaign were more common among the vaccinated. In addition, parents e.g. with higher socio-economic status may predominantly use private primary care, in which the threshold for obtaining laboratory confirmation is presumably lower than in public primary care. Even after adjustment, some residual confounding may still be present.

The role of exposure to previous influenza vaccine doses in the immunological response to subsequent doses has been debated [18]. In young children, two doses have been recommended as necessary for the first time exposure to secure proper priming and maturation of sufficient protection. For LAIV, however, the difference in protection provided by first time one or two doses is marginal [19]. The NVR with vaccination data since year 2009 allows stratified analyses of effectiveness by previously received seasonal influenza vaccine doses; past exposure to influenza vaccines appears to contribute to increased effectiveness in the two-year-old children during the season 2015/16, but due to the relatively small sample size, this difference did not reach statistical significance.

A good antigen match was expected for the quadrivalent LAIV before the start of the 2015/16 influenza epidemic, because the World Health Organization had recommended to change the influenza vaccine composition for both the A(H3N2)- and B-components. Also, the A(H1N1) strain of LAIV was changed due to concerns over its heat instability. Since subtype specific identification of viruses is seldom done in routine clinical practice, our study can reliably address only overall and influenza A VE. The numbers of observations of influenza B viruses were few in this age group and there was not sufficient power to detect VE.

Conclusion

During the influenza season 2015/16, both LAIV and TIV were effective against laboratory-confirmed influenza among two-year-old children. Finland will continue using LAIV as an alternative intervention to TIV without any official statement on preference. Our study also demonstrates that population-based national health registers are extremely valuable to generate routine data for measuring vaccine impact in a timely manner.

Conflict of interest

None declared.

Authors' contributions

Hanna Nohynek: conceptualised the paper, participated in the analysis of the data, wrote the first draft and finalised the manuscript.

Ulrike Baum: planned and performed the statistical analyses, participated in the writing of the paper.

Ritva Syrjänen: participated in the analysis of the data and reviewed the manuscript.

Niina Ikonen: performed the virological analyses and participated in the writing of the paper.

Jonas Sundman: was in charge of the data management and reviewed the manuscript.

Jukka Jokinen: conceptualised the design and data sources to be used for the study, supervised the statistical analyses, participated in the writing of the paper.

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Effectiveness of seasonal influenza vaccine for adults and children in preventing laboratory-confirmed influenza in primary care in the United Kingdom: 2015/16 end-of-season results

R Pebody [1], F Warburton [1], J Ellis [1], N Andrews [1], A Potts [2], S Cottrell [3], J Johnston [4], A Reynolds [2], R Gunson [5], C Thompson [1], M Galiano [1], C Robertson [6], R Byford [7], N Gallagher [4], M Sinnathamby [1], I Yonova [7 8], S Pathirannehelage [7], M Donati [1], C Moore [3], S de Lusignan [7 8], J McMenamin [2], M Zambon [1]

1. Public Health England, London, United Kingdom
2. Health Protection Scotland, Glasgow, United Kingdom
3. Public Health Wales, Cardiff, United Kingdom
4. Public Health Agency Northern Ireland, Belfast, United Kingdom
5. West of Scotland Specialist Virology Centre, Glasgow, United Kingdom
6. University of Strathclyde, Glasgow, United Kingdom
7. University of Surrey, Guildford, United Kingdom
8. Royal College of General Practitioners, London, United Kingdom

Correspondence: Richard Pebody (richard.pebody@phe.gov.uk)

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The United Kingdom (UK) is in the third season of introducing universal paediatric influenza vaccination with a quadrivalent live attenuated influenza vaccine (LAIV). The 2015/16 season in the UK was initially dominated by influenza A(H1N1)pdm09 and then influenza of B/Victoria lineage, not contained in that season's adult trivalent inactivated influenza vaccine (IIV). Overall adjusted end-of-season vaccine effectiveness (VE) was 52.4% (95% confidence interval (CI): 41.0–61.6) against influenza-confirmed primary care consultation, 54.5% (95% CI: 41.6–64.5) against influenza A(H1N1)pdm09 and 54.2% (95% CI: 33.1–68.6) against influenza B. In 2–17 year-olds, adjusted VE for LAIV was 57.6% (95% CI: 25.1 to 76.0) against any influenza, 81.4% (95% CI: 39.6–94.3) against influenza B and 41.5% (95% CI: –8.5 to 68.5) against influenza A(H1N1)pdm09. These estimates demonstrate moderate to good levels of protection, particularly against influenza B in children, but relatively less against influenza A(H1N1)pdm09. Despite lineage mismatch in the trivalent IIV, adults younger than 65 years were still protected against influenza B. These results provide reassurance for the UK to continue its influenza immunisation programme planned for 2016/17.

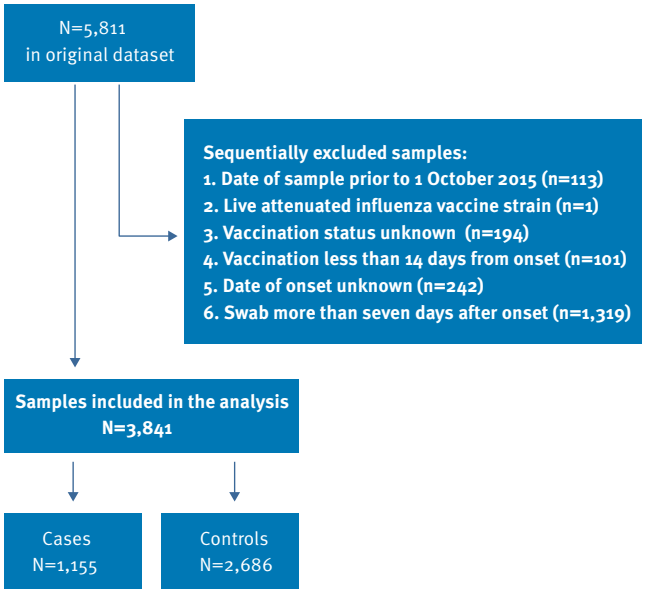
Introduction

The United Kingdom (UK) has had a long-standing selective inactivated influenza vaccination programme targeted at individuals at higher risk of severe disease such as the elderly, those with an underlying clinical risk condition and pregnant women. Following recommendations from the Joint Committee of Vaccination and Immunisation (JCVI) in 2012, the decision was taken for a phased introduction of a newly licensed live attenuated influenza vaccine (LAIV), ultimately offered LAIV in each season to all healthy children aged two to 16 years [1]. 2015/16 is the third season of the introduction of this new influenza vaccination programme; all healthy children aged two to four years and in school years 1 and 2 were offered a single dose of LAIV [2]. In Northern Ireland and Scotland and in selected pilot areas in England, all other older children of primary school age were also offered LAIV in 2015/16. Children aged two to 17 years in a clinical risk group were also offered LAIV, while children with a risk factor, in whom LAIV is contraindicated, were offered quadrivalent inactivated influenza vaccine (IIV). All children in a clinical risk group aged six to 23 months were offered IIV. The United States Centers for Disease Control and Prevention (US CDC) recently reported the observation that LAIV did not provide protection in children against circulating influenza strains in North America in the 2015/16 season [3]. This raised a question about the effectiveness of LAIV in children in the UK.

In the UK, the 2015/16 season started late, peaking in week 11 of 2016, with circulation initially dominated by influenza A(H1N1)pdm09 viruses. Impact mainly fell on younger adults resulting in large numbers of hospitalisations and admissions to intensive care units (ICU) [4].

Figure 1

Specimen inclusion and exclusion criteria, end-of-season 2015/16 influenza vaccine effectiveness evaluation, United Kingdom, 1 October 2015–1 May 2016 (n = 5,811)



Genetically, the haemagglutinin (HA) genes of A(H1N1) pdm09 viruses all belonged in subgroup 6B, the predominant clade circulating in the 2014/15 season. The later stages of the 2015/16 season were dominated by influenza B circulation, with the majority of viruses antigenically similar to B/Brisbane/60/2008, the influenza B/Victoria lineage component included in the 2015/16 northern hemisphere quadrivalent vaccine but not in the trivalent vaccine [4]. This raised questions about the protection provided by the 2015/16 trivalent vaccine, the main influenza vaccine offered to adults, and about the potential added value of switching to quadrivalent vaccine as the main vaccine of choice.

Following the mid-2015/16 season report of influenza vaccination effectiveness (VE) [5], this article presents the end-of-season estimates of influenza VE using well established systems across the four countries of the UK [6,7]. The aims of the investigation were to measure VE against laboratory-confirmed influenza by type, subtype and clade/lineage, and to determine the effectiveness of the vaccine in children two to 17 years of age according to type of vaccine, particularly in relation to LAIV, but also IIV. In addition, we estimated the effectiveness of both LAIV and IIV in children two to 17 years of age over the three seasons since the UK introduced the LAIV programme.

Methods

Study population and period

The test-negative case–control (TNCC) design was used to estimate VE. The study was undertaken in five sentinel general practice surveillance networks across the UK, details of which have been outlined previously [7]. The surveillance schemes were: Royal College of General Practitioners (RCGP), Research and Surveillance Centre (RSC), Specialist Microbiology Network (SMN) England and Wales, Northern Ireland and Scotland.

The main study took place from 1 October 2015 until 1 May 2016. The study population were patients presenting to their general practitioner (GP) during the study period with an acute influenza-like illness (ILI), who the physician consented verbally to be swabbed during the consultation. A patient with ILI was defined as an individual presenting in primary care with an acute respiratory illness with physician-diagnosed fever or complaint of feverishness. GPs were asked to swab a random sample of cases up to a total of 10 per week in any one practice. Cases were patients who tested positive for influenza A or B virus by real-time PCR. Controls were patients with the same symptoms who tested negative for influenza A and B. Further details of the laboratory methods are provided below.

During the consultation, the GP completed a standard questionnaire. It collected demographic, clinical and epidemiological information from patients including potential confounders such as sex, date of birth, underlying clinical risk factors, date of onset of ILI, date of sample collection (recommended within seven days of onset) and influenza vaccination history for the 2015/16 season including date of vaccination and route of administration (intranasal/injection). In England, residence in an area where a primary school LAIV immunisation programme took place was also recorded.

A further specific sub-analysis was undertaken among children two to 17 years of age for the period 1 October 2013 until 1 May 2016. This covered the period since the introduction of LAIV in the UK. All aspects of data collection, testing and analysis were comparable over this period and are as described above.

Laboratory methods

Sentinel GP surveillance networks sent the respiratory samples to the national laboratories as previously outlined [7]. Laboratory confirmation was made using comparable real-time PCR methods able to detect circulating influenza A and B viruses [8,9]. Positive samples were sent to the reference laboratories for genetic characterisation. Isolation of influenza viruses was tried from all PCR-positive samples using Madin-Darby canine kidney epithelial (MDCK) cells or MDCK cells containing the cDNA of human 2,6-sialtransferase (SIAT1) cells as described previously [10,11].

Antigenic characterisation was only undertaken at the PHE reference laboratory. Post-infection ferret antisera were used in haemagglutination inhibition (HI) assays with turkey red blood cells to antigenically characterise influenza A(H1N1)pdm09 and influenza B virus isolates with a haemagglutination titre ≥ 40 [12]. Reference virus strains used for HI assays for A(H1N1)pdm09

Figure 2

Phylogenetic tree of the haemagglutinin genes of sentinel influenza B isolates, United Kingdom, October 2015–May 2016 (n = 324)

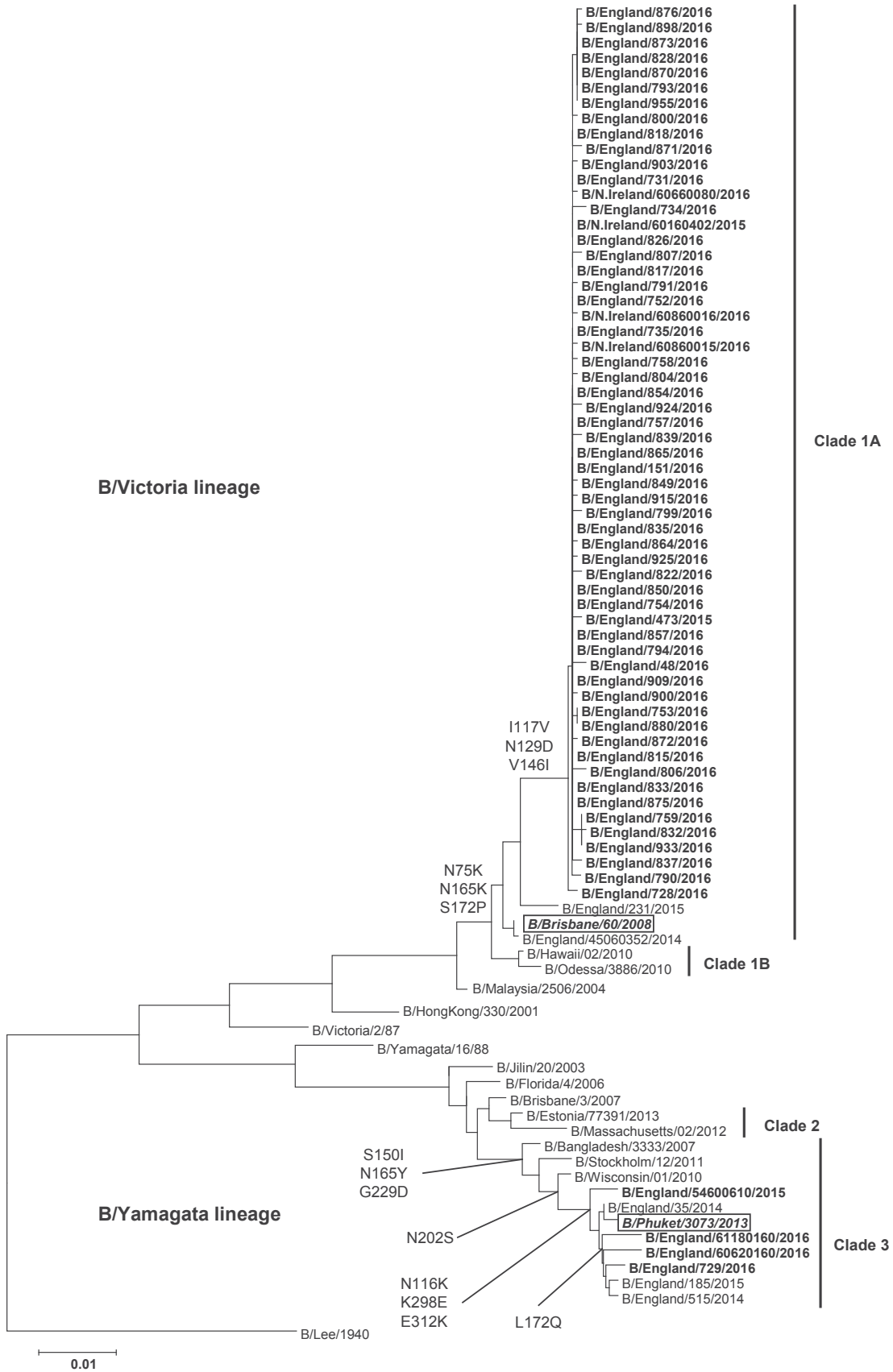


Table 1

Influenza B haemagglutinin sequences obtained from GISAID used in the phylogenetic analysis

Influenza virus isolate	Segment ID/ Accession number	Country	Collection date (year-month-day)	Originating laboratory	Submitting laboratory
B/Brisbane/3/2007	EPI154537	Australia	2007-Jan-01	Queensland Health Scientific Services, Queensland, Australia	WHO Collaborating Centre for Reference and Research on Influenza, Victoria, Australia
B/Stockholm/12/2011	EPI346827	Sweden	2011-Mar-28	Swedish Institute for Infectious Disease Control, Solna, Sweden	National Institute for Medical Research, London, UK
B/England/515/2014	EPI555201	United Kingdom	2014-Oct-22	Public Health England, London, UK	National Institute for Medical Research, London, UK
B/Estonia/77391/2013	EPI467120	Estonia	2013-Apr-08	Health Protection Inspectorate, Tallin, Estonia	National Institute for Medical Research, London, UK
B/Odessa/3886/2010	EPI271913	Ukraine	2010-Mar-19	Ministry of Health of Ukraine, Kiev, Ukraine	National Institute for Medical Research, London, UK
B/Phuket/3073/2013	EPI540675	Australia	2013-Nov-21	WHO Collaborating Centre for Reference and Research on Influenza, Victoria, Australia	National Institute for Medical Research, London, UK
B/Massachusetts/02/2012	EPI438406	United States	2012-Jan-01	New York Medical College, New York, US	Centers for Disease Control and Prevention, Atlanta, US
B/Wisconsin/01/2010	EPI271545	United States	2010-Feb-20	Wisconsin State Laboratory of Hygiene, Madison, US	Centers for Disease Control and Prevention, Atlanta, US
B/Hawaii/02/2010	EPI271558	United States	2010-Mar-25	State of Hawaii Department of Health, Pearl City, US	Centers for Disease Control and Prevention, Atlanta, US
B/Brisbane/60/2008	EPI172555	Australia	2008-Aug-04		Centers for Disease Control and Prevention, Atlanta, US
B/Florida/4/2006	EPI134356	United States	2006-Nov-01		Centers for Disease Control and Prevention, Atlanta, US
B/Bangladesh/3333/2007	EPI156050	Bangladesh	2007-Aug-18		Centers for Disease Control and Prevention, Atlanta, US

GISAID: Global Initiative on Sharing All Influenza Data; UK: United Kingdom; US: United States; WHO: World Health Organization.

viruses included A/California/7/2009 (vaccine strain) grown in embryonated chicken eggs and other A(H1N1) pdm09 England strains grown in embryonated chicken eggs or tissue culture cells. Reference virus strains used for HI assays for influenza B viruses included B/Phuket/3073/2013 (trivalent and quadrivalent vaccine strain) and B/Brisbane/60/2008 (quadrivalent vaccine strain) together with a panel of other egg- and tissue culture-grown influenza B viruses from both the B/Yamagata/16/88-lineage and the B/Victoria/2/87 lineage. The fold difference between the homologous HI titre for the corresponding vaccine strain and the HI titre for each clinical isolate was calculated to determine antigenic similarity of clinical isolates to the vaccine strain.

Nucleotide sequencing of the haemagglutinin (HA) gene was undertaken (primer sequences available on request) for a subset of influenza A(H1N1)pdm09 and B viruses selected to be representative of the range of the patients' age, date of sample collection, geographical location and, if performed, antigenic characterisation of the virus isolate, and phylogenetic trees were constructed with a neighbour-joining algorithm available in the Mega 6 software (http://www.megasoftware.net) [13]. The A(H1N1)pdm09 results have been previously presented [5]. HA sequences from reference strains used in the phylogenetic analysis for influenza B in this paper were obtained from GenBank: B/Malaysia/2506/2004 (CY038287), B/Jilin/20/2003 (CY033828), B/Yamagata/16/88 (CY018765), B/Victoria/2/87 (M58428), B/HongKong/330/2001 (AF532549) and from the EpiFlu database of the Global

Initiative on Sharing All Influenza Data (GISAID) (Table 1).

Statistical methods

Patients were defined as vaccinated if they had received the 2015/16 seasonal vaccine at least 14 days before first onset of ILI. Patients were excluded if they were vaccinated less than 14 days before symptom onset. If vaccinated, but date of vaccination was unknown, the median date of vaccination of those with known dates was taken instead. Patients with date of onset not known or onset more than seven days before swabbing were also excluded. A similar approach was used to undertake a pooled analysis for the 2013/14, 2014/15 and 2015/16 seasons.

The odds ratios (OR) obtained from multivariable logistic regression models were used to calculate VE with influenza laboratory results as the outcome and influenza vaccination status as the linear predictor. Influenza A(H1N1)pdm09- and influenza B-specific VE was also calculated. Samples positive for other subtypes were excluded as the numbers were too small, except for the three-season pooled analysis, which also included influenza A(H3N2). The adjusted estimates were set based on past seasons as age (age groups: 0–4, 5–17, 18–44, 45–64, ≥ 65 years), month of sample collection, residence in area where a primary school programme was in place, sex and surveillance scheme. We also explored whether being in a risk group for whom vaccination is recommended provided any evidence of confounding. For the three-year pooled analysis, year was also included in the model. All statistical analyses were carried out in Stata version 13 (StataCorp, College Station, Texas).

The HA sequences from England obtained in this study, which were also used in the phylogenetic analysis, were deposited in GISAID under the following accession numbers: EPI679258, EPI811543, EPI811551, EPI811554, EPI811562, EPI811570, EPI811578, EPI811586, EPI811594, EPI811598, EPI811606, EPI811614, EPI811622, EPI811626, EPI811629, EPI811637, EPI811645, EPI811648, EPI811656, EPI811664, EPI811671, EPI811675, EPI811683, EPI811691, EPI811699, EPI811707, EPI811715, EPI811723, EPI811726, EPI811734, EPI811742, EPI811750, EPI811758, EPI811766, EPI811774, EPI811782, EPI811788, EPI811796, EPI811799, EPI811807, EPI811815, EPI811823, EPI811831, EPI811839, EPI811842, EPI811845, EPI811853, EPI811856, EPI811864, EPI811868, EPI811876, EPI811884, EPI811891, EPI811894, EPI811898, EPI811906, EPI811909, EPI811915, EPI811916, EPI811924, EPI811932, EPI811940, EPI811944, EPI811952, EPI811958.

Results

Of the 5,811 swabbed individuals potentially eligible, 3,841 individuals were confirmed eligible and included in the study (Figure 1). The details of those included in the study are provided by swab result in Table 2, including those with missing data. There were a total of 2,686 controls, 351 (9.1%) influenza B detections, 770 A(H1N1)pdm09 detections (20.0%), 24 influenza A(H3N2) detections (0.6%) and 15 influenza A(untyped) detections (0.4%). Four samples tested positive for both A(H1N1)pdm09 and influenza B and one sample was positive for both A(H1N1)pdm09 and A(H3N2). Positivity rates differed significantly by age group, sex, risk group, month, scheme, vaccination status and area of primary school programme in England (Table 2).

Influenza A(H1N1)pdm09 and B strain characterisation from sentinel samples

Since week 40 in 2015, a total of 730 influenza viruses from this study have been characterised by the PHE Respiratory Virus Unit and the West of Scotland Virology Centre: 128 antigenically, 293 genetically and 309 through both methods. Only the PHE Respiratory Virus Unit undertook the antigenic analysis.

A total of 482 influenza A(H1N1)pdm09 viruses were characterised. All belonged in the genetic subgroup 6B, which had been the predominant genetic subgroup in the 2014/15 season. Some heterogeneity was seen in the HA of the current season's A(H1N1)pdm09 viruses, with some newly emerging genetic subgroups: the HA genes of the majority (93%) of A(H1N1)pdm09 viruses fell into genetic cluster 6B.1, characterised by the amino acid changes S84N, S162N (with gain of a potential glycosylation site) and I216T, with a subset in this cluster having the substitution A215G. Less than 6% of viruses fell into a second emerging cluster (6B.2) and had the amino acid substitutions V152T, V173I, E491G and D501E in the HA gene, or into a third minor cluster with substitutions N129D, R450K and E491G. A few viruses from this season did not have any of these changes or had only the substitution S84N, and clustered with A(H1N1)pdm09 viruses from season 2014/15 (6B subgroup). A tree showing the phylogenetic relationships for the A(H1N1)pdm09 has already been published [5]. Of 123 A(H1N1)pdm09 viruses isolated from sentinel samples between December 2015 and April 2016 and analysed by HI assay using an extended panel of ferret post-infection sera including a ferret post-infection antiserum to A/California/7/2009 (NIBSC, UK), 100% were antigenically similar to the A/California/7/2009 northern hemisphere 2015/16 A(H1N1)pdm09 vaccine strain. Using this extended panel of ferret post-infection antisera, no antigenic low reactors to A/California/7/2009 antisera were observed.

A total of 324 influenza B viruses were characterised: more than 96% of them belonged to the B/Victoria lineage in clade 1A, represented by B/Brisbane/60/2008 (the 2015/16 quadrivalent vaccine strain) (Figure 2). Viruses in this clade have N75K, N165K and S172P in their HA compared with the previous vaccine virus. Additional amino acid substitutions seen this season were I117V, N129D and V146I. A few (‹ 3%) UK 2015/16 B/Yamagata lineage viruses were detected, all belonging to genetic clade 3, with amino acid substitutions S150I, N165Y and G229D relative to a previous vaccine

Table 2

	Controls		Influenza B[a]		Influenza A(H1N1)[a]		Influenza A(H3N2)		Influenza A(untyped)		Total[a]	p value[b]
Age group (years)												
0–4	273	71.3	19	5.0	91	23.8	1	0.3	1	0.3	383	<0.0001
5–17	392	69.3	92	16.3	78	13.8	5	0.9	1	0.2	566	
18–44	1,022	65.9	170	11.0	348	22.4	7	0.5	5	0.3	1,551	
45–64	636	70.0	47	5.2	211	23.2	7	0.8	7	0.8	908	
≥ 65	346	84.6	19	4.6	39	9.5	4	1.0	1	0.2	409	
Missing	17	70.8	4	16.7	3	12.5	0	0	0	0	24	
Sex												
Female	1,627	72.4	188	8.4	417	18.5	12	0.5	8	0.4	2,248	<0.0001
Male	1,046	66.4	162	10.3	350	22.2	12	0.8	7	0.4	1,576	
Missing	13	76.5	1	5.9	3	17.6	0	0	0	0	17	
Surveillance scheme												
Northern Ireland	76	49.0	22	14.2	51	32.9	0	0	6	3.9	155	<0.0001
RCGP	1,148	64.0	179	10.0	449	25.0	19	1.1	0	0	1,793	
SMN	138	67.0	12	5.8	50	24.3	1	0.5	5	2.4	206	
Scotland	1,242	81.8	101	6.6	172	11.3	3	0.2	4	0.3	1,519	
Wales	82	48.8	37	22.0	48	28.6	1	0.6	0	0	168	
Risk group												
No	1,794	66.5	276	10.2	607	22.5	14	0.5	9	0.3	2,697	<0.0001
Yes	817	79.7	53	5.2	141	13.8	9	0.9	6	0.6	1,025	
Missing	75	63.0	22	18.5	22	18.5	1	0.8	0	0	119	
Interval onset–sample (days)												
0–1	292	67.6	41	9.5	95	22.0	2	0.5	2	0.5	432	<0.0001
2–4	1,351	66.1	216	10.6	463	22.6	14	0.7	5	0.2	2,045	
5–7	1,043	76.5	94	6.9	212	15.5	8	0.6	8	0.6	1,364	
Month												
October	304	98.7	1	0.3	1	0.3	1	0.3	1	0.3	308	<0.0001
November	396	96.1	6	1.5	8	1.9	2	0.5	0	0	412	
December	463	86.4	5	0.9	67	12.5	0	0	1	0.2	536	
January	541	68.7	26	3.3	217	27.6	3	0.4	2	0.3	787	
February	445	56.1	67	8.4	275	34.7	4	0.5	3	0.4	793	
March	366	48.0	197	25.8	190	24.9	7	0.9	5	0.7	763	
April	171	70.7	49	20.2	12	5.0	7	2.9	3	1.2	242	
Vaccination status (all ages)												
Unvaccinated	1,959	66.4	308	10.4	658	22.3	15	0.5	13	0.4	2,949	<0.0001
Vaccinated (14–91 days ago)	377	89.8	6	1.4	33	7.9	3	0.7	1	0.2	420	
Vaccinated (>91 days ago)	350	74.2	37	7.8	79	16.7	6	1.3	1	0.2	472	
Pilot area (SMN and RCGP only)												
No	1,185	63.8	181	9.7	470	25.3	20	1.1	2	0.1	1,858	0.057
Yes	91	72.2	9	7.1	24	19.0	0	0	2	1.6	126	
Missing	11	64.7	1	5.9	4	23.5	0	0	1	5.9	17	
Vaccine status (by route) (2–17 years)												
Not vaccinated	402	65.5	94	15.5	112	18.2	6	1.0	1	0.2	614	0.01
Injection	16	84.2	3	15.8	0	0	0	0	0	0	19	
Intranasal	89	77.4	4	3.5	22	19.1	0	0	0	0	115	
Missing	12	70.6	1	5.9	4	23.5	0	0	0	0	17	

RCGP: Royal College of General Practitioners Research and Surveillance Centre; SMN: Specialist Microbiology Network.
Note: Differences between cases and controls for all variables in this table were statistically significant.
[a] Four positive for influenza A(H1N1) and B; one positive for influenza A(H1N1) and A(H3N2). Duplicates are not included in row totals.
[b] Positive vs negative for influenza.

Table 3

Samples positive (cases; n = 1,155) and negative (controls; n = 2,686) for influenza A and B according to vaccination status and vaccine effectiveness estimates, United Kingdom, October 2015–May 2016

	Cases		Controls		Crude VE (95% CI)	Adjusted[a] VE (95% CI)
	Vaccinated	Unvaccinated	Vaccinated	Unvaccinated		
Influenza A or B	165	990	727	1,959	55.1 (45.9–62.7)	52.4 (41.0–61.6)
Influenza A(H1N1)	112	658	727	1,959	54.1 (43–63.1)	54.5 (41.6–64.5)
Influenza A/6B1[b]	45	232	651	1,739	48.2 (28.8–62.8)	48.9 (26.4–64.5)
Influenza B	43	308	727	1,959	62.4 (47.7–73.0)	54.2 (33.1–68.6)
Influenza B/ Victoria[b]	21	161	651	1,739	65.2 (44.6–78.1)	57.3 (28.4–74.6)

CI: confidence interval; RCGP: Royal College of General Practitioners Research and Surveillance Centre; VE: vaccine effectiveness.

[a] Adjusted for age group, sex, month, pilot area and surveillance scheme.

[b] Based only on data from RCGP and Scotland only.

Table 3 shows that the adjusted VE was 54.5% (95% CI: 41.6–64.5) against influenza A(H1N1)pdm09 and specifically 48.9% (95% CI: 26.4–64.5) for clade 6B1 viruses. The age-specific VE against influenza A(H1N1)pdm09 ranged from 48.5% (95% CI: 8.5–71.0) in those aged two to 17 years to 59.8% (95% CI: 35.8–74.8) in those aged 18 to 44 years (Table 4). There was no significant difference in VE against influenza A(H1N1) pdm09 by time since vaccination or period of vaccination (Table 4), overall or by age (adult/child).

strain. More recent substitutions observed this season included N116K, K298E, E312K and also L172Q seen in the majority of B/Yamagata clade 3 viruses.

Of 99 influenza B viruses isolated from sentinel sources between December 2015 and May 2016 and analysed by HI assay, 98 (99%) were characterised as belonging to the B/Victoria/2/87 lineage and were antigenically similar to B/Brisbane/60/2008, the influenza B/Victoria-lineage component of the 2015/16 northern hemisphere quadrivalent vaccines. One virus (1%) was characterised as belonging to the B/Yamagata/16/88-lineage and was antigenically similar to B/Phuket/3073/2013, the influenza B/Yamagata-lineage component of the 2015/16 northern hemisphere trivalent and quadrivalent vaccines.

Model fitting for vaccine effectiveness estimation

The variables incorporated in the multivariable model (month of sample collection, age group, sex, surveillance scheme and primary school programme area) were all significantly associated with swab positivity, and all except primary school programme area and sex were confounders for the vaccine effects (changed estimates by more than 5%). As with previous seasons' analyses [5-7], risk group was not included in the final model as it was not a confounder and data were missing for 119 samples (3.1%).

The crude and adjusted VE estimates against all confirmed influenza, influenza A(H1N1)pdm09 and influenza B for the 2015/16 season are given in Table 3. There were inadequate numbers to estimate VE against influenza A(H3N2). The adjusted VE was 52.4% (95% confidence interval (CI): 41.0–61.6) against all laboratory-confirmed influenza for all ages.

Table 3 shows that the adjusted VE was 54.5% (95% CI: 41.6–64.5) against influenza A(H1N1)pdm09 and specifically 48.9% (95% CI: 26.4–64.5) for clade 6B1 viruses. The age-specific VE against influenza A(H1N1) pdm09 ranged from 48.5% (95% CI: 8.5–71.0) in those aged two to 17 years to 59.8% (95% CI: 35.8–74.8) in those aged 18 to 44 years (Table 4). There was no significant difference in VE against influenza A(H1N1) pdm09 by time since vaccination or period of vaccination (Table 4), overall or by age (adult/child).

Table 3 also shows that the adjusted VE was 54.2% (95% CI: 33.1–68.6) against influenza B and specifically 57.3% (95% CI: 28.4–74.6) for viruses of the B/Victoria lineage. The age-specific VE against influenza B ranged from 76.5% (95% CI: 41.9–90.5) in those aged two to 17 years to −20.0% (95% CI: −259.1 to 59.8) in those aged 65 years and older (Table 4), although these age-specific differences in VE were not significant. There was no significant difference in influenza B VE by time since vaccination or by period of vaccination (Table 4).

The VE results by type of vaccine in children two to 17 years of age are given in Table 5. For children receiving LAIV, the overall VE against all laboratory-confirmed influenza was 57.6% (95% CI: 25.1–76) and specifically 81.4% (95% CI: 39.6–94.3) for influenza B and 41.5% (95% CI: −8.5 to 68.5) for influenza A(H1N1)pdm09. This compares to an overall VE of 77.8% (95% CI: 7.3–94.7) for children receiving IIV and a specific VE of 56.3% (95%CI: −121.6 to 91.4) against influenza B and 100% (95%CI: 13.3–100) against influenza A(H1N1)pdm09. By age group, overall LAIV VE in two to eight year-olds was

Table 4

Adjusted vaccine effectiveness estimates for influenza by age, time since vaccination, vaccination period and risk group, United Kingdom, October 2015–May 2016 (n = 3,841)

Factor	Level	Adjusted VE[a] by type % (95% CI)		
		A+B	A(H1N1) pdm09	B
Age (years)[b]	2–17	60.6 (34.4–76.3)	48.5 (8.5–71.0)	76.5 (41.9–90.5)
	18–44	55.3 (34.2–69.6)	59.8 (35.8–74.8)	45.9 (1.0–70.4)
	45–64	55.4 (34.6–69.5)	58.6 (36.9–72.8)	65.0 (15.1–85.6)
	≥65	29.1 (–34.1 to 61.8)	56.1 (7.2–79.3)	-20.2 (–259.1 to 59.8)
Period of vaccination[b]	Oct - Jan	50.0 (27.6–65.4)	54.3 (31.6–69.4)	35.9 (–70.5 to 75.9)
	Feb - April	53.0 (38.7–64.0)	53.6 (36.1–66.3)	56.9 (35.1–71.3)
Time from vaccination to onset[b]	<3 months	51.4 (29.9–66.3)	56.7 (34.9–71.3)	53.1 (–12.1 to 80.3)
	>3 months	52.7 (39.2–63.2)	53.9 (38.1–65.6)	53.4 (30.0–69.0)

CI: confidence interval; VE: vaccine effectiveness.

[a] Adjusted for age group, sex, month, pilot area and surveillance scheme.

[b] No significant evidence of interaction.

50.2% (95% CI: 1.6–74.8) and 63.9% (95% CI: –20.3 to 89.2) in nine to 17 year olds.

In 2013/14, the dominant circulating strain was influenza A(H1N1)pdm09, whereas in 2014/15, the dominant circulating strain was influenza A(H3N2), which had antigenically and genetically drifted from the vaccine strain, followed by influenza B mainly of the B/Yamagata lineage. Over the three seasons, the overall VE of LAIV was 53.1% (95% CI: 31.4–67.9) against all confirmed influenza, with a VE of 31.5% (95%CI: –50.4–68.8) for IIV (Table 6). The LAIV VE showed evidence of significant VE against laboratory-confirmed influenza B infection, borderline significance against influenza A(H3N2) and moderate, non-significant effectiveness against influenza A(H1N1)pdm09. Over the three-year period, albeit with small numbers, there was no evidence of significant effectiveness of IIV against influenza B or A(H3N2), but effectiveness of 100% (95% CI: 16.2–100) against influenza A(H1N1)pdm09.

Discussion

In the 2015/16 season, the UK completed the third season of the introduction of a universal paediatric LAIV programme. The 2015/16 season was characterised by late, prolonged influenza A(H1N1)pdm09 activity, with predominance of an emerging genetic HA subgroup, which was antigenically well matched to the vaccine strain, followed by circulation of influenza B viruses, predominantly of the B/Victoria lineage which was not represented in the 2015/16 trivalent inactivated influenza vaccine. The end-of-season VE was moderately good in adults for influenza A(H1N1)pdm09 and in adults younger than 65 years for influenza B, despite the B lineage mismatch for the trivalent influenza vaccine, the main vaccine used in adults. Overall VE for LAIV in children was also moderately good and specifically for influenza B, it was very good, although protection was less against influenza A(H1N1)pdm09. There was no evidence to suggest waning vaccine-derived protection or changes in circulating strains over the 2015/16 season.

We found an overall significant VE of 52.4% and specifically of 54.5% against influenza A(H1N1)pdm09, the dominant circulating strain this season. Although 2015/16 has seen the continued emergence of the new genetic subgroups 6B.1 and 6B.2, the antigenic characterisation indicates a good match to the 2015/16 influenza vaccine strain and no measurable differences between these two emerging groups, which reinforces the VE findings in this paper. These levels of effectiveness are consistent with those reported mid-season in 2015/16 [5], but also in earlier A(H1N1)pdm09 seasons, in particular in 2010/11 [14]. The 2015/16 A(H1N1)pdm09 VE results were also similar to the mid-season estimates reported from North America and elsewhere in Europe this season [15,16]. The continuing apparent antigenic and epidemiological match to the vaccine strain remains encouraging and supports the World Health Organization's recommendation that the vaccine for the 2016/17 northern hemisphere winter should include an A/California/7/2009-like vaccine strain [17].

In younger adults under 65 years of age, influenza B VE was over 50%. Almost all vaccinated adults in the UK can be expected to have received the 2015/16 trivalent inactivated (rather than the quadrivalent) influenza vaccine, which contained the B/Yamagata vaccine strain in 2015/16. Our results indicate that despite this lineage mismatch, the 2015/16 IIV in younger adults continued to provide important levels of protection against influenza B, findings which are consistent with earlier published literature [18]. On the other hand, we failed to find evidence of significant VE against influenza B in the elderly, although underpowered with only 19 positive detections and a low positivity of 4.6% in this age group. This is in contrast to the 2014/15 season, when influenza vaccines elsewhere in Europe provided effectiveness of 50.4% (95% CI: 14.6–71.2) against influenza B in those older than 65 years [19]; in that season, the dominant circulating strain was B/Yamagata and belonged to a clade that was antigenically similar to the vaccine virus that season. Evidence of cross-protection, as we seem to have seen in the younger adults this season, might have important implications for the potential incremental cost-effectiveness and recommendations for preferential use of quadrivalent vaccines in adults and highlights the importance of gathering further data in this area to better inform such decisions.

Table 5

Vaccine effectiveness estimates for influenza by type of vaccine in two to 17 year-olds, United Kingdom, October 2015–May 2016 (n = 729)

Type/subtype	Type of vaccine	Cases (unvaccinated; vaccinated)	Controls (unvaccinated; vaccinated)	Crude VE (95% CI)	Adjusted VE[a] (95% CI)
All	Intranasal	212; 26	402; 89	44.6 (11.6–65.3)	57.6 (25.1–76)
	Injectable	212; 3	402; 16	64.4 (–23.4 to 89.8)	77.8 (7.3–94.7)
Influenza A/(H1N1)pdm09	Intranasal	112; 22	402; 89	11.3 (–47.9 to 46.8)	41.5 (–8.5 to 68.5)
	Injectable	112; 0	402; 16	100 (13.3–100)	100 (13.3–100)[b]
Influenza B	Intranasal	95; 4	402; 89	81 (46.9–93.2)	81.4 (39.7–94.3)
	Injectable	95; 3	402; 16	20.7 (–177.8 to 77.3)	56.3 (–121.6 to 91.4)

CI: confidence interval; VE: vaccine effectiveness.

[a] Adjusted for age group, sex, month, pilot area and surveillance scheme.

[b] Cornfield's unadjusted estimate.

Among children two to 17 years of age, we observed an overall significant VE of 57.6% for the quadrivalent LAIV vaccine this season, specifically 81.4% for influenza B and 41.5% for influenza A(H1N1)pdm09, with a similar picture when examining the previous three seasons. Over the three seasons, the overall effectiveness of LAIV was higher compared with inactivated vaccine in that age group, specifically for influenza A(H3N2) and B, but lower in 2015/16 and specifically for influenza A(H1N1)pdm09. These findings are in contrast to those recently reported by the US CDC who found an overall VE of only 3% for LAIV in two to 17 year-old children with very low VE against influenza A(H1N1)pdm09, while the inactivated vaccine showed significant effectiveness [3]. The US first noted lower VE of LAIV against influenza A(H1N1)pdm09 in 2013/14, which on further investigation was considered related to reduced thermostability of the A/California/7/2009 vaccine strain [20]. This led to the replacement of the A(H1N1)pdm09 LAIV vaccine strain with the more recently emerged A/Bolivia/559/2013 vaccine strain for the 2015/16 season. Based on the 2015/16 VE findings from the CDC, the US Advisory Committee on Immunisation recommended a temporary suspension of use of LAIV for children in the US for the forthcoming 2016/17 season [3]. In addition to the UK findings presented here, Finland, in its first season of use of LAIV in pre-school age children, found overall levels of protection of 51%, similar to the UK [21].

The reasons why the observed levels of overall protection were higher in Europe than in the US, with apparent reduced protection against influenza A(H1N1)pdm09 compared to IIV, remain under investigation. Several hypotheses have been suggested. Firstly, are the observed differences real or the consequence of a methodological difference? If real, viral interference between the A(H1N1)pdm09 vaccine strain and the other influenza vaccine viruses in the quadrivalent LAIV vaccine might provide an explanation; such interference has been discussed previously [22] and might be reinforced by prior vaccination with LAIV and/or IIV in young children (which is at present much more likely in North America than Europe) or by repeat vaccination in-season, with the US offering two doses of influenza vaccine to children compared with one dose for healthy children in Europe. A further explanation is possible antigenic drift between the A/Bolivia/559/2013 vaccine strain in the 2015/16 LAIV vaccine and circulating A(H1N1)pdm09 strains in winter 2015/16, although antigenically, the virus is considered to be well matched. Finally, programmatic or logistical differences, e.g. related to cold chain or vaccine handling might play a role.

Further work is required to investigate these hypotheses, although UK programme evaluation results from 2013/14 and 2014/15 already suggest that the UK LAIV paediatric programme reduced influenza circulation when comparing pilot areas where children of primary school age were offered vaccine to those areas where they were not [23,24]. The UK VE results presented in this paper have been reviewed by the JCVI who strongly recommended not to change the current influenza immunisation strategy planned for 2016/17, but further work is required to better understand these recent observations in the light of the US findings and to potentially optimise vaccine composition.

Although waning protection post vaccination has recently been noted [25] and although 2015/16 was a particularly late influenza season with significant activity until late into the spring, there was no evidence to suggest either waning protection by time since

Table 6

Three-season vaccine effectiveness estimates for influenza by type of vaccine in two to 17 year-olds, United Kingdom, October 2013–May 2016 (n = 1,655)

Type/subtype	Type of vaccine	Cases (unvaccinated; vaccinated)	Controls (unvaccinated; vaccinated)	Crude VE (95% CI)	Adjusted VE[a] (95% CI)
All	Intranasal	414; 49	1,003; 189	37.2 (12.2–55)	53.1 (31.4–67.9)
	Injectable	414; 11	1,003; 29	8.1 (–85.7 to 54.5))	31.5 (–50.4 to 68.8)
Influenza A(H3N2)	Intranasal	129; 13	1,003; 189	46.5 (3.4–70.4)	46.7 (–6.9 to 73.4)
	Injectable	129; 5	1,003; 29	-34.1 (–252.4 to 49)	-22.0 (–274.8 to 60.3)
Influenza A/(H1N1)pdm09	Intranasal	159; 32	1,003; 189	-6.8 (–61 to 29.1)	35.6 (–4.4 to 60.3)
	Injectable	159; 0	1,003; 29	100 (16.2–100)	100 (16.2–100)[b]
Influenza B	Intranasal	125; 4	1,003; 189	83 (63.5–93.8)	86.9 (61.0–95.6)
	Injectable	125; 5	1,003; 29	-38.3 (–263.9 to 47.4)	24.8 (–153.3 to 77.7)

CI: confidence interval; VE: vaccine effectiveness.

[a] Adjusted for age group, sex, month, pilot area, surveillance scheme and year.

[b] Cornfield's unadjusted estimate.

vaccination or changes in effectiveness by vaccination period due to the emergence of new clades or lineages over the course of the season in the UK. Our findings are congruent with recent work which suggests that intra-seasonal waning is of lesser importance with influenza A(H1N1)pdm09 and influenza B compared with influenza A(H3N2) [25].

The paper has a number of strengths. It uses a well-established methodology, the TNCC, the results of which approximate well to more traditional case–control approaches [26]. Data completeness was very high and the integration of genetic characterisation data has allowed the estimation of clade- and lineage-specific VE. Caution is needed in the interpretation of the results in children two to 17 years of age owing to the small sample size, particularly in relation to IIV where only a small proportion of the paediatric control population with available information (16/507, 3%) were reported to be vaccinated, while for LAIV, 18% of controls were reported vaccinated.

Conclusion

In summary, notwithstanding the limitation of the small sample size, our findings together with those from Finland confirm encouraging overall levels of protection for LAIV. This protection is particularly effective against influenza B, though less against influenza A(H1N1)pdm09, a finding which in the light of observations in the US requires further investigation.

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Conflict of interest

MD declares lecture fees and travel grant/ conference fees from Sanofi-Pasteur MSD in 2016; SdeL declares no direct conflict of interest, however University of Surrey has received grant funding from two Innovative Medicine Initiatives programmes ADVANCE (SdeL is a work package lead) and FLUCOP. Surrey has also received grant funding from GSK to explore the feasibility of collecting European Medicine Agency listed influenza brand-specific side effects in near real time, SdeL is PI.

Authors' contributions

RGP led the drafting; FW, CR and NA led on the statistical analysis; JE, MG and CT led on the virological analysis; all co-authors contributed epidemiological and/or virological data, contributed to the design and interpretation of the results, reviewed drafts and approved the final version.

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Community-wide outbreak of haemolytic uraemic syndrome associated with Shiga toxin 2-producing *Escherichia coli* O26:H11 in southern Italy, summer 2013

C Germinario [1], A Caprioli [2], M Giordano [3], M Chironna [1], MS Gallone [1], S Tafuri [1], F Minelli [2], A Maugliani [2], V Michelacci [2], L Santangelo [3], O Mongelli [4], C Montagna [5], G Scavia [2], on behalf of all participants of the Outbreak investigation team [6]

1. Department of Biomedical Science and Human Oncology, Aldo Moro University of Bari, Bari, Italy
2. Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Rome, Italy
3. Paediatric Nephrology and Dialysis Unit, Children's Hospital Giovanni XXIII, Bari, Italy
4. Veterinary Public Health Office, Regione Puglia, Bari, Italy
5. Istituto Zooprofilattico Sperimentale della Puglia e Basilicata, Putignano, Italy
6. The members of the Outbreak investigation team are listed at the end of the article

Correspondence: Gaia Sofia Scavia (gaia.scavia@iss.it)

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In summer 2013, an excess of paediatric cases of haemolytic uraemic syndrome (HUS) in a southern region of Italy prompted the investigation of a community-wide outbreak of Shiga toxin 2-producing *Escherichia coli* (STEC) O26:H11 infections. Case finding was based on testing patients with HUS or bloody diarrhoea for STEC infection by microbiological and serological methods. A case–control study was conducted to identify the source of the outbreak. STEC O26 infection was identified in 20 children (median age 17 months) with HUS, two of whom reported severe neurological sequelae. No cases in adults were detected. Molecular typing showed that two distinct STEC O26:H11 strains were involved. The case–control study showed an association between STEC O26 infection and consumption of dairy products from two local plants, but not with specific ready-to-eat products. *E.coli* O26:H11 strains lacking the *stx* genes were isolated from bulk milk and curd samples, but their PFGE profiles did not match those of the outbreak isolates. This outbreak supports the view that infections with Stx2-producing *E. coli* O26 in children have a high probability of progressing to HUS and represent an emerging public health problem in Europe.

Introduction

Haemolytic uraemic syndrome (HUS) is a rare disorder characterised by microangiopathic haemolysis, platelet consumption, and multi-organ damage (mainly to the kidneys) [1]. In its typical form, it occurs after a prodromal diarrhoea, usually due to Shiga toxin (Stx)-producing *Escherichia coli* (STEC) infection. HUS is the most common cause of acute renal failure in childhood and occurs in ca 15% of children with STEC O157 infections [1]. Although STEC O157 is the predominant cause of paediatric HUS worldwide [1], cases associated with infections with STEC belonging to non-O157 serogroups have been increasingly reported [2-5].

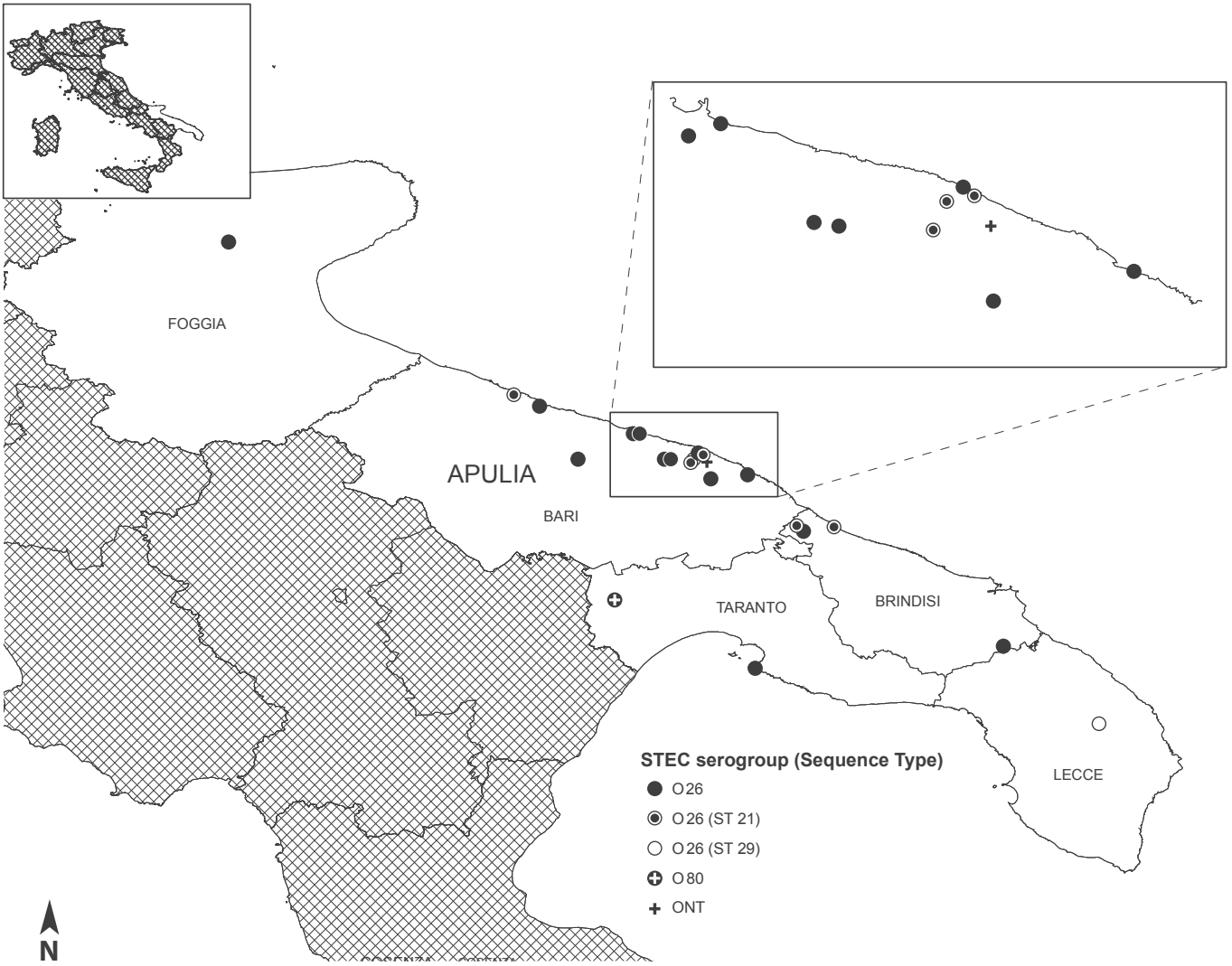
In Italy, surveillance of HUS in children (<15 years) was established in 1988 through the National Registry of HUS, carried out by the Italian Society for Paediatric Nephrology in cooperation with the National Reference Laboratory for *E. coli* [4]. Between 1988 and 2012, an average of 33 sporadic cases of HUS per year were observed in Italy, with a mean annual incidence of 0.4 cases per 100,000 residents aged 0–15 years. The STEC serogroups most frequently reported were O157 (35%), O26 (26%), O145 (12%), O111 (10%) and O103 (5%) [6].

The outbreak

Between 4 June and 9 August 2013, seven paediatric cases of HUS resident in the Apulia region (19,345 km2, ca 4 million inhabitants) or with a history of recent travel to the area were reported to the National Registry of HUS. This represented an excess with respect to the three to five cases per year annually reported in the Apulia region since 1988 [4], and laboratory investigation showed evidence of infection with STEC O26 for four of the five cases whose clinical samples were examined. An outbreak limited to the Apulia region was suspected and an alert was issued to the regional health authorities. Investigations were started to find additional cases, identify the sources of infection, and limit the spread of the outbreak. This report describes the epidemiological, clinical, and microbiological features of the outbreak.

Figure 1

Geographical distribution of recorded cases of haemolytic uraemic syndrome, Apulia region, Italy, 1 June to 30 September 2013 (n=22)



ONT: O non-typeable; STEC: Shiga toxin-producing *Escherichia coli*.

Methods

Case definition and case finding

A probable case was defined as a patient presenting with HUS, or with suspected HUS, or with bloody diarrhoea between 1 June and 30 September 2013, resident in, or with a history of travelling to the Apulia region during the 15 days before the onset of illness. A confirmed case was defined as a patient with diarrhoea or HUS, and laboratory evidence of infection with STEC O26. HUS cases were defined according to Tozzi et al. [4] as patients with evidence of renal failure, intravascular haemolysis, and thrombocytopenia (platelet count‹100,000/mm3).

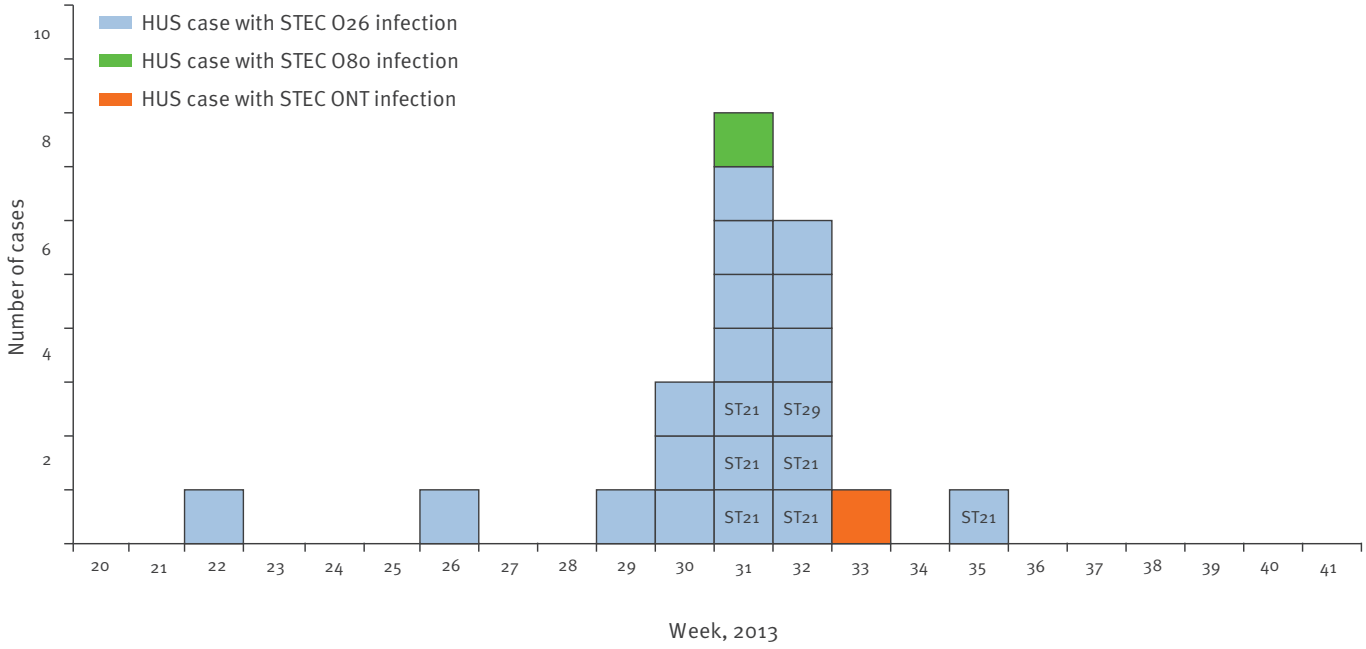
In the Apulia region, the active case finding was carried out by alerting hospitals and emergency rooms to promptly report to the regional surveillance system for infectious diseases any case of bloody diarrhoea, or HUS, or suspected HUS and to submit stool and serum samples for the laboratory diagnosis of STEC infection. The case-finding was extended at the national and international level by posting alerts through the dedicated information systems coordinated by the National HUS Registry and the European Centre for Disease Prevention and Control (ECDC).

Laboratory diagnosis of Shiga toxin 2-producing *Escherichia coli* infection

Stool samples were inoculated in buffered peptone water (BPW) and incubated at 37°C for 18 hours. DNA was extracted from 1 mL of the culture with the InstaGene Matrix (Bio-rad Laboratories, Hercules, CA, US) and tested by a real-time PCR assay to detect the presence of Stx- (*stx1* and *stx2*) [7] and intimin (*eae*)-coding genes [8]. PCR-positive samples were streaked

Figure 2

Distribution of cases of haemolytic uraemic syndrome by infection with different Shiga toxin-producing *Escherichia coli* strains, Apulia region, Italy, 1 June to 30 September 2013 (n=22)



HUS: haemolytic uraemic syndrome; ONT: O non-typeable; STEC: Shiga toxin 2-producing *Escherichia coli*.

Boxes represent HUS cases and are coloured according to the different STEC O serogroups. Where available, the sequence type (ST) of the infecting STEC O26 strain is indicated in the box.

onto MacConkey agar plates and colonies resembling *E. coli* tested for the presence of *stx* and *eae* genes by PCR [9]. The *stx* and/or *eae*-positive strains were tested with O antisera against the main STEC serogroups (Statens Serum Institut, Copenhagen, Denmark) by slide agglutination. Serotyping of STEC belonging to other serogroups was kindly performed by F. Scheutz, at the Statens Serum Institut, Copenhagen, Denmark. Stools were also examined for the presence of free Stx by the Vero cell cytotoxicity assay [4]. Serum samples were tested for antibodies to the lipopolysaccharide (LPS) of five major STEC serogroups (O157, O26, O103, O111, and O145) by ELISA [10].

Characterisation of the Shiga toxin 2-producing *Escherichia coli* O26 strains

The flagellar (H-antigen) *fliC* alleles were detected by real-time PCR, as described by Madic et al. [11]. The *stx* gene subtyping was carried out by PCR, as described by Scheutz et al. [12]. PFGE (Pulsed-Field Gel Electrophoresis) was performed as previously described [9]. Similarity of PFGE profiles was evaluated using the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium), using the UPGMA algorithm with tolerance and optimisation set at 1.5%.

Multilocus sequence typing (MLST) was performed using the scheme developed by Wirth et al. [13]. Sequence types (STs) were determined using the tool available at the University of Warwick [13].

Epidemiological investigation

Parents of confirmed cases were interviewed using the HUS Registry questionnaire, after giving their informed consent. Age, sex, type and time of onset of clinical symptoms, and food and environmental exposures for STEC infection in the 7 days before illness onset were annotated. Other information included dietary habits, food and water consumption, exposure to livestock, presence of household contacts with diarrhoea, exposures to potential environmental sources of STEC, and travel in the 2 weeks before the onset of symptoms.

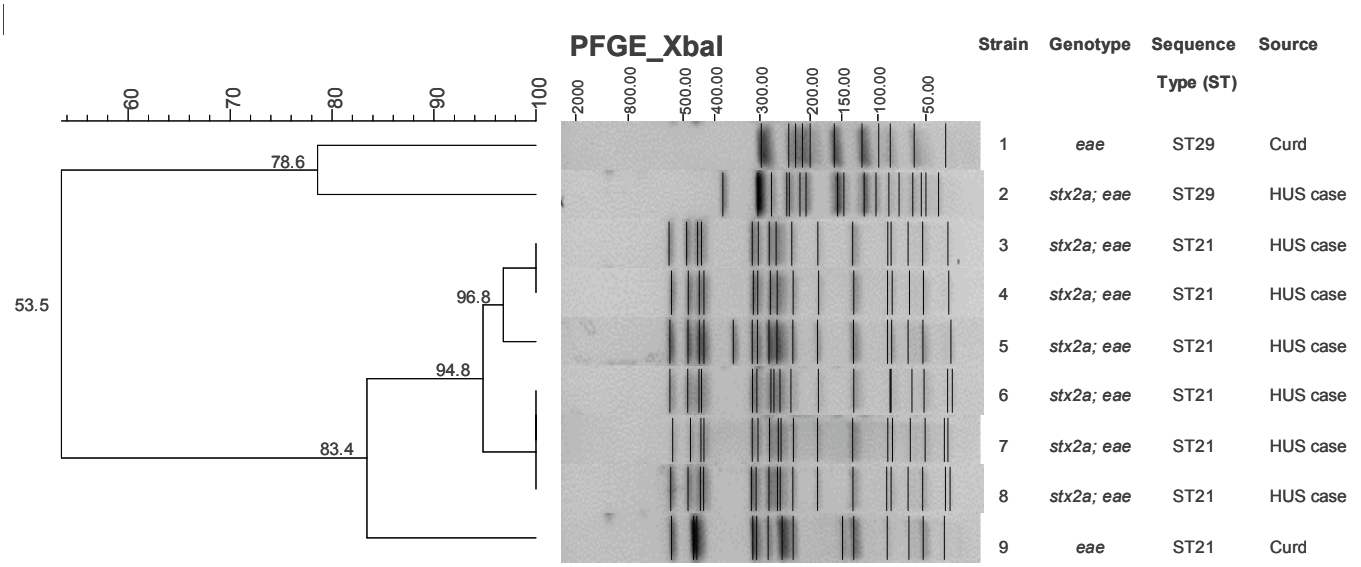
Food trace-back investigation

Trace-back investigations were mainly focused on the retail outlets that sold dairy products and vegetables to the families of the cases in the two weeks before the onset of symptoms. Based on the interviews, it was hypothesised that these products might be implicated in the transmission of STEC infection to patients. Indeed, they were the only items consumed by most of the cases, for which the mode of preparation and consumption would not have eliminated any possible contamination with STEC.

The identified dairy plants were inspected and their staff interviewed by local health authorities regarding

Figure 3

Dendrogram of the degree (%) of similarity between pulsed-field gel electrophoresis profiles of XbaI-digested genomic DNA from strains of *Escherichia coli* O26 isolated from cases of haemolytic uraemic syndrome and dairy products, Apulia region, Italy, 1 June and 30 September 2013



processing practices and sources of raw materials. The dairy farms that supplied milk to the plants were identified and visited as well. Samples of ready-to-eat dairy products, curd, raw milk, fruit and vegetables, and ground beef were collected and tested for the presence of STEC O26 according to the ISO/TS 13136:2012 method for the detection of STEC in food. Briefly, 25 g of each sample were enriched in BPW at 37 °C for 18h. DNA was extracted from one mL of the enrichment culture with the InstaGene Matrix and tested by real-time PCR for the presence of *stx1*, *stx2*, and *eae* genes. Positive DNA samples were further tested for the *wzx* gene associated to the O26 *E. coli* antigen [7]. Positive enrichment cultures were subjected to an O26-specific immunomagnetic separation followed by plating on MacConkey Agar. Colonies were tested for the presence of *stx* and/or *eae* genes by PCR amplification [9].

Environmental investigation

Marine water samples were collected from seaside locations that had been attended by some of the cases. Water samples (250 mL) were subjected to filtration through membranes of mean pore size 0.45 µm. Membranes were then transferred to BPW for enrichment at 37 °C for 18h. DNA extraction and real-time PCR assays for STEC virulence genes were carried out as described for stool and food samples.

Case–control study

A case–control study, limited to the 15 laboratory confirmed cases resident in the Apulia region, was conducted to identify exposures associated with STEC O26 infection in the 10 days before illness onset. Controls, up to five for each case, were children who were reported not to have had diarrhoea in the month preceding the interview, randomly selected among the patients of the family paediatricians of the cases. Controls were matched by sex, age and area of residence. Exposures possibly associated with STEC infection and reported by cases during the hypothesis generation were investigated. Cases and controls were interviewed face-to-face and by telephone, respectively. Exposures associated with STEC O26 infection were analysed by exact univariate and multivariate logistic regression. For subjects reporting consumption of dairy products, univariate and multivariate analyses were carried out to identify associations between STEC O26 infection and the dairy plant of origin of the artisan products of bovine origin. The level of significance was set at $p<0.05$. Data were analysed by Stata 11 MP (StataCorp LP, College Station, Texas).

Results

Case finding and diagnosis of Shiga toxin 2-producing *Escherichia coli* infection

Between 1 June and 30 September 2013, 17 children with HUS resident in the Apulia region were admitted to the regional paediatric nephrology centre participating in the National HUS Registry. Two of the 17 cases were siblings and fell ill 10 days apart. The active case-finding revealed five additional children with HUS with a history of travel to the Apulia region: four were diagnosed in other Italian hospitals and one in a Swiss hospital. Figure 1 shows a map of the Apulia region with the location of the HUS cases at the onset of prodromal symptoms.

Stool and serum samples were obtained from 19 HUS cases, and serum only from the remaining three. The mean interval between onset of enteric symptoms and stool collection was 9 days (range 5–14 days). Stool

Table 1

Evidence of Shiga toxin-producing *Escherichia coli* infection in 22 cases admitted to hospital with haemolytic uraemic syndrome between 1 June and 30 September 2013 and resident in or with a history of travel to Apulia region, Italy in the 15 days before illness onset

Laboratory diagnosis: Type of evidence	No. of cases
Infection with STEC O26 (confirmed cases):	20
Isolation of *E. coli* O26:H11 (*stx2a*+, *eae*+) and O26 LPS antibodies	7
Free faecal Stx and/or *stx2* genes, and O26 LPS antbodies: 4 cases	4
O26 LPS antibodies only	9
Infection with other STEC:	2
Isolation of *E. coli* O80:H2 (*stx2f*+, *eae*+)	1
Isolation of *E. coli* ONT (*stx2* group+, *eae*+)	1

LPS: lipopolysaccharide; ONT: O non-typeable; STEC: Shiga toxin 2-producing *Escherichia coli*; Stx: Shiga toxin.

examination yielded the isolation of STEC O26:H11 strains positive for the *stx2a* and *eae* genes from seven cases. STEC strains belonging to serotypes O80:H2, positive for *stx2f* and *eae*, and O non-typeable (NT) positive for *stx2* group and *eae* were isolated from two other cases. Four other cases had the enrichment cultures positive for *stx2* genes in PCR and/or had free faecal Stx. Serum antibodies against the LPS of *E. coli* O26 were detected in 20 cases. The two patients with STEC belonging to other serogroups did not have antibodies to any of the LPS tested (Table 1). For the six cases with serum antibodies against the LPS of *E.coli* O26 and STEC and Stx-negative faeces, the mean interval between onset of symptoms and stool collection was 10.5 days, longer than that of cases with Stx-positive stools (7 days). Overall, the evidence of infection with STEC O26 was obtained for 20 cases: 16 were resident in the Apulia region and four were travel-related. STEC O26 was isolated from five residents and two travel-related cases.

The active case finding also allowed the identification of 20 cases of bloody diarrhoea whose stools were negative for salmonella and campylobacter and were examined for the presence of STEC. A STEC O157 strain was isolated from one of these patients while the faeces of the other 19 were negative for *stx* and *eae* genes. Stool samples were also obtained from 26 of 40 household contacts of 12 confirmed cases. No STEC strains were isolated, but one sample collected by an adult and one from a child, both presenting with gastroenteric symptoms, tested positive in PCR for the *stx2* and the *eae* gene, respectively.

In the 20 confirmed cases with STEC O26 infection, the onset of symptoms occurred between 4 June and 8 September 2013 (Figure 2).

Case characteristics

The age of the 20 confirmed cases ranged from 11 to 78 months (mean: 24 months; median: 17 months). Eleven cases were female. All confirmed cases developed HUS. Prodromal diarrhoea was reported for 18 cases, and in 10 of them it was bloody. The median interval between onset of diarrhoea and diagnosis of HUS was 9 days (range 1–20 days). Vomiting, abdominal pain, and fever (›38 °C) were reported for 16, 9, and 6 cases, respectively. Other clinical details were available for 19 children. On admission, six cases presented neurological symptoms, primarily seizures (n=5). Seven cases had haematuria and 10 cases had oliguria or anuria. Four cases underwent haemodialysis and plasma exchange, and four patients haemodialysis only. Blood transfusions were administered to 17 patients and 13 also received plasma infusion. Severe neurological sequelae as of 18 months after the onset of HUS were reported for two cases. One of these children also presented a light chronic renal failure.

Source hypothesis generation

Parents of children were interviewed to generate hypotheses about sources of infection but no obvious link was identified that could explain a significant number of them. Of the 19 confirmed cases that could be interviewed, four had household members with diarrhoea in the 15 days before the onset of illness and six reported contact with farm animals. As for food exposure, of the 18 patients for which the information was available, most cases reported consumption of cooked bovine meat (78%), pasteurised or UHT milk (61%), yoghurt (72%), artisanal dairy products (mainly ricotta and mozzarella cheese) of bovine origin (100%), fresh fruit (61%) and watermelon (83%), with multiple brands and/or food retailers involved. Interviews also revealed that two seaside beaches were attended by two and four cases, respectively.

Trace-back investigations and laboratory examination of food and environmental samples

The dairy products of bovine origin consumed by 16 confirmed cases had been prepared by at least six different plants (Table 2). These plants were inspected between 20 August 2013 and 13 September 2013, as well as other 14 dairy plants supplying the retail outlets attended by the cases' families. Recommendations for implementing hygiene measures and good manufacturing practices were issued. The activity of one plant showed gross hygiene failures and was suspended. Pasteurisation of milk for dairy product production was prescribed to three other plants. Visits were also carried out to 31 dairy farms that supplied raw milk to the plants. A total of 218 samples of raw milk and dairy products of bovine origin were collected and tested for the presence of STEC. Sixty-five samples of fruit and vegetables, in particular watermelon, and five beef samples were also collected at retail and wholesale outlets identified through the trace-back investigations and tested for STEC. All the fruit, vegetable, and beef

Table 2

Presence of *Escherichia coli* O26 and/or its virulence genes in bulk milk or curd samples collected in the Apulia region, Italy, 20 August to 13 September 2013

Dairy plant[a]	Confirmed cases associated with the dairy plant	Number and type of samples associated with the dairy plants				
		Samples positive for *stx*, *eae* and wzx_{O26} genes		Samples with *E. coli* O26 isolation		
	n	n	Type of matrix	n	Type of matrix	Characteristics of the strain
A	3	3	1 curd, 2 bulk milk[b]	1	curd	*E. coli* O26 *stx*-, *eae*+
B	2	1	bulk milk[b]	0	0	0
C	7	0	0	0	0	0
D	2	0	0	0	0	0
E	1	6	4 curd, 2 bulk milk	2	milk, curd	*E. coli* O26 *stx*-, *eae*+
F	1	1	bulk milk	1	milk	*E. coli* O26 *stx*-, *eae*+
Other dairy farms	Not possible to determine	2	bulk milk[c]	0	0	0
Total	16	12	5 curd, 7 bulk milk[c]	4	2 milk, 2 curd	*E. coli* O26 *stx*-, *eae*+

E. coli: Escherichia coli.

[a] Samples were collected directly at the plant or at dairy farms supplying milk to the plant.

[b] One bulk milk sample was from a farm that supplied both plant A and B.

[c] Two samples were positive for *stx* genes only.

Samples were from dairy plants linked with confirmed cases of Shiga toxin-producing *Escherichia coli* O26 infection or dairy farms supplying milk to the plants.

samples were negative. The enrichment cultures of 12 bulk milk or curd samples were positive for *stx* genes and, in 10 samples, for the *eae* and *wzxO26* genes. *E. coli* O26:H11 strains lacking the *stx* genes but positive for the *eae* gene were isolated from two curd and two bulk milk specimens (Table 2).

Fifteen marine water samples collected at the two seaside beaches attended by the cases proved negative for the presence of *stx* and *eae* genes.

Characterisation of the *Escherichia coli* O26 strains

The Stx-positive and Stx-negative *E. coli* O26 strains isolated respectively from cases and from dairy products were characterised by MLST and PFGE. All the strains belonged to the clonal complex ST29, but two different STs were distinguished: six strains from cases and one strain from food belonged to ST21, while one human and three food strains were ST29. PFGE analysis (Figure 3) of the strains isolated from cases showed similar profiles (between 94.8% and 100% similarity) for the six ST21 strains and a clearly different profile for the ST29 strain. The PFGE profiles of the two Stx-negative strains isolated from curd were not related with the two profiles of the human strains, and the two strains from milk were untypable due to degradation of DNA during the procedure.

Case–control study

Fifteen confirmed cases and 52 matched controls were included in the case–control study. No differences in the mean age (t = 0.2; p = 0.80) and in the distribution by sex (Chi-squared = 0.01; p = 0.91) were observed between cases and controls. In the univariate analysis (Table 3), STEC O26 infection was significantly associated with the consumption of products from dairy plants A and C but was associated neither with any individual dairy product from these plants nor with any other food items. Multivariate analysis confirmed the association for both plant A (odds ratio (OR): 42.7; 95% confidence interval (CI):2.4–750.5; p = 0.01) and plant C (OR:21.3; 95% CI:3.0–152.5; p < 0.01).

Discussion

This report describes the largest outbreak of STEC-associated HUS ever observed in Italy. The STEC serotype involved was O26:H11, which represents the most common cause of STEC non-O157 infections in Europe [2,14] and has been frequently associated with HUS worldwide [3,4,15-18]. In Italy, the proportion of HUS cases associated with STEC O26 infection has increased since the late 1990s, and currently exceeds that of STEC O157 [2].

This particular STEC serogroup seems to be evolving [5], with a shift from the *stx1* to the *stx2* genotype in the strains associated with severe illness that occurred over the last two decades [2,5,19]. Such a phenomenon has public health relevance, since Stx2-producing *E. coli* O26 strains can cause a disease that is as severe as that caused by STEC O157 [15,19-22]. Two of the cases involved in this episode reported severe neurological sequelae.

At the time of writing this report, to our knowledge, the episode herein described represented the second community-wide outbreak of HUS caused by Stx2-producing

E. coli O26 after that involving 16 HUS cases in France in 2005, linked to the consumption of unpasteurised cow's cheese (camembert) [16,23]. Another severe community-wide outbreak of HUS mainly associated with STEC O26 infection was been reported in Romania in early 2016, with at least 15 children involved [24].

In the Italian outbreak, as in other STEC community-wide outbreaks [10,25-28], cases occurred over a large geographic area and a prolonged period of time and were observed in the framework of a HUS surveillance system, confirming that the emergence of HUS clusters represents an important sentinel event for outbreak recognition [2,10,25,26]. In this episode, an active case finding was promptly implemented after the alert to find new cases, improving the sensitivity and the promptness of case reporting. The enhanced regional surveillance system facilitated the outbreak investigation as well as the adoption of public health measures. Moreover, the existence of national HUS surveillance systems allowed a timely finding of STEC O26 cases resident in other Italian regions and in Switzerland, and who had visited Apulia.

A prompt and accurate laboratory diagnosis of STEC infection is of the utmost importance in HUS cases, to assess the STEC serotype/genotype involved. In 13 out of the 20 cases involved in this episode, the diagnosis of STEC O26 infection was based only on the detection of LPS antibodies, confirming the importance of LPS serology in identifying STEC O157 and non-O157 infections in HUS patients [4,10,16]. Molecular typing of the seven STEC O26:H11 isolates from cases showed that two distinct STEC O26 strains were involved in the outbreak, with the one belonging to ST21 playing a major role. The concomitant presence of two different STEC O26:H11 strains and the two cases of HUS due to different STEC serotypes in the same area and period of time suggest the possibility of a multiple-aetiology outbreak [29]. Outbreaks with different non-O157 STEC strains, including STEC O26, have been reported in the United States [29], Belgium [30] and France [16,23]. As in our case, one of them involved two STEC strains belonging to serotypes O26:H11 and O80:H2 [16,23] and another one, two STEC O26 strains displaying different PFGE profiles [29].

Multiple-aetiology outbreaks have been frequently associated with exposures to environmental sources [29]. Some of the cases shared exposure to the same seaside locations, but water samples collected at those places proved negative for STEC, although the size of the samples may have been too small to allow the detection of the pathogen. However, in this outbreak the spread of cases in a large geographic area makes environmental sources unlike.

Although the origin of human infections with Stx2-producing *E. coli* O26 strains has rarely been identified [31], at least two episodes involving cases of HUS and associated with consumption of unpasteurised milk or dairy products have been reported in Austria [32] and France [16,23]. In our investigation, STEC O26 infection was significantly associated with the consumption of dairy products from two local plants and a drop in the occurrence of cases was observed after the adoption of control measures involving those plants. Neither the association of STEC O26 infection with specific products nor a laboratory evidence of STEC contamination in the final ready-to-eat dairy products could be demonstrated. However, we cannot exclude that a contaminated raw material with a prolonged shelf life, such as a frozen ingredient, could have been continuously used in local plants to prepare different fresh, ready-to-eat products, even though no evidence of such use emerged from the visits to the dairy plants.

The possible involvement of dairy products was also suggested by the evidence of STEC contamination in some bulk milk and curd samples from different plants and by the isolation of *stx*-negative *E. coli* O26:H11 strains from four of these samples. The loss and transfer of *stx* genes by *E. coli* O26 has been demonstrated during human infections [33], and Stx2-positive and negative variants of the same STEC O26:H11 strain, as defined by PFGE analysis, have been isolated from both HUS cases and cheese samples in the French camembert-associated outbreak [23]. Conversely, in the present episode the PFGE profiles of the *stx*-negative *E. coli* O26:H11 strains from milk and curd did not match those of the outbreak isolates.

Another interesting feature of this outbreak is that, despite the enhanced surveillance, we were unable to identify cases of STEC O26 infection other than young children with HUS. In outbreaks due to STEC O157, conversely, severe diarrhoea is generally observed in all the age bands, with young children being more prone to develop HUS [1,25]. The apparent absence of adult cases of infection could be explained by a lack of exposure to the source of infection, even though the epidemiological investigation did not show any suspect food dedicated to small children or other possible STEC risk factors restricted to the young. Another possibility is that the outbreak source was contaminated at a very low level, albeit sufficient to cause disease in young children, the most susceptible age group. Stx2-producing *E. coli* O26 is considered a highly virulent STEC [5]. Nonetheless, an analysis of the literature confirms that the reported outbreaks [16,32] and severe cases [15,19-22] have generally involved only young children. Moreover, in HUS surveys [4], children with STEC O26 infection have been reported to be younger than those with STEC O157 infection. Altogether, these observations allow us to speculate that Stx2-producing *E. coli* O26 might exert a particular virulence towards young children, which could be the reflection of a better fitness of these pathogens in a particular intestinal environment, eventually resulting in an increased colonisation of the gastrointestinal tract.

Table 3

Results of the case–control analysis of exposures in an outbreak of Shiga toxin-producing *Escherichia coli* O26 infection, Apulia region, Italy, 2013

Exposure	Cases (n = 15)	Controls (n = 52)	Matched OR (95% CI)	*p*-value
Meat				
Chicken	11	33	1.6 (0.4–7.7)	0.5
Frankfurter sausages	4	8	2 (0.4–9.2)	0.3
Hamburger	4	21	0.6 (0.1–2.8)	0.4
Meatballs	4	19	0.5 (0.1–2.4)	0.4
Pork	5	19	0.9 (0.2–3.3)	0.8
Sausages	5	26	0.5 (0.1–1.9)	0.3
Veal	12	28	3.1 (0.7–19.2)	0.1
Fruit and vegetables				
Fresh fruit	8	32	0.7 (0.2–2.7)	0.6
Green leafy vegetables	3	4	2.9 (0.4–19.3)	0.2
Fruit juice	8	30	0.8 (0.2–3.2)	0.8
Watermelon	12	39	1.3 (0.3–8.5)	0.7
Other vegetables	7	22	1.2 (0.3–4.4)	0.8
Dairy products				
Pasteurised milk	5	15	1.2 (0.3–4.8)	0.7
UHT milk	4	24	0.4 (0.1–1.6)	0.2
Yogurt	11	31	1.9 (0.5–9.0)	0.3
Burrata cheese	3	5	2.4 (0.3–13.9)	0.3
Mozzarella cheese	8	22	1.6 (0.5–5.9)	0.5
Ricotta cheese	8	32	0.7 (0.2–2.6)	0.5
Other fresh cheeses	3	28	0.2 (0.0–0.9)	0.2
Any dairy product from				
Plant A	3	1	10.3 (1.5–930.2)	<0.01
Plant B	2	5	1.4 (0.1–10.1)	0.7
Plant C	7	5	13.9 (2.2–43.4)	<0.01
Plant E	1	2	1.8 (0.0–36.3)	0.6
Other food				
Ice cream	10	41	0.5 (0.1–2.5)	0.3

CI: confidence interval; OR: odds ratio; UHT: ultra-high temperature processing.

Results are from the univariate analysis.

In conclusion, the present outbreak supports the view that infections with Stx2-producing *E. coli* O26 in children have a high probability to progress to HUS and represent an emerging public health problem in Europe [5]. This further underlines the importance of maintaining national and local surveillance systems for HUS for an early detection and response to STEC O26 infections.

Members of the Outbreak investigation team

Other participants of the outbreak investigation team: Francesco Desiante, Giulia Calabrese, Carmen Martino, Maria Filomena Gallone, Anna Morea (Department of Biomedical Science and Human Oncology, Aldo Moro University of Bari, Italy), Rosangela Tozzoli, Stefano Morabito (Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Rome, Italy), Antonio Parisi (Istituto Zooprofilattico Sperimentale della Puglia e Basilicata, Putignano, Italy), Tommaso Depalo, Vincenza Carbone, Annalisa Resta (Paediatric Nephrology and Dialysis Unit, Children's Hospital Giovanni XXIII, Bari, Italy), Pierfrancesco Pinto (Veterinary Public Health Office, Regione Puglia, Bari, Italy), Massimo Ricci (Regional Agency for Prevention and Environment Protection, Brindisi, Italy), Alessandra Gianviti (Children's Hospital Bambino Gesù, Rome, Italy), Gianluigi Ardissino (IRCCS Ospedale Maggior Policlinico, Clinica De' Marchi, Milan, Italy).

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Conflict of interest

None declared.

Authors' contributions

Diagnosis and clinical management of cases: M Giordano, L Santangelo. Contribution to the epidemiological investigations: C Germinario, MS Gallone, S Tafuri, G Scavia; Laboratory diagnosis of STEC infection: M Chironna, A Caprioli, F Minelli, A Maugliani. Molecular typing: V Michelacci, M Chironna. Trace-back investigations and implementation of the control measures: O Mongelli. Laboratory investigations on food: C Montagna. Contribution to the writing of the paper: G Scavia, A Caprioli, C Germinario. The members of the outbreak investigation team contributed to the clinical, epidemiological, laboratory, and trace-back investigations.

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