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Rapid emergence and antigenic diversification of the norovirus 2012 Sydney variant in Denmark, October to December, 2012

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The norovirus (NoV) season in Denmark in late 2012 was characterised by an increase in the number of NoV infections caused mainly by the 2012 Sydney variant, but also by the 2009 New Orleans variant. Analysis of approximately 85% of the capsid gene from 10 Sydney 2012 and 9 New Orleans 2009 isolates showed rapid antigenic diversification of the Sydney 2012 variant shortly after its emergence. We also present new primers useful for transmission tracking.

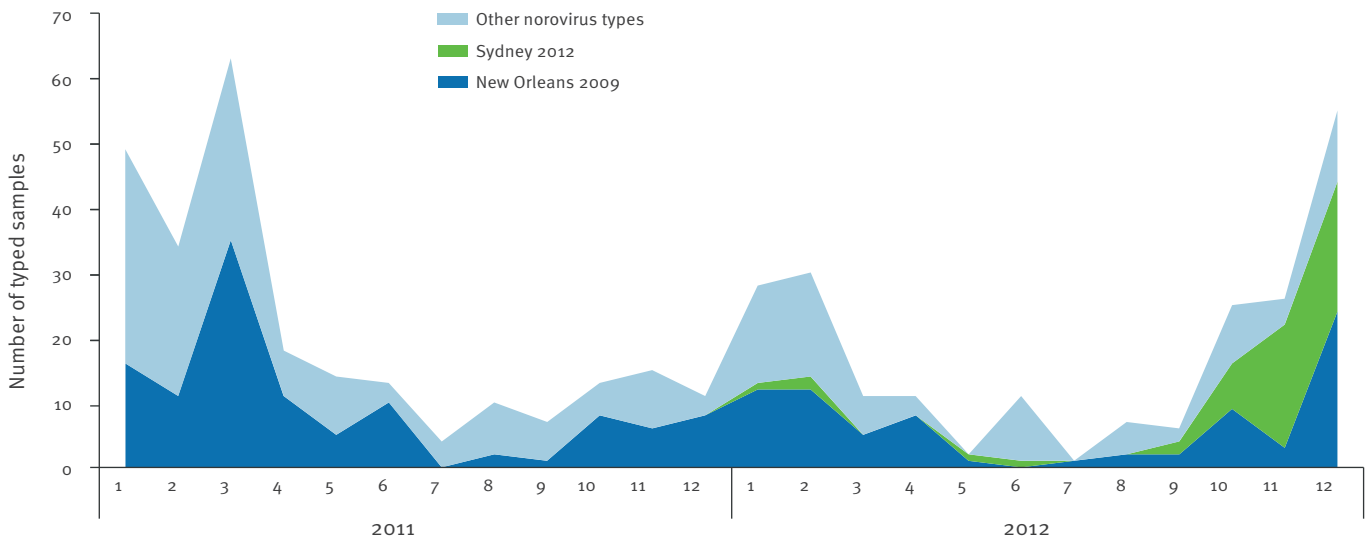
Emergence of the norovirus 2012 Sydney variant in Denmark

In Denmark, we initially identified the norovirus (NoV) GGII.4 2012 Sydney variant in a geographically localised outbreak in November 2012 and simultaneously observed a rapid increase in the proportion of NoV infections caused by this variant. This emerging variant has been reported in two recent European studies [1,2]. In light of these, we re-examined our data and found earlier occurrences of the Sydney 2012 variant. We also used new primers, developed to amplify GGII.4 variants, which enabled characterisation of about 85% of the NoV capsid gene.

A subset of NoV-positive samples obtained from routine diagnostics and outbreaks were selected for genotyping from months in which most NoV cases with each variant were observed: 251 of 1,056 NoV-positive samples in 2011 and 213 of 670 NoV-positive samples in 2012. We analysed NoV polymerase and/or capsid

Figure 1

Norovirus types in Denmark 2011 (n=251) and 2012 (n=213)



Collection dates of the samples analysed further in this study were (month/year/variant): 03/11, 12/11, 01/12, 02/12, 12/13 (New Orleans 2009) and 10-12/12 (Sydney 2012).

Table

Primers used for RT-PCR amplification and sequencing of the norovirus capsid gene

Primer name	Orientation	Used in	Sequence (5´ to 3´)	Positions	Size (bp)
GGII.4 F1	F	1 PCR	GCACGTGGGAGGGCGATCG	5043 to 5061	–
GGII.4 R1	R	1 PCR	GCCAATCCAGCAAAGAAAGCTCCAG	6711 to 6735	1,692
GGII.4 F2	F	2 PCRs	CAGCCAACCTCGTCCCAGAGGTC	5128 to 5150	–
GGII.4 R2	R	2 PCRs	CACGTCTACGCCCCGTYCCATTTCC	6675 to 6699	1,571
GGII.4 F3(Seq)	F	Sequencing	CACCACTTAGGGCYAAYAATGCTGG	5635 to 5659	–
GGII.4 R3(Seq)	R	Sequencing	CCAGCATTRTTRGCCCTAAGTGGTG	5635 to 5659	–
GGII.4 F4(Seq)	F	Sequencing	GATGTCACCCACATTGCAGGTTCTCG	5649 to 5974	–
GGII.4 R4(Seq)	R	Sequencing	CGAGAACCTGCAATGTGGGTGACATC	5649 to 5974	–

bp: base pairs; F: forward; R: reverse; RT-PCR: reverse transcription polymerase chain reaction.
Positions are indicated relative to the GU445325.2 reference sequence.

sequences obtained by reverse transcription polymerase chain reaction (RT-PCR) using standard typing primers [3-8]. Genotypes were assigned to one of three categories (Figure 1), according to the capsid and/or polymerase sequences. In cases where only polymerase gene sequences were available, exclusively top BLASTN hits (National Center for Biotechnology Information) against the 2012 Sydney variant reference sequence or, alternatively, genotype identification via the NoV typing tool [9] were used for variant designation. We found no evidence of recombination between any GGII polymerase and II.4 capsid genes among 132 double (both polymerase and capsid) genotyped samples from 2011 and 2012, and therefore assigned all non-II.e polymerase gene sequences (2012 Sydney variant) to the category other NoV types (Figure 1).

The results show that the 2009 New Orleans variant was the single most dominant variant during 2011 and until April 2012, accounting for 150 of 331 (45%) typed samples, whereas the 2012 Sydney variant was only sporadically detected in routine diagnostics and outbreak samples from January 2012 to October 2012, after which it rapidly emerged as the dominant variant in both surveillance and outbreak samples, accounting for 46 of 106 (43%) typed samples from October 2012 to December 2012 (Figure 1). However, during this period, the New Orleans 2009 variant still accounted for 36 out of 106 (34%) typed samples, making it too early to predict whether the Sydney 2012 variant will entirely replace the New Orleans variant.

Further characterisation of both variants was performed through phylogenetic analysis of nucleotide and the deduced amino acid sequences – using neighbor joining with Jukes–Cantor model of substitution (nucleotides) and the number of differences (amino acids) respectively, in MEGA5 [10] – of nearly complete capsid gene sequences obtained through RT-PCR with primers described in this report (Table).

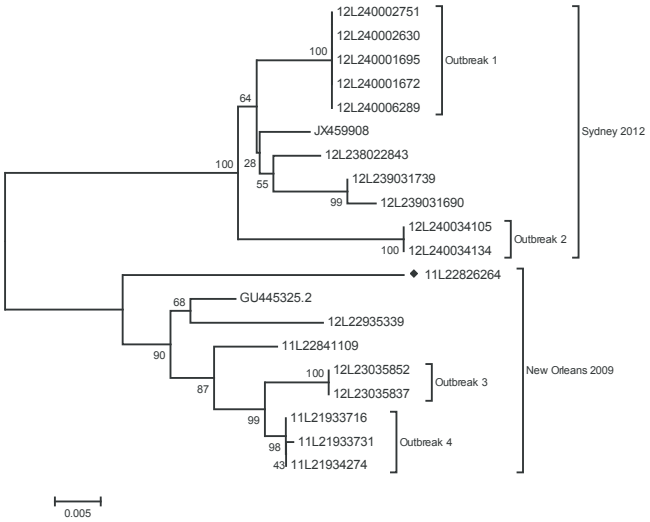
In total, 10 Sydney 2012 variants (obtained from October to December 2012) and nine New Orleans 2009 variants (obtained from March 2011 to December 2012) were selected from the months with the highest number of cases observed for each variant for analysis of the capsid gene*. Included in this set were 12 samples from four outbreaks (Figure 2). All outbreaks occurred in a geographically localised area and the following number of samples were sent from each outbreak: outbreak 1 (18 samples from nine persons), outbreak 2 (seven samples from three persons), outbreak 3 (three samples from three persons) and outbreak 4 (eight samples from seven persons) The remaining samples of each variant were from our routine diagnostics and did not belong to any known outbreak. The primers were able to amplify the capsid region of both variants, which will make them a valuable tool in transmission tracking of both variants. For both variants, 100% identical sequences were observed within each of the four outbreaks (apart from outbreak 4, where two nucleotide differences were observed in one sequence), which indicates a probable point source of infection and also shows that sequencing of the capsid region using these primers is a reliable way of identifying a probable point source of infection caused by the 2012 Sydney variant.

Antigenic diversification of norovirus variants

In order to assess the functional divergence of the two variants, we translated the almost-complete capsid gene sequences (n=19) into amino acids, and found five different branches for the Sydney 2012 variant and six different branches for the New Orleans 2009 variant (Figure 3). We also investigated the amino acid variations occurring in the predicted GII.4 blockage epitope sites [11] and found three distinct patterns of amino acid substitutions compared with the reference sequences for both variants (Figure 4). Although more amino acid substitutions occurred in the New Orleans

Figure 2

Neighbor-joining (Jukes–Cantor) phylogenetic tree of approximately 85% of the complete capsid gene nucleotide sequences and the Sydney 2012 (JX459908) and New Orleans 2009 (GU445325.2) reference sequences



The following isolates were analysed (sample collection day/month/year in parentheses): 11L21933716 (01/03/11),11L21933731 (18/03/11), 11L21934274 (21/03/11), 11L22826264 (16/12/11), 11L22841109 (23/12/11), 12L22935339 (20/01/12), 12L23035852 (24/02/12), 12L23035837 (24/02/12), 11L240026757 (18/12/12) and 10 Sydney 2012 isolates: 12L238022843 (15/10/12), 12L239031739 (17/11/12), 12L239031690 (19/11/12), 12L240001672 (03/12/12), 12L240001695 (03/12/12), 12L240002751 (04/12/12), 12L240002630 (04/12/12), 12L240006289 (05/12/12), 12L240034134 (22/12/12), 12L240034105 (23/12/12).

A very diverse New Orleans 2009 sequence is indicated by a black diamond.

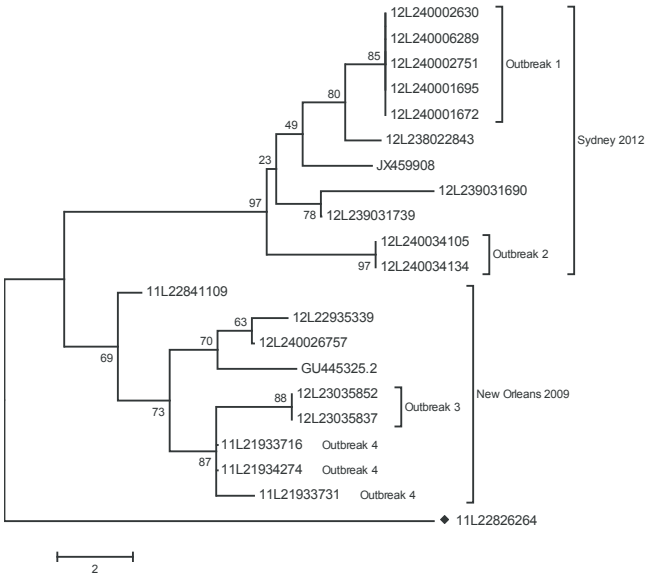
variants (five substitutions) than in the Sydney 2012 variant (three substitutions), the time interval from the first to the last New Orleans 2009 variant was 647 days, compared with only 68 days for the Sydney 2012 variant, showing that the Sydney 2012 variant is displaying a potentially diverse antigenic repertoire shortly after its emergence in Denmark. Interestingly, one of the New Orleans 2009 sequences (obtained just 17 days before the first Sydney 2012 variant was identified in Denmark) was found to be very distinct in both the phylogenetic analysis and alone accounted for two of the five amino acid substitutions in the antigenic sites of all New Orleans variants analysed (Figure 4). This could indicate the presence of a strong selection pressure on the 2009 New Orleans variants for functional variation to avoid accumulated herd immunity, while the newly introduced 2012 Sydney variant can circulate and diversify much more freely in the population.

Conclusion

In summary, we have documented the rapid emergence of the Sydney 2012 variant as the dominant NoV type in Denmark. Although it is still too early to predict whether the Sydney 2012 variant will replace the New Orleans 2009 variant, our analyses of the capsid gene demonstrate that this variant has the potential for strain replacement as it is rapidly diversifying within the Danish population. In addition, we present and demonstrate the successful use of new primers, which can amplify approximately 85% of the capsid (and the hypervariable P2) region. The primers can therefore be used to perform a detailed comparison of sequences and thereby assist in transmission tracking of the new 2012 Sydney variant.

Figure 3

Neighbor joining (number of differences) phylogenetic analysis of translated capsid sequences

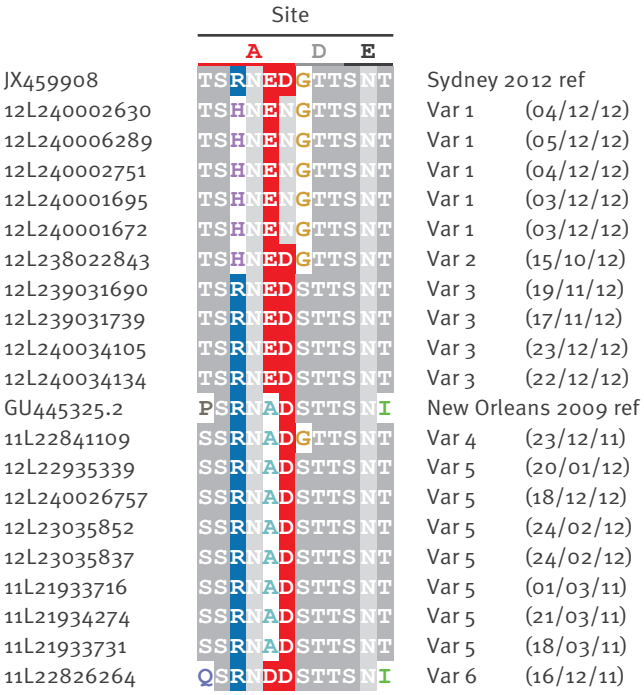


The following isolates were analysed (sample collection day/month year in parentheses): 11L21933716 (01/03/11),11L21933731 (18/03/11), 11L21934274 (21/03/11), 11L22826264 (16/12/11), 11L22841109 (23/12/11), 12L22935339 (20/01/12), 12L23035852 (24/02/12), 12L23035837 (24/02/12), 11L240026757 (18/12/12) and 10 Sydney 2012 isolates: 12L238022843 (15/10/12), 12L239031739 (17/11/12), 12L239031690 (19/11/12), 12L240001672 (03/12/12), 12L240001695 (03/12/12), 12L240002751 (04/12/12), 12L240002630 (04/12/12), 12L240006289 (05/12/12), 12L240034134 (22/12/12), 12L240034105 (23/12/12).

A very diverse New Orleans 2009 sequence is indicated by a black diamond.

Figure 4

Variation in the predicted antigenic blockade epitope sites (A, D and E) for the New Orleans 2009 and Sydney 2012 variants (var 1–6)



Observed epitope variants, sample collection dates (day/month/year) and reference sequences are indicated on the right of the figure. Positions of the epitopes are indicated relative to the translated capsid (VP1) New Orleans reference sequence (GU445325.2): A site (left to right): 294, 296, 297, 298, 368, 372; D site (left to right): 393, 394, 395; E site (left to right): 407, 412, 413.

*Addendum

An addendum for this article was published on 7 March 2013, listing the GenBank accession numbers for the capsid genes used in the phylogenetic analysis.

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Conflict of interest

None declared.

Authors' contributions

J Fonager: Conceived the idea for the study and performed the phylogenetic analysis and antigenic characterisations and drafted the first version of the paper, made revisions, and approved the final version of the paper. TK Fischer: Designed the project together with J Fonager, provided constructive comments and revised the first draft of the paper critically and approved the final version of the paper. LS Hindbæk: Contributed considerably with the laboratory analyses and approved the final version of the paper.

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Pet rats as a source of hantavirus in England and Wales, 2013

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We report the detection of a strain of Seoul hantavirus (SEOV) in pet rats in England and Wales. The discovery followed an investigation of a case of haemorrhagic fever with renal syndrome in Wales. Hantavirus RNA was detected via real-time reverse transcription-polymerase chain reaction (RT-PCR) and classic RT-PCR in pet rats belonging to the patient. Sequencing and phylogenetic analysis confirmed the virus to be a SEOV that is similar, but not identical, to a previously reported United Kingdom strain from wild rats.

In January 2013, a male patient in north Wales suffering from acute kidney injury and clinically presenting with haemorrhagic fever and renal syndrome, tested seropositive (IgG 1:10,000) for Seoul (SEOV) and Hantaan (HTNV) hantavirus using indirect immunofluorescence (Euroimmun, Germany). A previous blood sample from October 2012, taken for unrelated purposes, was retrospectively obtained and tested. This sample demonstrated no antibody to hantavirus thus confirming serconversion as defined by Heyman et al., 2007 [1]. Epidemiological assessment identified the patient's two pet agouti rats (*Rattus norvegicus*) as a possible source of the hantavirus.

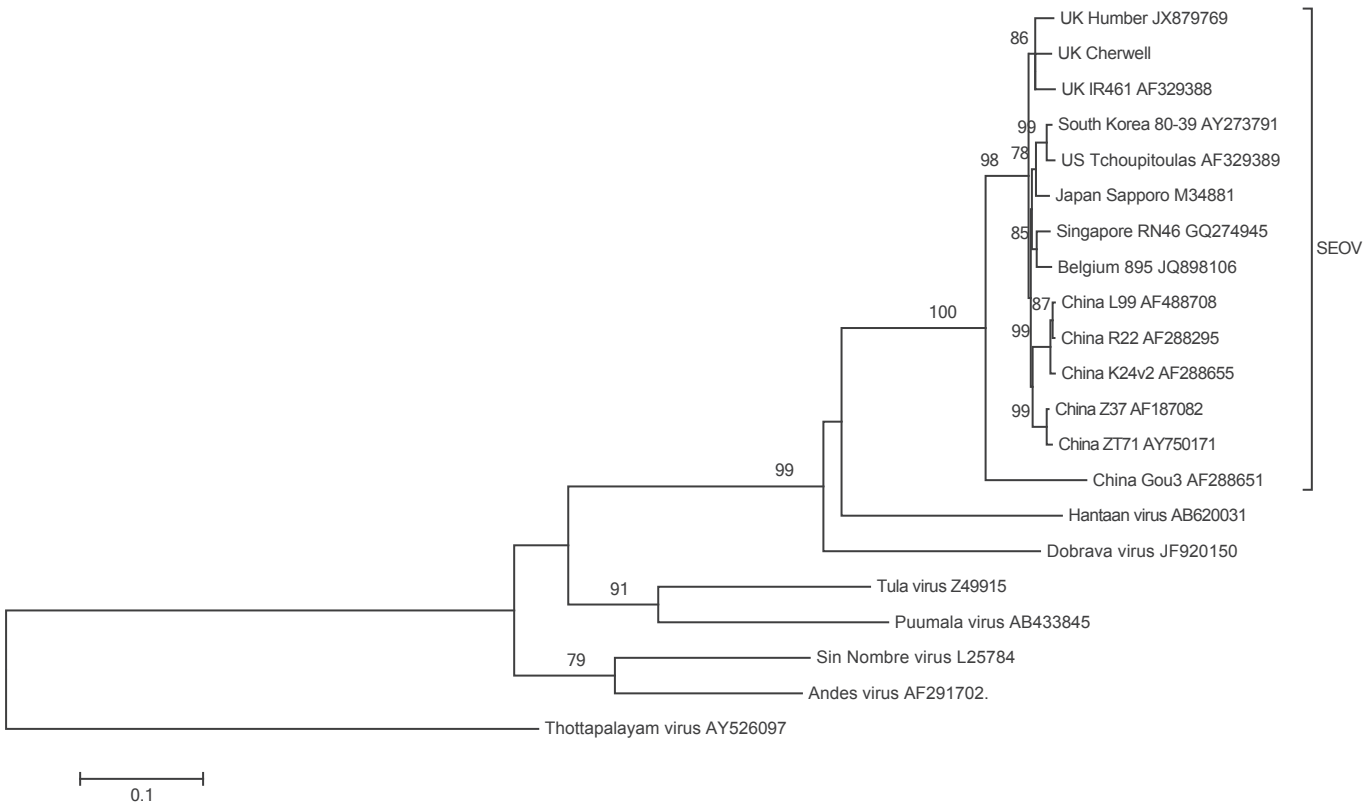
Virus investigation

Due to the patient's serious clinical condition the pet rats were in the care of the original breeder in Oxfordshire, south England. They were housed in a separate building to that of the breeder's pet rat colony. Blood samples from both rats, and urine from one of the two rats, were obtained and processed for RNA extraction using RNeasy kit (Qiagen). The extract was tested using a modified version of a previously published real-time reverse transcription-polymerase chain reaction (RT-PCR) assay for the dual detection of HTNV and SEOV [2]. The modifications adopted were: (i) use of a single Minor Groove Binder (MGB) -probe with a degenerate single base change (5' FAM-TCAATGGGRATACAACT-3') in place of the two non-degenerate published MGB-probes and (ii) use of the SuperScript III Platinum One-step qRT-PCR kit (Invitrogen) in accordance with the manufacturer's specifications. In-house validation confirmed that these changes had no detrimental impact on assay sensitivity and reduced the cost of the test.

The urine and blood samples tested positive and results were rapidly fed back to the incident control team, which included representatives from Health Protection Agency (HPA, Porton and Colindale), Thames Valley Health Protection Unit (HPU), Public Health Wales (PHW), Environmental Health (EH) and Animal Health and Veterinary Laboratories Agency (AHVLA). Due to uncertainties regarding the prevalence of this virus within the pet rat population in the United Kingdom (UK), the nature of its transmission, and the potential seriousness of human disease in this particular instance, the owner's consent was obtained to euthanase the two pet rats and remove them for further testing at the HPA Porton. One of the rats was processed as previously described [3] and viral RNA sourced directly from lung tissue was subjected to additional characterisation of the virus through standard Sanger sequencing on a 3130xl sequencer (Life Technologies). Sequencing of the S segment was achieved, confirming the virus was indeed a strain of SEOV similar, but not identical, to previously isolated UK SEOV strains: Humber (wild rats) [3] and IR461 (laboratory rats) [4]. We have provisionally designated this strain 'Cherwell'. Alignments of sequences from the virus strains were

Figure

Phylogenetic analysis of S segment sequence of the Seoul hantavirus Cherwell strain derived from a pet rat, England, February 2013



SEOV: Seoul hantavirus; UK: United Kingdom; US: United States.

Horizontal distances represent the number of nucleotide differences. Bootstrap confidence limits exceeding 70% are shown for each branch node. The phylogenetic tree was based on S segment sequences of previously published SEOV strains and other major hantavirus species as well as the sequence derived from the pet rat (*Rattus norvegicus*). The available geographical origin of the viral sequences, the viruses' names and respective GenBank accession numbers figure on the tree.

conducted using ClustalW and the molecular evolutionary genetics analysis (MEGA5) programme suite [5] was used to perform phylogenetic analysis. Comparisons between Cherwell and Humber S segments highlighted a total of 47 nucleotide differences, 36 within the open reading frame (ORF) resulting in one amino acid difference (methionine to isoleucine) at position 247. Phylogenetic analysis using the neighbour-joining method in MEGA5, with bootstrap values (2,000 replicates), placed the Cherwell S segment within the same group as the corresponding segments of Humber and IR461 (Figure). The sequence (1,769 nucleotides) was released to GenBank under accession number KC626089.

Following confirmation of hantavirus infection in these two rats, blood samples were obtained with the owner's permission, from 21 of the breeding colony rats and processed in the same way. Guidance was provided to the breeder to minimise the potential risk of infection while caring for the remaining rats, should they be positive. On the day of sampling, 7/21 rats had detectable RNA specific to HTNV/SEOV in blood. Preliminary sequence data indicates the same Cherwell strain in these rats.

Investigation of human contacts with rats

A blood sample was obtained from the patient's partner in Wales; this was negative for IgG antibodies. Blood samples were also obtained from the breeder and her spouse in England. The breeder, who had most contact with the rats in the breeding colony, had a low positive titre to HTNV and SEOV (IgG 1: 100). The breeder's spouse tested strongly positive with an IgG titre of 1: 10,000 to HTNV and SEOV, strongly suggesting hantavirus infection. Retrospective investigation of his medical records showed that he had been admitted to hospital in late 2011 with an undiagnosed viral illness resulting in acute renal impairment and thrombocytopenia. It is now considered highly likely that this was due to hantavirus infection; an archived blood sample from this admission was retrospectively tested

and demonstrated an IgG titre of 1: 1,000 to HTNV and SEOV. Detailed clinical findings of these cases will be published shortly [6]. In summary, of four people exposed to this particular population of rats, two had been clinically ill with renal impairment and were strongly seropositive, one had a low level of antibody with no clinical illness, and one was seronegative.

Control measures

Transmission of hantaviruses to humans most often occurs through breathing in aerosols of excreta from infected rodents [7]. Large quantities of infectious virus are intermittently excreted in the urine, saliva and faeces of infected rodents. The unique finding in this investigation of a strain of SEOV in pet rats, rather than wild rats, posed a challenge for infection control and involved a multi-disciplinary panel including medical/scientific experts from HPA, HPU, PHW, EH, and veterinarians from AHVLA. As part of this investigation the pet rats were euthanised, with owner's consent, in order to further scientific understanding of hantavirus infection in pet rats. Recommendations for management of any future incidents would be made on a case for case basis. Further studies are planned to gather evidence on the prevalence of this virus in the pet rat community which will inform future risk assessment and the provision of appropriate public health guidance. Interim guidance on minimising the infection to the pet rat community has been prepared [8] and will continue to be updated as the investigation progresses.

Discussion and conclusions

In January 2013, we reported the isolation of a UK strain of SEOV (Humber) from wild rats in north-east England [3]. We now report a second SEOV strain from the UK which is similar, but genetically distinct from the Humber isolate and laboratory-associated IR461 isolate. Further research will continue to investigate the relationship of these three strains. The pet rats identified in this investigation are part of a wider pet rat community which partakes in national and international shows and fosters international sharing of rats. Currently, the prevalence of SEOV in the UK pet rat community is unknown, but if SEOV infection was widespread, there would be implications for the wider (non-UK) pet rat community. The HPA is continuing to investigate this newly recognised source of hantavirus infection in collaboration with the AHVLA. Should overall findings indicate that further health protection advice is necessary, the HPA will work with the relevant partners to provide this.

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Conflicts of interest

None declared.

Authors' contributions

Lisa Jameson, Barry Atkinson and Roger Hewson were responsible for the virological analysis and the interpretation of laboratory results. Surabhi Taori, Jane Osborne and Tim Brooks coordinated the national public health response and were responsible for clinical diagnosis. Peter Levick, Charlotte Featherstone and Guda van der Burgt performed the blood sampling. Noel McCarthy and Judy Hart coordinated the local public health response. Amanda Walsh contributed to the national public health response and prepared public health guidance for dissemination. Lisa Jameson wrote the draft manuscript and all authors revised and approved the final version.

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A *Clostridium perfringens* outbreak traced to temperature-abused beef stew, Norway, 2012

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On 21 January 2012, the Norwegian Food Safety Authority was informed about gastrointestinal illness among 111 swimming club members, who were staying at a hotel in Trondheim. A hotel dinner on 20 January was their only common meal. Kitchen staff were interviewed, and food leftovers and kitchen environment were sampled. A case was defined as a swimming team member staying at the hotel from 20 to 22 January, who fell ill with diarrhoea, abdominal pain or nausea during this period. A total of 43 cases were identified, with median duration of symptoms of 35 hours. *cpe*-positive *Clostridium perfringens* (3.8 x 10^8 CFU), but not *Bacillus cereus*, was isolated from beef stew eaten by cases. *cpe*-negative *C. perfringens* was detected in a sample from the kitchen floor. SDS-PAGE showed indistinguishable protein profiles among *C. perfringens* cultures isolated from the beef stew, but slightly different profiles from the culture isolated from the kitchen floor. Cohort analysis showed that eating beef stew and rice was significantly associated with illness. No pathogens were detected in the rice. The temperature control of the stew, but not of the rice, was poor. Our results strongly indicate that cases were infected by *Clostridium perfringens* in beef stew that had inadequate temperature control during preparation.

Outbreak description

On the evening of 21 January 2012, the Norwegian Food Safety Authority, district office of Trondheim and Orkdal, was alerted to an outbreak of gastrointestinal disease among participants from several swimming clubs attending a swimming competition in Trondheim. Initial information was passed on in a private conversation between a swimming coach and an employee of the Food Safety Authority, who happened to be present at the swimming competition. Symptoms had started early that day. In response, the Food Safety Authority conducted a few enquires the same evening, which showed that the sick swimmers had stayed at the same hotel and that dinner at this hotel the previous evening (20 January) was the only known meal common to all the swimming teams, clearly indicating a probable food-borne outbreak associated with this meal. The Food Safety Authority launched an investigation, according to standard procedures, aimed at preventing possible continuation of the outbreak, describing the outbreak, identifying the source and causal agent, and if possible advise on preventive measures. Initial information allowed further investigations to focus on factors specifically associated with the suspected hotel dinner. We describe here the investigation and results of what turned out to be among the largest *Clostridium perfringens* outbreaks reported in Norway, adding to the current evidence base of *C. perfringens* outbreaks.

Outbreak population data

A total of 111 people from six swimming clubs from different parts of Norway, including swimmers, coaches and tour leaders, aged 12–55 years, stayed at the hotel from Friday 20 to Sunday 22 January, while they attended a swimming competition in Trondheim that weekend. In addition to the swimming team members, approximately 50 other guests stayed at the hotel that weekend. For those other guests, information on food consumption and possible illness was not available, and they were therefore not included in the outbreak investigation. Consequently, the outbreak population was defined as all members of the six swimming teams staying at the hotel from 20 to 22 January.

Members from all six swimming teams had dinner at the hotel on 20 January at approximately 9 p.m., whereas members from only two of the swimming teams had lunch at the hotel that day. Except for these meals at the hotel, the members from the six teams had no other known common meal or other common contacts, neither during 20 January, nor during the preceding month.

Case definition

An outbreak case was defined as a member of a swimming team staying at the hotel from 20 to 22 January, who fell ill with diarrhoea, abdominal pain or nausea during this period.

Kitchen inspections and guidance

Food control officers inspected the hotel kitchen several times. On 23 January, the number and group affiliations of guests and list of hotel meals eaten were collected, and food control officers observed the basic hygiene status of the kitchen. Kitchen staff were asked to describe all dishes served at the dinner on 20 January and all relevant procedures for preparing and handling of foods served at this meal. The investigation specifically addressed factors associated with the growth of spore-forming pathogens in foods: *C. perfringens* and *Bacillus cereus* – such as time and temperature aspects of chilling and reheating, and temperature during serving – because preliminary descriptions of the symptoms and outbreak setting provided reasons to suspect one of these pathogens as the causative agent. On this inspection, available food leftovers from the dinner – beef stew and boiled rice – were sampled. During a second inspection of the kitchen on 26 January, more detailed data on food handling were collected, and the kitchen was swabbed. On meeting with the kitchen manager on 31 January, preliminary microbial results were presented and control officers gave guidance on hygiene, food safety and preventive measures.

Background

C. perfringens is a spore-forming bacterium widely distributed throughout the environment, which may cause food-borne disease [1]. *C. perfringens* enterotoxin (CPE) encoded by the *cpe* gene is the major virulence factor, causing tissue damage of intestinal epithelial cells in an infected person and leading to self-limiting diarrhoea and abdominal pain as main symptoms. The incubation period is 6–24 hours (usually 10–12 hours) [2]. The duration of illness is mostly reported to be a maximum of 24 hours. Longer duration has been reported from at least one outbreak (mean: 2.3 days; range: 1–10) [3]. The infective dose is estimated to be 10^6–10^7 cells [1,4].

Vegetative cells of *C. perfringens* grow between 15 °C and 50 °C, with optimal growth around 43–45 °C [1]. The generation time may be as low as 7–8 minutes under optimal growth conditions [5]. *C. perfringens* spores in food will survive boiling. If food handling includes long time intervals at temperatures permitting rapid growth, the content of *C. perfringens* may rise to a level that causes risk of food-borne infection.

The majority of environmental *C. perfringens* strains are *cpe*-negative [6]. *cpe*-positive *C. perfringens* strains are shown to produce spores with a higher heat resistance [1] and will therefore be selected for in kitchen environments. For these reasons, food-borne outbreaks due to *C. perfringens* are most often associated with foods subjected to poor temperature control, produced in commercial kitchens [1,7], as demonstrated by reported outbreaks [8,9]. Direct or indirect person-to-person faecal–oral transmission is not considered to be an important transmission route [1,2].

C. perfringens is traditionally considered to be a frequent cause of food-borne infection in Norway [4] and other industrialised countries [1], but as the symptoms are usually mild, outbreaks are often not reported. Among 242 food-borne outbreaks with a recognised causal microbial agent reported in Norway during 2005 to 2011, eight outbreaks (3%) were caused by *C. perfringens*, of which the largest included 45 cases (personal communication, B. Heier, Norwegian Institute of Public Health, 4 May 2012).

Methods

Sampling and microbiological analysis

When the hotel kitchen was inspected on 23 January, the only available leftovers from the dinner on 20 January were beef stew and boiled rice: these were sampled. After the meal, these leftovers had been stored at room temperature for a few hours (information on the exact time was not available) and then placed in a refrigeration room at 4 ºC. Samples of leftovers were analysed for *C. perfringens* and B. cereus, for the reasons stated above. Analysis for detection of C. pefringens was performed as described by the Nordic Committee on Food Analysis (NMKL) (anaerobic cultivation at 37 °C for 24 hours on membrane *C. perfringens* (m-Cp) agar and blood agar) [10]. Analysis for detection of B. cereus was performed as described by NMKL (aerobic cultivation on blood agar at 37 °C for 24 hours) [11].

In order to asses pathogenic potential of the *C. perfringens* flora, four isolated *C. perfringens* cultures were selected arbitrarily from primary culture plates and further analysed by PCR, for detection of *cpe*. Colonies were isolated using AVDAMAX beads (Edge Bio, Gaithersburg, United States), according to the manufacturer's protocol. DNA was suspended in 10 mM Tris-HCl at pH 7.5–7.8. Two primers: CAAGTCAAATTCTTAATCCT and CATCACCTAAGGACTGTTCT were used. Amplification was carried out with initial denaturation at 95 °C (3 min.) and then 30 cycles with 1 min. at 92 °C, 1 min. at 50 °C, and 1 min. at 72 °C, and finally 7 min. at 72 °C.

For the purpose of source tracing and characterisation of the general load of *C. perfringens* in the kitchen environment, 20 points on the surfaces of walls, floors, working desks and equipment in the kitchen were sampled by swabbing on 26 January. Some of the sampling points had been washed before sampling. The swabs were smeared on blood agar plates. Protocols for detection of *C. perfringens* by incubation on blood agar and for PCR detection of *cpe* were identical to those described above.

The protein profile of four selected cultures of *C. perfringens* isolated from the beef stew and one culture isolated by swabbing the kitchen were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [12]. After incubation of the cultures in lactose broth at 37 ºC in 12 hours and centrifugation at 13.000 rpm at 5 min., supernatant was precipitated

with 80% $(NH_4)\ ^2SO_4$. The profiles were compared by visual inspection.

Epidemiological investigation

A self-administered questionnaire was prepared, including questions on demography, consumption on food items eaten during dinner at the hotel 20 January, as well as illness and symptoms with onset between 20 and 22 January. Food items included in the questionnaire were based on a list supplied by the hotel kitchen manager. The questionnaire was distributed by email on 24 January, via contact persons for each swimming team, to all the 111 members who had stayed at the hotel during the competition in Trondheim. For children aged under 16 years, the questionnaires were addressed to their parents, and they were asked to assist in answering the questions. One week later, the contact persons were asked to remind those who had not yet responded. From the returned questionnaires, basic descriptive epidemiological parameters were investigated and a cohort analysis was performed.

Results

Details of hotel dinner and food storage

The hotel dinner on 20 January was served from 6 p.m. to 9.30 p.m. as a buffet with six cold dishes (cheese, ham, cured meat sausage, liver paste ('leverpostei'), green salad and bread) and two hot dishes (beef stew and rice). The rice had been boiled immediately before the meal, whereas the beef stew had been prepared the day before, cooled to room temperature and placed in a refrigerated room at 4 °C overnight and reheated before serving. During cooking, chilling, storage, reheating and service, the stew was kept in trays containing approximately 15 L. The duration of storage and temperature of the food during cooling, reheating and serving of the stew had not been recorded. The kitchen had no clear procedures for control of these aspects of food handling, and could not provide data on relevant temperature tests to validate general procedures.

Microbial analysis

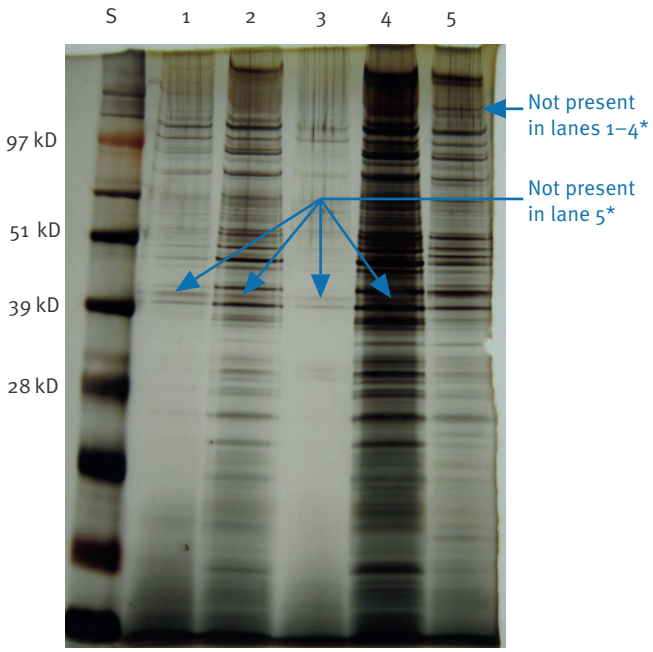
From the beef stew, 3.8 x 10^8 colony-forming units (CFU) of *C. perfringens* per gramme of food was found; B. cereus was not detected. Further investigation of cultures from four selected *C. perfringens* colonies isolated from beef stew showed the presence of *cpe* in all these cultures. Neither B. cereus nor *C. perfringens* was detected in the rice.

Among 20 swab samples taken from different parts of the kitchen, *cpe*-negative *C. perfringens* was detected in one culture from the floor; the remaining 19 samples were negative. *cpe*-positive *C. perfringens* was not detected in any of the samples from the kitchen environment.

From four *cpe*-positive *C. perfringens* cultures isolated from the stew, SDS PAGE analysis showed an indistinguishable protein profile among these cultures, whereas the profile of the single *cpe*-negative *C. perfringens* culture isolated from the sample from the kitchen floor showed several protein bands not present in the profile from the cultures isolated from the stew (Figure).

Figure

SDS-PAGE protein profiles from *Clostridium perfringens* cultures isolated from beef stew and kitchen environment, Trondheim, Norway, 2012



SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

* Protein bands representing differences between lanes 1–4 versus lane 5.

Gel from SDS-PAGE protein profile analysis of four *Clostridium perfringens* colonies isolated from beef stew with indistinguishable profiles (lanes 1–4) and one *C. perfringens* isolated from the kitchen environment with a different profile (lane 5). Lane S shows the reference marker: SeeBlue Plus2 Pre-Stained Standard, Invitrogen.

Descriptive epidemiological results

Of the outbreak population comprising 111 individuals, 61 (55%) responded and returned the questionnaires. The median age of the respondents was 16 years (range:12–55). A total of 43 respondents met the case definition, giving an attack rate of 70% (43/61) among the respondents.

The median age of the cases was 16 years (range: 12–47). The median incubation period (counting from the start of the dinner on 20 January, at 9 p.m.) was 10 hours (range: 3–28 hours). The most frequent

Table

Relative risk of gastrointestinal disease among questionnaire respondents (n=61), by food exposure at hotel dinner, outbreak of *Clostridium perfringens* infection, Trondheim, Norway, 2012

Food item	Exposed		Unexposed		Relative risk	95% CI
	Number of cases who ate food item/total	Attack rate (%)	Number of cases who did not eat food item/total	Attack rate (%)		
Beef stew	42/47	89	1/14	7	12.51	1.89–82.91
Rice	41/49	84	2/12	17	5.02	1.41–17.90
Green salad	28/40	70	15/21	71	0.98	0.70–1.37
Bread	30/46	65	13/15	87	0.75	0.56–1.01
Cured meat sausage	3/6	50	40/55	73	0.69	0.30–1.55
Ham	5/17	29	38/44	86	0.34	0.16–0.72
Cheese	6/21	29	37/40	93	0.31	0.16–0.61
Liver paste	1/5	20	42/56	75	0.27	0.05–1.55

symptoms were diarrhoea and abdominal pain: both were reported from 39 of the 43 cases, followed by nausea (n=31), headache (n=15) and vomiting n=5). The median duration of symptoms was 35 hours (range: 8–96). No cases reported having consulted a physician. A total of 10 cases reported to have withdrawn from scheduled participation in swimming events because of the illness, and 10 other cases reported having participated in all the swimming events as scheduled, but with lower performance than expected, due to the illness.

Cohort analysis

Cohort analysis of the 61 respondents showed that both hot dishes served at the hotel dinner on 20 January – beef stew (relative risk (RR): 12.51; 95% CI: 1.89–82.91) and rice (RR: 5.02; 95% CI: 1.41–17.90) were significantly associated with illness. One case did not eat beef stew and two cases did not eat rice. Among 47 persons who ate the stew, all but one also ate rice. For all six cold dishes served at the dinner, the RR was ‹1 (Table).

Food safety and public health action

In accordance with legislation and normal procedures of the Norwegian Food Safety Authority, the hotel kitchen management was advised to implement routines to prevent similar incidents, including procedures for control of time and temperature during food handling, cleaning procedures, and to ensure that these routines were understood and followed by all staff.

Discussion

This outbreak of 43 cases of gastrointestinal disease, occuring within a period of 25 hours among the swimming team members at the hotel, strongly suggested a common source and possible food-borne outbreak. The setting – a communal meal prepared in a commercial kitchen [1] – clinical symptoms and incubation period [2] were typical of *C. perfringens* infection. *C. perfringens* in high numbers, exceeding the assumed infection dose, was isolated from beef stew eaten by all cases but one. The presence of *cpe* and indistinguishable protein profiles among all four of the isolated *C. perfringens* cultures that were tested indicated that the microbial flora of the stew was dominated by a single *cpe*-positive *C. perfringens* strain. These findings together strongly indicated *C. perfringens* as the causative agent. Conditions in which the leftovers were stored after the dinner and before sampling (three days after consumption) would have allowed further growth of *C. perfringens*. Therefore, the concentration and heterogeneity of *C. perfringens* in the stew at the time of consumption is unknown, but considering the short generation time under optimal conditions, the concentration probably increased substantially during preparation and handling before consumption.

Since none of the cases visited a physician, no stool samples were taken. Detection of *C. perfringens* in stool samples from one or more cases might have supported the identification of the causative agent.

The univariate cohort analysis demonstrated significant association between disease and eating beef stew. The only exposure other than consumption of stew showing a RR ›1 was consumption of rice. Preparation and handling of the stew before serving was not satisfactory, providing conditions (time and temperature) that permitted rapid growth of *C. perfringens*, whereas for the rice, this was not the case. Reheating before serving would not have killed the bacterial spores. The pathogenic bacteria were found in high concentrations in the stew but not in the rice. These findings strongly suggest that the beef stew was the source of the pathogens. A RR ›1 for the rice in the univariate analysis can

be explained as a confounding effect, attributed to the fact that all persons but one who ate the stew also ate the rice, and is therefore compatible with a hypothesis of the stew as source of the pathogens. Had a multivariate analysis been performed, confounding effects might have been better clarified. Due to lack of resources, however, this was not performed.

The case definition was chosen not to include vomiting. Only five of those who were ill reported vomiting, which is consistent with reports of other authors, thus we regard this as a variable rare symptom of *C. perfringens* infection [1]. Inclusion of vomiting in the case definition would not have altered the epidemiological results and conclusions substantially.

The symptoms experienced by the cases were mild (mainly diarrhoea, abdominal pain and nausea) and the median incubation period was 10 hours. These clinical observations correspond well with the typical characteristics of *C. perfringens* infection [2]. The median duration of symptoms (35 hours; range 8–96), however, deviates from the typical duration of symptoms for *C. perfringens* infection – generally assumed to be less than 24 hours [1,2]. Duration of symptoms exceeding 24 hours has been described in one other outbreak of *C. perfringens* infection, in which the median duration was reported be to two days [3]. Elderly or immunosuppressed persons may experience longer duration of symptoms [13]. Among the cases in the outbreak described here, there was, however, no indication of immunosuppression – on the contrary, most cases were young athletes, assumed to be in good health condition. We cannot envisage other specific factors that could explain the unusually long duration of symptoms among the cases in this outbreak. The clinical data in our study were self reported, subject to individual judgment and possible recall bias, and should therefore be considered with caution. We consider it however unlikely, that the long duration of symptoms observed in our study can be attributed exclusively to bias. Thus our findings and the report from Eriksen et al. [3] suggest that the normal range for duration of symptoms for *C. perfringens* infection among the general population should be considered to exceed 24 hours, possibly up to two or three days.

No samples from the kitchen environment contained *cpe*-positive *C. perfringens* and only one contained *cpe*-negative *C. perfringens*. Thus the sampling did not reveal any substantial reservoir of *C. perfringens* spores in the kitchen. Furthermore, as the kitchen had been washed before sampling, the absence of *cpe*-positive *C. perfringens* in the samples cannot be considered fully representative of the status of the kitchen environment during preparation of the meal. The sampling from kitchen environment gave therefore no indication of the mode or source of transmission for the contamination of the beef stew.

When inferring epidemiological data from respondents to the whole outbreak population, representativeness should be considered. We did not have data on age and sex of non-responders, but if we assume that the attack rate among all 111 swimming team members was the same as that among the 61 respondents (70%), the total number of cases would have been as high as 77. However, as those affected by food poisoning are more likely to respond to a questionnaire than those who are healthy, such assumption may lead to an overestimation. The response rate of 55% may be modest, but we consider that it did not substantially undermine the clear conclusions derived from the descriptive data and cohort analysis. And if all non-respondents were healthy, the attack rate among all swim team members would still have been high (at 39%). Counting only the 43 reported cases, this outbreak still ranks among the largest outbreaks of *C. perfringens* infection ever reported in Norway.

A large proportion of outbreaks of *C. perfringens* infection are probably never recognised or reported. Several factors contributed to the recognition and elucidation of different aspects of this outbreak. Firstly, initial information about a possible outbreak was reported to the Food Safety Authority by coincidence. Since none of the cases consulted a physician, it is doubtful whether authorities would have been informed about the outbreak in any other way. Had the Food Safety Authority not been alerted, this outbreak would have been undetected. Secondly, the outbreak occurred among swimming teams, facilitating case finding and collection of information from those staying at the hotel. Thirdly, food leftovers were available and sampling led to detection of the presumed causative agent.

The strengths of this investigation are: (i) findings of a homogenous strain of *cpe*-positive *C. perfringens* in high concentration in beef stew eaten by most cases; (ii) high and significant association between disease and consumption of beef stew in the cohort analysis; and (iii) reports of suboptimal handling of beef stew permitting growth of *C. perfringens* before serving. Several limitations must however also be acknowledged – these include: (i) a modest response rate in the epidemiological investigation among swimming team members and possible recall bias; (ii) only univariate analysis was carried out in the cohort study and therefore no adjustment could be made for possible confounding factors; (iii) food leftovers were not sampled until three days after consumption; (iv) lack of detection of the outbreak *C. perfringens* strain in the kitchen environment; and (v) there were no stool samples from cases.

The food safety and public health action carried out was in accordance with the procedures of the Norwegian Food safety Authority for dealing with food-borne outbreaks. Due to the high infective dose, person-to-person transmission is assumed to be only a theoretical possibility for *C. perfringens*, compared with that

for many other important food-borne pathogens. There was therefore no need for specific measures to prevent secondary infections among cases' close contacts.

In conclusion, the outbreak described is one of the largest *C. perfringens* outbreaks reported in Norway. Although the incubation period and symptoms were typical for *C. perfringens* infection, the duration of the symptoms was markedly longer in this outbreak compared with that described in most reports, suggesting that the range for duration of symptoms for *C. perfringens* infection should be reconsidered.

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The number of deaths among infants under one year of age in England with pertussis: results of a capture/recapture analysis for the period 2001 to 2011

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Pertussis activity in England in 2012 was at its highest level for more than 12 years, leading to an increased number of deaths, especially among infants who were too young to be vaccinated. To support decision making on the introduction of maternal immunisation as an outbreak response measure to prevent these early deaths, we analysed reported deaths amongst infants of less than one year of age during the period from 2001 to 2011 with a capture/recapture analysis. We used log linear regression to allow for interactions. Reported deaths were obtained from the Hospital Episode Statistics for England, death registered by the Office of National Statistics and the enhanced surveillance of laboratory-confirmed pertussis conducted by the Health Protection Agency. There were a total of 48 deaths recorded; of these 41 had a disease onset before being fully protected by vaccination. Around half of these deaths (23) were recorded in all three datasets and 10 in only one. Due to the high coverage of the datasets the estimated number of deaths missed was small with 1.6 (95% confidence interval (CI): 0.5–4.5) deaths. The total average incidence was 0.721 (95% CI: 0.705–0.763) per 100,000 maternities. We concluded that under ascertainment of deaths from diagnosed pertussis cases is small.

Introduction

An increase in laboratory-confirmed cases of pertussis in England and Wales was observed from the third quarter of 2011, predominantly in adolescents and adults. This increase continued into 2012, leading to the highest recorded disease levels for more than 12 years [1], and extended into infants under three months who are at highest risk of severe complications, hospitalisation and death. This recent rise may partly be due to increased awareness amongst health professionals, leading to an improved ascertainment of cases. The reported rise in young infant cases, however, for whom ascertainment is more consistent through time, is likely to be indicative of a true increase in disease transmission. A national outbreak was declared in April 2012 [2] to initiate a nationally coordinated response to the ongoing increased pertussis activity, primarily to minimise further infant hospitalisations and deaths.

Increased pertussis activity has also been reported in other countries in recent years [3–5]. The reasons for these recent increases are not yet fully understood and a number of factors may be important. Improved ascertainment, the replacement of whole cell with acellular vaccines [6], genetic changes in *Bordetella pertussis* and waning protection have all been implicated [7,8].

Increased mortality among infants who are too young to be fully vaccinated would be expected in association with a true rise in pertussis activity; in England and Wales this has been observed in 2012 with 13 deaths in infants with laboratory-confirmed pertussis reported by the end of November, exceeding the highest annual number of deaths in the last 10 years. As an outbreak control measure, the Departments of Health in the United Kingdom (UK), has recommended pertussis-containing vaccine for pregnant woman at 28 to 38 weeks gestation. Immunisation in pregnancy aims to induce passive immunity in the neonate through transplacental transfer of antibodies in the last trimester. It would also contribute to a cocooning effect by preventing disease in the mother, thereby indirectly protecting the infant against pertussis infection from birth.

To inform decision making regarding the introduction of such a programme, it is key to estimate the likely number of deaths to be prevented. The first step in this is to understand the completeness of ascertainment of pertussis-attributable deaths. It has previously been shown using the capture/recapture approach over the period from 1994 to 1999 that fatal pertussis cases were under-ascertained in England using the routine data sources, namely; laboratory-confirmed cases, deaths registered by the Office of National Statistics (ONS) and the Hospital Episode Statistics (HES) [9]. However over the last decade there might have been changes in recording and diagnostic practice. We therefore repeated the capture/recapture analysis for the period from 2001 to 2011.

Methods

Data sources

Deaths due to pertussis were extracted from three different datasets: the Hospital Episode Statistics (HES), the Office of National Statistics (ONS) and the enhanced surveillance (ES) of laboratory-confirmed pertussis conducted by the Health Protection Agency (HPA) Immunisation, Hepatitis and Blood Safety Department (IHBSD). The ES is the only system that is based on laboratory-confirmed cases only; in the HES and ONS datasets cases may be clinically confirmed.

The HES dataset holds all records for hospitalised patients in England, including detailed information on diagnosis, operative procedures and discharge method. Patients with ICD10 code (the Tenth revision of the International Statistical Classification of Diseases and Related Health Problems: ICD-10) indicating whooping cough (codes beginning with A37) and an indication of death (recorded as method of discharge = 4 and/or discharge destination = 79) were extracted.

The ONS mortality data is a record of all deaths in England and Wales. For each death there is a death certificate which records personal details and cause of death recorded by ICD-10 completed by the treating medical doctor or a coroner. Certificates recording the ICD-10 code beginning with 'A37' in the field 'underlying cause of death' were extracted.

The ES system managed by the HPA IHBSD is based on all cases of pertussis that have been laboratory confirmed by the HPA Bordetella Reference Laboratory and/or reported to the HPA by a National Health Service (NHS) diagnostic laboratory in England and Wales. Confirmed cases are followed up by a surveillance questionnaire to the general practitioner to obtain information on vaccination status, hospitalisation and mortality. All deaths following a diagnosis of pertussis held on this database were extracted.

Matching

Cases in the three datasets were matched on sex, date of birth, name and date of death. Not all data items were available in all three datasets but each case could be unambiguously assigned to a matched or non-matched status.

Statistical analysis

Due to limitations in the availability of HES data, the analysis was restricted to the years from 2001 to 2011 and only performed for deaths recorded in England. The number of maternities in England for those years was based on the recorded maternities in England and Wales [10] (2001–2011), assuming that 95% of them were in England (based on the distribution in 2010). The analysis was performed for deaths in pertussis cases aged under one year, under six months and under 66 days. The ‹66 day cut-off excluded any infant potentially protected by at least one dose of pertussis-containing vaccine on the assumption that the earliest age at which the first dose is given is eight weeks (56 days) and that a minimum of 10 days is required to derive any vaccine-induced protection (56+10=66 days). Log linear Models were fitted to the data [11], and two-way

Table 1

Annual deaths due to pertussis in infants aged under one year and proportion captured by three surveillance systems, England, 2001–2011 (n=48)

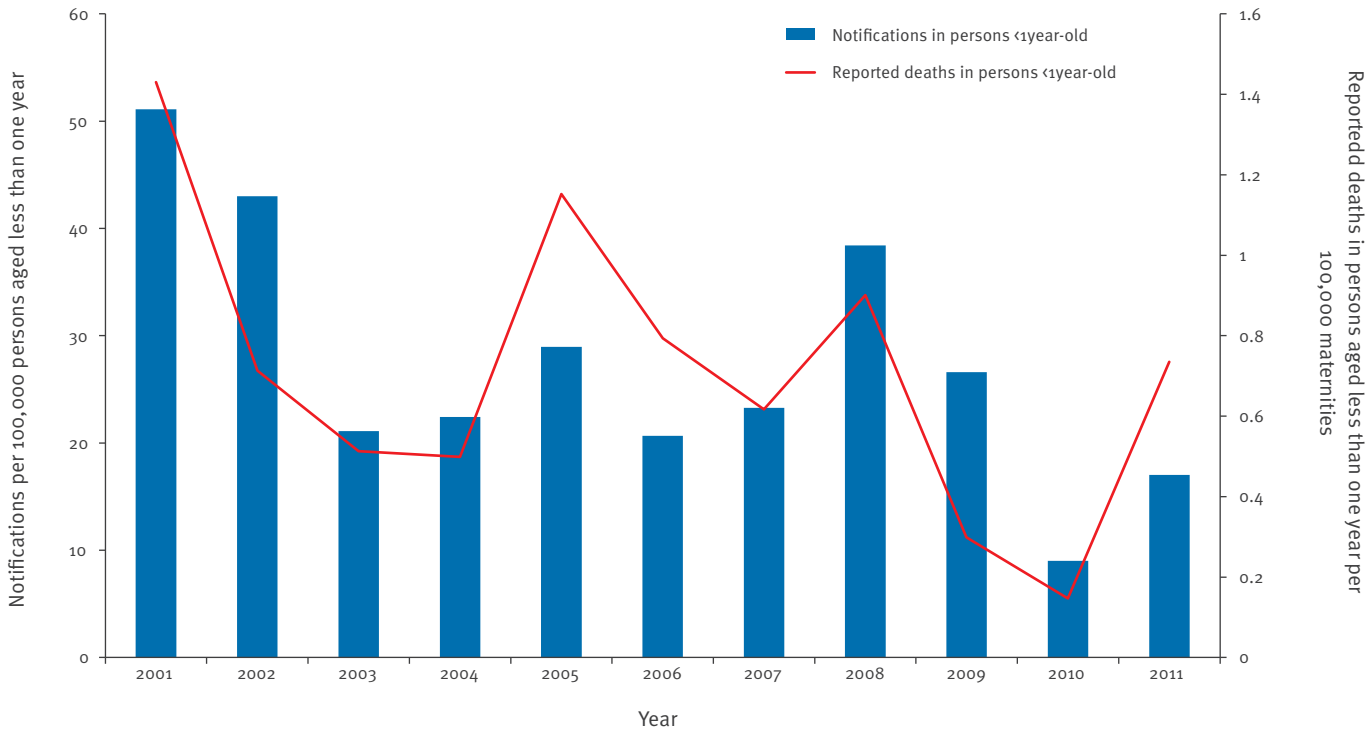
	Total	Office of National Statistics	Enhanced Surveillance[a]	Hospital Episode Statistics
2001	8	5	6	5
2002	4	4	1	2
2003	3	2	0	2
2004	3	3	3	3
2005	7	5	7	4
2006	5	1	4	3
2007	4	4	4	4
2008	6	6	6	5
2009	2	2	2	2
2010	1	1	1	1
2011	5	3	5	3
Total	**48**	**36**	**39**	**34**

The Enhanced Surveillance[a], Hospital Episode Statistics and Office of National Statistics are three surveillance systems capturing deaths due to pertussis.

[a] Coordinated by the Health Protection Agency.

Figure 1

Reported pertussis deaths in England and notifications in England and Wales for persons aged less than one year per 100,000 maternities and 100,000 persons under one year-old respectively, 2001–2011



interactions between the terms were investigated. Confidence intervals (95% CI) were obtained by profile likelihood. All analyses were performed in R 2.14.1.

Results

There were 48 deaths in infants under one year of age identified in at least one dataset between 1 January 2001 and 31 December 2011; 23 (46%) were in females. The number of deaths fluctuated between years, with the highest number in 2001 and the lowest in 2010 (Table 1). The annual mortality reflected the cyclical pattern in pertussis incidence in under one year-olds (Figure 1 and [12]). The distribution by age at death shows a clustering in the first two months of life (Figure 2) with 36 deaths occurring at this age; and of the 11 cases who died between two and six months of age, five had onset or specimen date before 66 days giving a total of 41 deaths (36+5) in infants too young to be protected by a first dose of vaccine. Vaccination history was obtained for three of the remaining seven deaths; only one infant was vaccinated, nine days before specimen date.

The HPA ES recorded 39 deaths, ONS recorded 36 and 34 were recorded in HES, however almost half of the deaths were recorded in all three datasets (23/48) and only 21% were recorded in just one (6 in ES, and 2 in ENS and HES respectively) (Figure 3). Due to the low number of cases recorded in only one dataset the estimated number of missed deaths was low. The interaction between the HES and ONS databases was significant (p=0.03, and 0.039 respectively) for all under one year-olds and all under six months of age; this interaction was therefore included for these two groups.

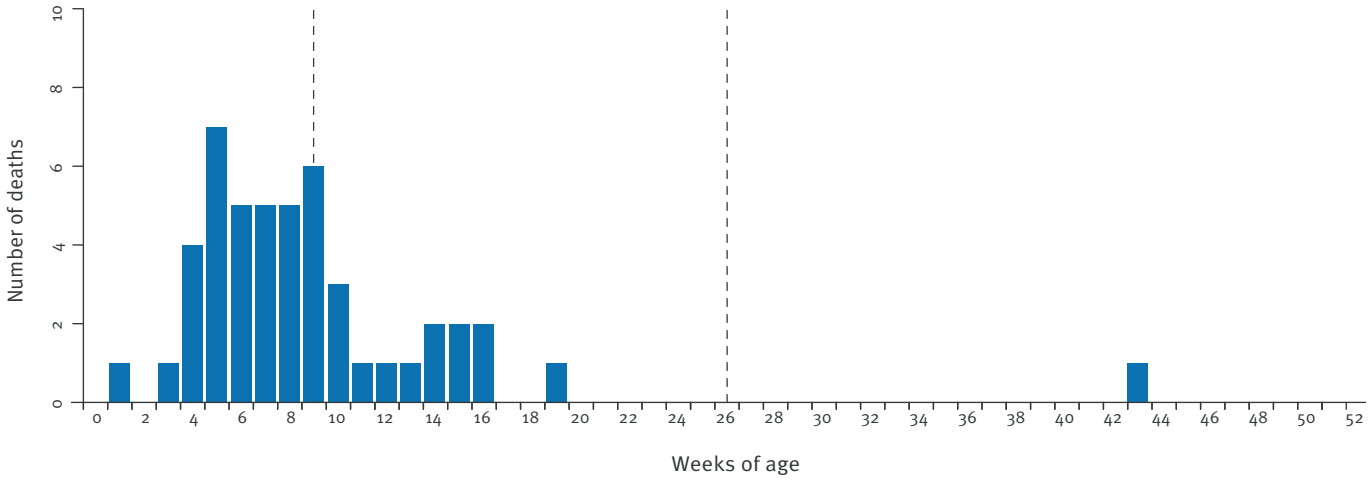
The total number deaths that were missed was estimated as 1.6 (95% CI: 0.5–4.5) for under one year-olds, 1.7 (95% CI: 0.5–4.7) for those under six months of age and 0.4 (95% CI: 0.1–1.2) for those who died before reaching the age of 66 days (Table 2) over the full period. The estimated average risk of death including the missed cases was 0.721 per 100,000 maternities for all under one year-olds, 0.708 for all under six months of age and 0.601 for those under 66 days of age (including all those with known onset before 66 days). Expressed as an annual average number of deaths in England and Wales over the period 2001 to 2011 and based on the 716,040 maternities for England and Wales in 2011 [1], it is estimated that there were 5.2 (95% CI: 5.0–5.5) deaths in under one year-olds, 5.1 (95% CI: 4.9–5.4) deaths for those <6 months of age and 4.3 (95% CI: 4.3–4.4) for those aged under 66 days.

Discussion

Our study covered a period during which notified pertussis incidence was at an all time low, with sustained high vaccine coverage for the infant course, currently

Figure 2

Number of reported pertussis deaths in infants aged less than one year, by age at death, England, 2001–2011 (n=48)

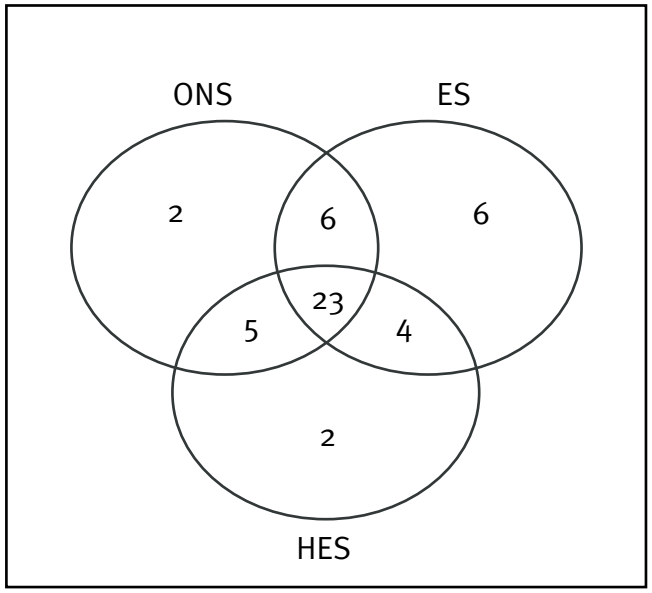


The dotted lines indicate the ages of 66 days and six months respectively.

The 66 day cut-off is the earliest time an infant can be potentially protected by at least one dose of pertussis-containing vaccine on the assumption that the earliest age at which the first dose is given is eight weeks (56 days) and that a minimum of 10 days is required to derive any vaccine-induced protection (56+10=66 days).

Figure 3

Venn diagram of the number of reported deaths due to pertussis in infants less than one year-old for three datasets obtained by three respective surveillance systems, England, 2001–2011 (n=48)



ES: Enhanced Surveillance coordinated by the Health Protection Agency; HES: Hospital Episode Statistics; ONS: Office of National Statistics.

The ES, HES and ONS are three surveillance systems capturing deaths due to pertussis.

at 93% by first birthday, and a pre-school booster included in the programme since 2001. Yet, an average of five deaths a year due to pertussis were estimated in England and Wales, the majority in infants too young to have received protection from a first dose of vaccine. The annual number of deaths fluctuated depending on whether it was a peak year reflecting the vulnerability of very young unvaccinated infants to changes in pertussis transmission [12]. The highest annual number of deaths was in 2001 (8 cases) which has been exceeded in 2012 with 13 deaths reported by the end of November: a reflection of the large increase in pertussis transmission since the third quarter of 2011.

Our analysis shows that ascertainment of deaths in infants with diagnosed pertussis is fairly complete when using information from the three available data sources. However, there are limitations in using known fatal cases to estimate pertussis-attributable mortality. Inevitably, the analysis does not include deaths that are not diagnosed or registered as being related to pertussis. While this number cannot be estimated in our study, it was found that even when the infant has laboratory-confirmed disease the death certificate may not mention pertussis (10 of 39 laboratory-confirmed cases, Figure 3). This confirms an earlier study in which only two of seven infants with notified pertussis who died from the acute illness respiratory complications had pertussis recorded on the death certificate [13]. Pertussis has also been implicated in cases of sudden infant death syndrome, although the relationship remains speculative [14,15]. Of the 48 deaths in

Table 2

Capture/recapture analysis of reported deaths due to pertussis in infants less than one year-old, England, 2001–2011 (n=48)

Age at death	Total observed deaths	Estimated deaths missed n (95% CI)	Total corrected deaths n (95% CI)	Total deaths observed % (95% CI)	Interaction between surveillance systems	Estimated average deaths per 100,000 maternities n (95% CI)
< 1 year	48	1.6 (0.5–4.5)	49.6 (48.5–52.5)	96.7 (91.4–98.9)	ONS[a] HES p=0.035	0.721 (0.705–0.763)
< 6 months	47	1.7 (0.5–4.7)	48.7 (47.5–51.7)	96.5 (90.9–98.9)	ONS[a] HES p=0.039	0.708 (0.690–0.751)
<66 days	41[a]	0.4 (0.1–1.2)	41.4 (41.1–42.2)	99.0 (97.2–99.8)	None	0.601 (0.597–0.613)

HES: Hospital Episode Statistics; ONS: Office of National Statistics.

[a] Includes the five deaths for which cases had onset of disease before 66 days of age.

our study, nine had no laboratory confirmation and diagnosis was thus made on clinical grounds. It is possible that some of these cases may have been wrongly attributed to pertussis, resulting in an overestimate of mortality. However, on balance, given the lack of sensitivity of laboratory confirmation and the atypical presentation of pertussis in infants who may not develop the characteristic whoop and paroxysmal cough [16], an underestimate of mortality seems more likely.

We expressed infant mortality as deaths per 100,000 maternities rather than live births in order use the data in a cost-effectiveness analysis of a maternal pertussis immunisation policy. Maternities differ from live births in that vaccinating pregnant women will provide additional benefit in the event of the birth of a twin/triplet but have no benefit in the case of a stillbirth. Expression of deaths per 100,000 maternities is thus a more accurate measure of the infant mortality potentially preventable by a maternal immunisation policy.

A capture/recapture analysis was previously performed for England using the same three datasets for the years 1994 to 1999 [9]. This suggested that only 70% (33 of an estimated 46) of deaths were ascertained, in contrast to our estimate of 97%. The previous estimate included a year for which there was no information from HES, as hospital data was only available up to March 1998. Excluding this year changes the results with only 0.91 deaths missed of a total of 22. This gives an estimated 96% of all known deaths observed, similar to the percentage estimated from our study. The absolute number of observed deaths of 33 in six years in the earlier study gives only a slightly higher annual average (5.5) compared with our estimate of 4.4 (48 infant deaths observed in 11 years) despite a considerably higher notification rate in the 1990s [12]. This suggests that laboratory confirmation or clinical recognition of pertussis may have improved in recent years.

In conclusion, under ascertainment of known pertussis deaths is small when using our three national data sources though undiagnosed fatal cases may still be occurring. More deaths occur from pertussis than from any other disease covered by the current routine childhood immunisation programme. Mortality in vulnerable infants too young to be protected by vaccination reflects the overall population transmission of pertussis and interventions to protect young infants from exposure or development of disease if exposed are needed if pertussis mortality is to be reduced. The only intervention currently available that can protect infants from birth in all settings is immunisation in pregnancy. Such an approach can potentially protect the infant by the transfer of maternal antibodies and also reduce the likelihood of exposure to an infectious mother.

Acknowledgements

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Authors' reply: Application of Bayesian methods to the inference of phylogeny for enterovirus surveillance

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To the editor:

We would like to thank Roberts et al. for their comments on our paper entitled 'Laboratory-based surveillance in the molecular era: the TYPENED model, a joint data-sharing platform for clinical and public health laboratories' [1]. We fully agree with the remarks on the potential strength of adding more advanced analytical tools once the molecular surveillance network is established. In fact, this is what we have done previously, as part of studies of the evolution of noroviruses and enteroviruses [2,3]. For enteroviruses, we focused those studies on enterovirus 71 (EV71), for the reasons mentioned by Roberts et al. We compared the evolutionary trajectory of EV71 in the Netherlands with that from a systematic collection of EV71 strains detected in southern China. This analysis showed a clear difference in the circulating strains, with evidence that the EV71 subgenogroups B and C circulating in Asia have evolved further than those in Europe and the United States [3,4]. The severity of outbreaks of EV71 infection associated with the EV71 lineages circulating in Asia raises the question if these viruses need to be regarded as an emerging public health problem [5]. The analysis also showed that previous peaks in reporting reflected true epidemics [3]. Further studies showed that recombination was part of the driving force for those epidemics [6]. We agree that such studies give a much deeper understanding of the epidemiology of enteroviruses, and would strongly support its development in an international context. The rapid transition from culture-based diagnostic methods to molecular diagnostics in clinical virology in our country was a trigger to do this, and will most likely also affect other surveillance systems such as those for influenza or food-borne bacterial pathogens [7]. However, at present, this transition has not been made in most countries globally. Our article aimed to describe the model applied in the Netherlands for an approach to molecular surveillance, in which clinical and public health laboratories collaborate by defining common goals. This model was developed to facilitate the transition, and includes comparative evaluation of surveillance trends obtained by classical and molecular virological methods 1. If outcomes are agreed upon, we will further develop the system, which would include the more advanced analytical approaches suggested by Roberts et al.

Conflict of interest

None declared.

Authors' contributions

Marion Koopmans drafted the response letter after discussions and input from the authors of the article in question.

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Letter to the editor: Application of Bayesian methods to the inference of phylogeny for enterovirus surveillance

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To the editor:

We read with interest the article by Niesters et al. describing a pilot study to share enterovirus sequence data within the Netherlands for epidemiological investigation [1]. As the authors note, nucleotide sequence data can extend the identification of virus serotype to the tracing of disease transmission patterns through phylogenetic analysis. The article referred to use of a web-based typing tool for enteroviruses and noroviruses based on BLAST analysis followed by a neighbor-joining phylogeny. We believe such analyses can be further enhanced through the inclusion of temporal and geographical discrete variables for the inference of phylogeny (phylogeography). In particular, the application of Bayesian inference to phylogeography offers several advantages, such as the capacity to explicitly account for parameter uncertainty, reducing potential model bias, especially where data are scarce. This approach was referred to in the same issue of *Eurosurveillance* by Carriço et al. for bacterial molecular epidemiology [2] and has been successfully applied to virological studies for avian influenza A(H5N1) and rabies [3]. Carriço et al. make reference to the computational demands of such methods [2] and indeed, this is an important consideration. From our experience, the use of commodity graphics processing units combined with appropriate parallel threading software extensions can overcome some of the limitations imposed by high computational demand at low cost [4].

Australia established an Enterovirus Reference Laboratory Network – primarily for poliovirus surveillance but also to detect other enteroviruses of public health significance, such as enterovirus 71 (EV71) – through the sharing of enterovirus sequence data. EV71 infection often manifests benignly as hand, foot and mouth disease in infants but has been associated with fatal neurological disease, particularly in the Asia-Pacific region [5]. The correlation of specific EV71 subgenogroups with increased neurological presentation, such as C4 infection recently in Cambodia, China and Vietnam, highlights the benefit of a phylodynamic analysis that accounts for temporal and geographical parameters. We feel that the application of Bayesian methods to the inference of phylogeny will play an important role in the elucidation of global chains of enterovirus transmission.

Conflict of interest

None declared.

Authors' contributions

Jason A Roberts conceived the idea to respond to the recent articles on molecular epidemiology in Eurosurveillance and drafted the letter. The text was revised and edited by Andrew Hung and Bruce R Thorley, and all authors approved the final version of the letter.

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WHO recommendations on the composition of the 2013/14 influenza virus vaccines in the northern hemisphere

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The World Health Organization (WHO) published recommendations on the composition of the trivalent vaccines for use in the 2013/14 influenza season in the northern hemisphere [1].

WHO recommended that the 2013/14 trivalent influenza vaccines contain the following:

- an A/California/7/2009 (H1N1)pdm09-like virus[a];
- an A(H3N2) virus antigenically like the cell-propagated prototype virus A/Victoria/361/2011[b];
- a B/Massachusetts/2/2012-like virus.

WHO recommends that quadrivalent vaccines containing two influenza B viruses contain the above three viruses and a B/Brisbane/60/2008-like virus[c].

[a] A/Christchurch/16/2010 is an A/California/7/2009-like virus;
[b] A/Texas/50/2012 is an A(H3N2) virus antigenically like the cell-propagated prototype virus A/Victoria/361/2011;
[c] B/Brisbane/33/2008 is a B/Brisbane/60/2008-like virus.

WHO also recommended that A/Texas/50/2012 is used as the A(H3N2) vaccine component because of antigenic changes in earlier A/Victoria/361/2011-like vaccine viruses (such as IVR-165) resulting from adaptation to propagation in eggs.

As in previous years, national or regional authorities approve the composition and formulation of vaccines used in each country. National public health authorities are also responsible for making recommendations regarding the use of the vaccine.

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