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Continued seasonal circulation of enterovirus D68 in the Netherlands, 2011–2014

A Meijer (Adam.Meijer@rivm.nl), K S Benschop¹, G A Donker², H G van der Avoort¹
1. Centre for Infectious Disease Research, Diagnostics and Screening, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
2. NIVEL Primary Care Database, Sentinel Practices, Utrecht, The Netherlands

Enterovirus D68 (EV-D68) continued to circulate in a seasonal pattern in the Netherlands, after the outbreak in 2010. Outpatient EV-D68 cases, mainly in the under 20 and 50–59 years age groups, presented with relatively mild respiratory disease. Hospital-based enterovirus surveillance identified more severe cases, mainly in children under 10 years of age. Dutch partial VP1 genomic region sequences from 2012 through 2014 were distributed over three sublineages similar to EV-D68 from the outbreak in the US in 2014.

After the 2010 outbreak, enterovirus D68 (EV-D68) continued to circulate in a seasonal pattern in the Netherlands. Here, we report the results of the monitoring of EV-D68 circulation in the Netherlands from week 1 2011 through week 40 2014.

EV-D68 has been sporadically detected since its first description in 1962, up to 2008 [1,2]. From 2008 onwards, EV-D68 outbreaks occurred worldwide, including in 2010 in the Netherlands [2–5]. The largest outbreak is currently occurring in Northern America, causing substantial hospitalisation of children with severe respiratory disease in the United States (US) [3,6]. Many of these children have underlying disease, such as asthma [3,6]. Previous outbreaks described in the literature reported mainly on hospitalised patients [3].

In the Netherlands, retrospective analysis of enteroviruses detected from the general practitioner (GP) sentinel surveillance of influenza-like illness (ILI) and other acute respiratory infections (ARI) showed that circulation of EV-D68 occurred at least since 1996 up to the upsurge of 2010 [5]. EV-D68 cases had significantly more dyspnoea and bronchiolitis compared to EV-D68-negative patients with ILI or ARI notified in the same week [5]. In the Dutch national enterovirus surveillance aimed at exclusion of poliovirus circulation, EV-D68 was rarely detected, mainly because the focus has been on enteroviruses detected in stool specimens [7]. Since 2010, we continued to monitor EV-D68 circulation in the Netherlands through both surveillance schemes.

Specimen collection
The methods used for specimen collection and for enterovirus detection and VP1 genomic region sequence analysis have been described [5,7,8]. For phylogenetic analysis using MEGA6 [9] all available VP1 sequences (covering nucleotides 132 through 471 relative to the VP1 gene of the Fermon strain) as of 12 October 2014 were downloaded from GenBank. The phylogeny was reconstructed using maximum likelihood and 1,000 bootstrap iterations with new Dutch sequences included (GenBank accession numbers KM975324-KM975350). Numbering of the major clusters (1, 2 and 3) has been described [5] and is synonymous to major clusters B, C and A respectively described by Tokarz et al. [10].

Results
Figure 1 and Table 1 summarise EV-D68 detections through the GP-based sentinel ILI and other ARI surveillance and the national enterovirus surveillance in the Netherlands, in specimens with collection dates from week 1 2011 through week 40 2014. Over the whole period, 27 EV-D68 cases were identified in a seasonal pattern; one in autumn 2011, 10 in autumn-winter period 2011/12, five in autumn-winter period 2012/13, and 11 since summer 2014 (Figure 1). The start of detections in 2014 was earlier compared to the start of detections in 2012 (12 and six weeks earlier in the enterovirus and ILI/ARI surveillance respectively) and in 2013 (15 and 11 weeks earlier in the enterovirus and ILI/ARI surveillance respectively) (Figure 1). By year, the proportion EV-D68 among enteroviruses analysed was much higher (median 25%; range 0–38%) in the ILI/ARI surveillance compared to the enterovirus surveillance (median 0.5%; range 0.3–1.4%) (Table 1). However, by year, the percentage of enterovirus detections among ILI/ARI cases was low, on average 1.7% (range 1.4–2.1%) (Table 1).
Due to increased awareness of the importance of enteroviruses in respiratory infections, laboratories participating in the Dutch national enterovirus surveillance also submitted enteroviruses associated with respiratory illness for typing after 2010; all 11 EV-D68 detections were in respiratory specimens. The age distribution in outpatients over the whole period was not different from that reported before, over the period 1996 through 2010 [5]; cases occurred mainly in the under 20 and in the 50–59 years age groups (Table 2). The male/female ratio was 1.3 (Table 2). In the national enterovirus surveillance, however, EV-D68 was mainly detected in the under 10 years age group and the male/female ratio was 0.8 (Table 2).

The age distribution in 2014 was similar to that for the whole period for both surveillance schemes (data not shown). EV-D68 positive outpatients presented with ILI as well as other ARI, with most prominent symptoms being fever and cough (Table 2). Similar to the situation in Northern America in 2014, the hospitalised cases experienced severe respiratory disease (Table 2).

### Table 1

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of clinical specimens tested</th>
<th>Number of enterovirus positive specimens (% of specimens tested)</th>
<th>Number of enterovirus D68 positive specimens (% of enterovirus positive specimens)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ILI/ARI surveillance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>1,369</td>
<td>19 (1.4)</td>
<td>0</td>
</tr>
<tr>
<td>2012</td>
<td>1,126</td>
<td>24 (2.1)</td>
<td>7 (29)</td>
</tr>
<tr>
<td>2013</td>
<td>1,292</td>
<td>19 (1.5)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>2014 (through week 40)</td>
<td>792</td>
<td>13 (1.6)</td>
<td>5 (38)</td>
</tr>
<tr>
<td><strong>Enterovirus surveillance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>Unknown</td>
<td>362</td>
<td>1 (0.3)</td>
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<tr>
<td>2012</td>
<td>Unknown</td>
<td>498</td>
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<td>2013</td>
<td>Unknown</td>
<td>309</td>
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</tr>
<tr>
<td>2014 (through week 40)</td>
<td>Unknown</td>
<td>414</td>
<td>6 (1.4)</td>
</tr>
</tbody>
</table>

ARIs: acute respiratory infections; ILI: influenza-like illness.

*In enterovirus surveillance the number of enterovirus isolates or enterovirus positive clinical specimens submitted to the National Institute for Public Health and the Environment (RIVM) for VP1 typing is represented.*
EV-D68 cases were detected all over the country; no localised outbreak was detected. Phylogenetic analysis of the VP1 genomic region showed that the Dutch EV-D68 from 2014, and from 2012 and 2013 as well, clustered with the US 2014 outbreak sequences in major group 1 in two sublineages and in major group 3 in one of the sublineages (Figure 2). Other Dutch EV-D68 from 2011 through 2014 clustered in other sublineages of major group 3. The sequences in the two sublineages of major group 1 had highly similar amino acid signatures, whereas sequences in the sublineages of major group 3 had clearly different amino acid signatures, with most differences located in the immunogenic BC and DE loops (Figure 3).

**Discussion**

Although rarely detected worldwide, our combined previous and current results over the period 1996–2014 show that EV-D68 seems to circulate every year in a seasonal pattern in the northern hemisphere in predominantly the autumn through early winter period, causing relatively mild respiratory illness in a number of individuals large enough to be picked up by the GP ILI/ARI surveillance [5]. The national enterovirus surveillance shows that a number of EV-D68 cases are admitted to hospital each year with more severe respiratory disease. Clinical presentation ranging from mild to severe respiratory disease is in line with our previous findings, and has been described before [1–6]. None of the patients described in this paper had symptoms of neurological disease or paralysis. A causative link between EV-D68 infection and paralysis has not been established to date [11]. Given the acute flaccid paralysis rate (AFP) indicator used by the World Health Organization for optimal polio surveillance (1–2 cases per 100,000 children below 15 years of age) one can expect that during a large EV-D68 outbreak, also several AFP patients will be shedding EV-D68. The present outbreak in Northern America provides an opportunity to investigate the link.

The difference in age distribution of EV-D68 cases between the ILI/ARI surveillance and the national enterovirus surveillance in our dataset is biased by the fact that 95% of enteroviruses identified by enterovirus surveillance are from children [7]. The male/female ratio of 1.3 among EV-D68 cases in the ILI/ARI surveillance was slightly lower compared to 1.5 over the period 1996 through 2010, but showing the usual male predominance among enterovirus infected persons [5]. Hence, the female predominance among EV-D68 cases in the national enterovirus surveillance is unusual, but likely the result of the low number of cases.

The number of hospitalised EV-D68 cases identified through the national enterovirus surveillance in the Netherlands is likely underestimated. When first described, EV-D68 was found to be relatively acid resistant and was distinguished from the acid-sensitive human rhinovirus type 87 (HRV87) on this basis [12]. However, in 2002, HRV87 was reclassified as EV-D68 based on phylogenetic analysis [13]. Many RT-PCR diagnostic tests for enteroviruses as well as rhinoviruses are targeted at the 5' untranslated region of the genome [8]. Many of these tests are capable of detecting EV-D68 despite mismatches in primers and probes with the EV-D68 target sites, although with varying sensitivity depending on reagents and equipment used for RT-PCR [8]. This might also result in a false negative or a false rhinovirus-positive result [8].

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ILI/ARI surveillance (N = 16)</th>
<th>Enterovirus surveillance (N = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age groups (years)</strong></td>
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</tr>
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<td>&lt; 10</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>10–19</td>
<td>3</td>
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<td>20–29</td>
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<td>30–39</td>
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<tr>
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<tr>
<td>50–59</td>
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<td>70–79</td>
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<td>≥ 80</td>
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<td>Male</td>
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<td>5</td>
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<td><strong>Diagnosis</strong></td>
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<tr>
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<tr>
<td>Fatigue</td>
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<tr>
<td>Headache</td>
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<tr>
<td>Myalgia</td>
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<tr>
<td>Dyspnoea</td>
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<tr>
<td>Diarrhoea</td>
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<tr>
<td>Underlying disease[^c^]</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>No clinical data reported[^c^]</td>
<td>0</td>
<td>5</td>
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</tbody>
</table>

[^a^] ILI: influenza-like illness.
[^b^] A prodromal stage of three or four days.
[^c^] In ILI/ARI and enterovirus surveillance, underlying disease is reported in a free text item on the specimen form.
Figure 2
Phylogenetic analysis of partial VP1 genomic region sequences of enterovirus D68, nucleotides 132 through 471 relative to the VP1 genomic region of the Fermon strain, covering the BC and DE immunogenic loops in the VP1 protein.

*GenBank ID: AFO81348.1.

Figure 3.

One enterovirus D68 from 2013 could only be identified by sequencing of the 5' untranslated region diagnostic RT-PCR product and is therefore not included in Figures 2 and 3. The maximum likelihood tree is shown with the percentage bootstrap support for branching events after 1,000 iterations indicated at the nodes. Major phylogenetic groups as described in references 5 and 10 are indicated on the right of the tree. Dutch sequences covering the period 2011–2014, and sequences from the 2014 outbreak in the US are enlarged.

**Sequences from the US, 2014**

Blue labels indicate sequences from the Netherlands:
- 2011 (n=1)
- 2012 (n=9)
- 2013 (n=4)
- 2014 (n=13)

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Figure 3

Analysis of partial VP1 amino acid sequences of enterovirus D68 in the Netherlands covering the period 2011–2014 and of enterovirus D68 from the 2014 outbreak in the United States.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Strain ID</th>
<th>Major Group 1</th>
<th>Major Group 2</th>
<th>Major Group 3</th>
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</table>

X: mixed Q/R amino acids.

Major groups as identified in Figure 2 are indicated on the right of the alignment. Numbering of amino acid residues is relative to the start of the VP1 reading frame of the Fermon strain. Amino acids common to the Fermon strain are indicated with a dot in the alignment. The putative BC and DE loops are indicated by boxes on the aligned amino acid sequences.

One enterovirus D68 from 2013 could only be identified by sequencing of the 5’ untranslated region diagnostic RT-PCR product and is therefore not included in Figures 2 and 3.
on respiratory specimens, these tests might therefore wrongly identify an EV-D68 virus as a rhinovirus, and further investigation by typing in the national enterovirus surveillance protocol will not be performed [7,8]. Furthermore, a number of Dutch laboratories have started to type enteroviruses themselves and share data through the national enterovirus surveillance, although this is done with delay and infrequently. These laboratories participate in VIRO-TypeNed (formerly called TYPENED) [14] to provide a year-round surveillance and current efforts are directed at updating VIRO-TypeNed with EV-D68 detections.

Previous work has indicated that co-circulation of the different phylogenetic lineages of EV-D68 is the result of increased variability of the VP1 genomic region, i.e. the BC and DE loops, leading to reduced cross-neutralising antibodies raised against viruses of the major groups [5,10,15]. Variation of the highly conserved internal ribosome entry site in the 5' untranslated region, present in major group 1 and 2 viruses, has been suggested to be associated with increased virulence [10]. However, the US 2014 outbreak viruses are located in major groups 1 and 3, and similar viruses have been detected in the Netherlands, but associated with mild disease. Nevertheless, underlying disease like asthma seems to be an important factor for development of severe disease following EV-D68 infection [6]. Further in depth analysis of the EV-D68 full genomes from mild and severe cases and linked virological, clinical and epidemiological information should provide further insight in the factors determining severity of EV-D68 infection.

Acknowledgements
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Conflict of interest
None declared.

Authors’ contributions
Adam Meijer, Harrie van der Avoort and Kimberley Benschop collected data. Gé Donker coordinated the sentinel GP network collecting specimens. Adam Meijer performed the analysis of the data and wrote the first draft of the paper. All other authors reviewed the manuscript critically, and comments and suggestions were incorporated in the final version by Adam Meijer.

References
The quick spread of an Ebola outbreak in West Africa has led a number of countries and airline companies to issue travel bans to the affected areas. Considering data up to 31 Aug 2014, we assess the impact of the resulting traffic reductions with detailed numerical simulations of the international spread of the epidemic. Traffic reductions are shown to delay by only a few weeks the risk that the outbreak extends to new countries.

Introduction

The 2014 Ebola outbreak currently involves three countries with widespread and intense transmission in the West African region (Guinea, Liberia and Sierra Leone) and four others where initial case(s) or localised transmission have been reported (Nigeria, Senegal, Spain and the United States), reaching a total of 8,997 cases and 4,493 deaths in the official report of 15 October 2014 [1].

With the number of cases exponentially increasing in the affected area, several agencies and governments are calling for massive coordinated interventions aimed at the surveillance and containment of the epidemic [2]. Scaling up the international response appears necessary for providing financial support, supply of technical resources and expertise, and delivery of essential services to the affected area [2]. The need to consider an international framework lies also in the possible further international spread of the epidemic [3]. In response to such concerns and in an attempt to reduce the risk of case importation, several countries and airlines have adopted travel restrictions to and from the affected area. These include the suspension of flights by a number of carriers, air/sea/land border closures, restrictions for non-residents, suspension of visa issuance, and entry screening. Travel bans could potentially hamper the delivery of medical supplies and the deployment of specialised personnel to manage the epidemic [4]. Although international public health and relief agencies and representatives have been urgently calling for lifting such travel bans [4-6], these disease-avoidance mechanisms remain in place at the time of writing, and are being considered. In light of their potentially harmful effects, the benefits of travel restrictions need to be carefully evaluated.

Air travel data is a critical source of information that has been recently analysed to characterise the degree of connectivity of the affected area to the rest of the world [7,8]. Air travel and human mobility data have also been integrated in large-scale computer microsimulations that, taking explicitly into account the local evolution of the epidemic in the affected countries, quantify the risk for international spread of Ebola virus disease (EVD) out of Africa in the short term [9]. Hypothetical simulation scenarios considering an 80% reduction of passenger traffic flow out of the region indicate that further international spread is delayed by only a few weeks. Here, we use the model to quantify the effect that the travel restrictions implemented during August 2014 by countries and airlines have on the global spread of Ebola. By comparing the differences between simulations with and without travel restrictions, we can make quantitative estimates of the effectiveness of such restrictions on reducing the importation of new Ebola cases to countries outside of West Africa. Our goal is to inform the debate over the utility of travel bans to slow the spread of Ebola.
<table>
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<th>Travel-related measure</th>
<th>Travel-related measure/Authorities/Companies</th>
<th>Starting date of intervention$^\text{a}$</th>
<th>Target area</th>
<th>Additional details$^\text{bc}$</th>
</tr>
</thead>
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<tr>
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<td>Three European airlines</td>
<td>From 6 Aug 2014 to 28 Aug 2014</td>
<td>Liberia Sierra Leone</td>
<td>See SI</td>
</tr>
<tr>
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<td>Two Asian airlines</td>
<td>From 6 Aug 2014 to 14 Aug 2014</td>
<td>Guinea Kenya</td>
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<tr>
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<td>Six African airlines</td>
<td>From 6 Aug 2014 to 26 Aug 2014</td>
<td>Guinea Liberia Sierra Leone</td>
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<td>Liberia Nigeria Sierra Leone</td>
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<tr>
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<tr>
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<tr>
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<td>13 Aug 2014</td>
<td>Nigeria</td>
<td>Ban of all flights, closure of land borders</td>
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<td>Ban of all flights from the affected countries</td>
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<td>Banned travellers from affected countries</td>
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<td>Suspended the issuance of visas</td>
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<td>Ban of all flights</td>
</tr>
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<td>Guinea Liberia Sierra Leone</td>
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<tr>
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<td>21 Aug 2014</td>
<td>Guinea Liberia Sierra Leone</td>
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<td>Guinea Liberia Sierra Leone</td>
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</tr>
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</tr>
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</tr>
<tr>
<td></td>
<td>Guinea Bissau</td>
<td>Before 26 Aug 2014</td>
<td>Guinea Liberia Sierra Leone</td>
<td>Ban of all flights, closure of land borders</td>
</tr>
<tr>
<td></td>
<td>Togo</td>
<td>Before 26 Aug 2014</td>
<td>Guinea Liberia Sierra Leone</td>
<td>Ban of all flights</td>
</tr>
</tbody>
</table>

Si: supplementary information.

$^\text{a}$ Depending on the information available, this can be either the date of intervention or the date of the bulletin/news.

$^\text{b}$ Closure of land borders is for all travellers irrespective of citizenship.

$^\text{c}$ Border closure is generally for citizens of the target countries and travellers coming from the affected area, with the exception of nationals of the destination country.

The list is obtained from publicly available sources extracted from the search [“ebola” AND “travel”] on Twitter on 1 September 2014. Additional searches of news published on the Internet were performed to confirm and complement the initial list. More detailed information and references are provided in the supplementary information available at [http://www.mobs-lab.org/ebola-eurosurvsup.html](http://www.mobs-lab.org/ebola-eurosurvsup.html)
Methods

We used 2013 flight itinerary data providing travel volumes of passengers flying between any origin–destination pair of commercial airports in the world (International Air Transport Association (IATA), www.iata.org; Official Airline Guide (OAG), www.oag.com). Starting from the airport of origin, each itinerary reports all connecting airports to reach the final destination and the airline companies handling the connecting flights along the given route. We collected publicly available information on the travel restrictions related to Ebola-affected regions up to 31 August 2014. We considered both travel bans implemented by national authorities and flight discontinuations by individual airlines (Table). Restrictions are heterogeneous in terms of start date and target country in the affected area (e.g. some concern the entire Western Africa area and others just one of its countries). Flight suspensions by airline company A targeting the set of countries C were considered by removing from the flight database all itineraries (and associated travel volumes) to C where A was the dominant airline. Then, travel bans and border closures implemented by country B targeting the set of countries C were considered by singling out all itineraries connecting B with C (in both directions) and reducing by a factor r the associated travel volumes, with r_neighbours = 80% for the affected area’s neighbouring countries and r_others = 90% for all other countries, to model residual human mobility and non-compliance to policies. The resulting overall traffic reduction for each country was obtained by combining the effect of flight discontinuation and country level travel bans. We further required that the overall reduction could not be larger than r. This additional constraint is meant to model additional types of possible movements not captured by the air travel data (e.g. cross-border ground movement) and also adaptation to the restrictions (e.g. rearrangements of flight itineraries to other airline companies) for which detailed data are not currently available.

Figure 1

Modelled effect of travel restrictions on the risk of Ebola case importation for individual countries

The delay in the risk of case importation induced by the applied travel restrictions is shown for each country versus the overall reduction of the country’s air traffic. The delay was calculated as the time after which the risk of case importation in the scenario with travel restrictions was equal to the value reached on 30 September 2014 in the baseline case. For clarity, only countries having a non-negligible risk of importation (> 0.5%) are shown in the plot. The size of the dots is proportional to the country’s population. Colours indicate the continents.
We used the Global Epidemic and Mobility model [10,11] applied to the EVD outbreak [9] to simulate case importation events in 220 countries around the world. The model [9] accounts for EVD transmission in the general community, in hospital settings, and during funeral rites [12]. Basic reproductive numbers for each of these settings were inferred through a Monte Carlo likelihood analysis considering more than 3,500,000 simulations that sampled the disease model parameter space and the case data on the EVD outbreak up to 27 August 2014. Other epidemiological parameters were taken from the literature [9,12,13]. The spatio-temporal epidemic evolution is modelled using individual-level dynamics where transitions are mathematically defined by chain binomial and multinomial processes to preserve the discrete and stochastic nature of the processes. Individuals in the latent state are allowed to follow the same mobility patterns and international travel behaviour as those who are not infected. Travel probabilities are calculated based on the integrated flight database and mechanically simulated travel and commuting patterns. More details on the model and on the parameters’ inference procedure are provided in [9] and in the supplementary information* (http://www.mobs-lab.org/ebola-eurosursup.html).

To assess the effect of current travel restrictions on the risk of case importation, we compared the international spread of the EVD epidemic obtained from numerical simulations of the model with and without the travel reductions. We focus on short-term projections and calculate the probability of case importation per country (and per continent) predicted for 30 September 2014 in the baseline scenario without travel restrictions. The probability of importation at that date is still relatively small for most of the countries and detailed values for different dates can be found in [9]. We then compute the time delay needed to reach the same value of case importation probability per country (or continent) once the travel restrictions shown in the Table are implemented.

Results

The modelled travel restrictions impacted airline passenger volume to countries worldwide in a very heterogeneous manner (Figure 1, reporting results for countries with a case importation probability larger than 0.5% as of 30 September 2014). Notably, flight suppressions and border closures did not affect solely the countries implementing such measures but they also had considerable repercussions on others (e.g. India and the Philippines following the suppression of Emirates Airline flights). With few exceptions, African countries were predicted to experience traffic reductions greater than 70% due to generalised travel bans.

The total estimated reduction of 60% of airline passenger traffic connecting the West Africa region currently most affected by Ebola to the rest of the world was shown to be insufficient to prevent the exportation of Ebola cases. The observed traffic reductions were shown to delay the risk of case importation per country

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**Figure 2**
Modelled overall delays predicted for Ebola case importation by continent, following the application of the travel restrictions.

Grey bars below the circles indicate the overall travel reduction per continent resulting from the currently applied travel restrictions. The size of the discs is related to the duration of the delay.
from a few days to a few weeks (Figure 1). The majority of the countries (56%, mainly in Central Europe, Asia and the Americas) would not experience a delay longer than one month. At the continental level, the delay was predicted to be negligible for the Americas, and at most one month for the African continent (Figure 2). Results confirmed previous empirical evidence from past epidemics of other infectious diseases and were in agreement with mathematical modelling studies of the relationship between the exponential growth rate of an epidemic in a source region and the exportation to other regions [14-18]. Those can be summarised with the simple rule of thumb that a 50% travel reduction produces a delay equal to the doubling time of the number of cases.

**Discussion**

Although the current travel restrictions postpone the spread of EVD to other continents by at most a few weeks, they can impose heavy logistical constraints on the management of the epidemic in the countries severely hit by the disease and ill-equipped to cope with its alarming rapid spread [4-6]. If not offset by massive humanitarian operations, they can cause major short-ages of food, energy and essential resources, with the potential to severely compromise local economies [19].

Similar to what happened during the severe acute respiratory syndrome (SARS) outbreak in 2003 [20], adverse effects on local economies of the same countries implementing the bans may also occur, as a reduced connectivity and the increased apprehension may induce a considerable reduction in the demand for service industries (business travel, tourism and associated services).

International agencies suggest that currently unaffected countries should invest in health system preparedness, strengthening their own capacity to detect and contain newly imported cases [21]. These measures are expected to substantially reduce the risk of importation. Indeed, while the relatively long latency period of EVD may allow exposed individuals to travel long distances, infectiousness occurs at symptom onset only, so that potentially infectious individuals can be clinically recognised. The mode of transmission is expected to minimise the risk of spread during a flight [21].

It is also worth mentioning that delays in the global spread of the outbreak may have to be evaluated with respect to the development timeline of pharmaceutical interventions. For instance, Ebola vaccines are being fast-tracked, and field trials are planned, probably in healthcare workers at high risk of exposure to the virus in the affected areas [22].

The results presented here need to be considered in light of the assumptions and limitations of the modelling approach used. We considered all travel restrictions obtained from publicly available sources that were implemented up to the end of August 2014, but this list may not be complete and not all information could be verified with the original sources. In the presence of uncertainty (e.g. vague information or inconsistency between different news) we assumed the scenario with the strongest traffic reduction in order to provide the best-case scenario in terms of resulting delay. An additional world-wide fear-induced decrease of tourist and business travel to the region has been observed [23,24] in September and has probably further increased the delay in case importation, although only logarithmically with the magnitude of the traffic reduction [15,16].

The simulation presented was based on the study of the current West African outbreak described in Gomes et al. [9], which contains estimates of the incubation period and generation time based on past Ebola outbreaks. Recent estimates for the current outbreak have been published by Hollingsworth et al., and Althaus et al. [13,25]. Updated results on the risk of the epidemic spread are regularly posted on our website http://www.mobs-lab.org/ebola.html to account for the most recently published epidemiological information. We note that, although these parameters affect the absolute value of the probability of importation, they do not affect the relative delay depending on the epidemic growth rate [15,16].

Detailed data on unmeasured movements during the epidemic and on possible rearrangements of air travel volumes following decisions of airline companies to suspend flights are not available to be implemented directly into the model. For this reason, we took these aspects into account by considering a maximum of 90% overall traffic reduction (80% for countries bordering the currently affected area), representing the maximum ability of a country to implement the border closures. A sensitivity analysis exploring smaller values of these upper bounds (70% for neighbouring countries and 80% for the others) yielded delays in the risk of case importations reduced to five weeks for the African countries with the largest overall reductions (supplementary information*).

**Conclusion**

This study indicates that travel bans are only delaying the further international spread of the Ebola outbreak in West Africa for a limited time, at the risk of compromising connectivity to the region, mobilisation of resources to the affected area and sustained response operations, all actions of critical value for the immediate local control of EVD and for preventing its further geographical spread. Any decision making process on this issue must take into account complex cost-benefit analyses of travel bans.

*Note*

Supplementary information made available by the authors on an independent website is not edited by *Eurosurveillance*, and *Eurosurveillance* is not responsible for the content. The
Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014

M. Monaco1,2, T Giani2,3, M Raffone1,4, F Arena3, A Garcia-Fernandez1, S Pollini3, Network EuSCAPE-Italy5, H Grundmann6, A Pantosti (annalisa.pantosti@iss.it)1, G M Rossolini3,7,8
1. Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy
2. MM and TG have equally contributed to this work
3. Department of Medical Biotechnologies, University of Siena, Siena, Italy
4. Federico II University Hospital, Neaples, Italy
5. The network EuSCAPE-Italy participants are listed at the end of this article
6. Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, the Netherlands
7. Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy
8. Clinical Microbiology and Virology Unit, Florence Careggi University Hospital, Florence, Italy

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Consecutive non-replicate clinical isolates (n=191) of carbapenem non-susceptible Enterobacteriaceae were collected from 21 hospital laboratories across Italy from November 2013 to April 2014 as part of the European Survey on Carbapenemase-producing Enterobacteriaceae (EuSCAPE) project. *Klebsiella pneumoniae* carbapenemase-producing (KPC-KP) represented 178 (93%) isolates with 76 (43%) respectively resistant to colistin, a key drug for treating carbapenemase-producing Enterobacteriaceae. KPC-KP colistin-resistant isolates were detected in all participating laboratories. This underscores a concerning evolution of colistin resistance in a setting of high KPC-KP endemicity.

We report the widespread and rapid dissemination of resistance against colistin, a key drug for treatment of carbapenemase-producing Enterobacteriaceae, among *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* (KPC-KP) in Italy. As part of the European Survey on Carbapenemase-producing Enterobacteriaceae (EuSCAPE) project, consecutive non-replicate clinical isolates of carbapenem non-susceptible (resistant or intermediate) Enterobacteriaceae (n=191) were collected from 21 Italian hospital laboratories between November 2013 and April 2014. Most isolates 178 (93%) were KPC-KP, with 76 (43%) respectively resistant to colistin. This report details the findings and discusses potential implications for infection control.

**Background**

Carbapenem-resistant Enterobacteriaceae (CRE) emerged in recent years as one of the most challenging group of antibiotic-resistant pathogens. Related mortality rates are high due to limited treatment options, and some strains have the potential for rapid dissemination in healthcare settings [1,2]. In Europe, CRE have been reported from virtually all countries, but in some countries, namely Greece and Italy, they have spread rapidly and are presently endemic in many hospitals [3,4]. Resistance to carbapenems in Enterbacteriaceae is largely due to production of enzymes (carbapenemases) inactivating these antibiotics, hence the definition of carbapenemase-producing Enterobacteriaceae (CPE).

In Italy, the dramatic increase of carbapenem-resistant *Klebsiella pneumoniae* has been documented by the European Antimicrobial Resistance Surveillance Network (EARS-Net) which showed that the percentage of invasive isolates of carbapenem-resistant *K. pneumoniae*, that was until 2009 lower than one to 2%, increased to 15% in 2010 to reach 35% in 2013 ([5] and unpublished data). Data provided by Micronet (http://www.sim.iss.it/micronet.htm), a sentinel epidemiological surveillance network based on computerised daily collection of microbiological data from the laboratory information systems of 27 laboratories nationwide, confirmed the increase in the percentage of carbapenem-resistant *K. pneumoniae* in samples from different anatomical sites, including lower respiratory secretions and urine [6]. In addition, analysis of resistance determinants and clonality, revealed that the Italian CRE epidemic was mostly sustained by KPC-KP of clonal complex 258, with only a minority of different clones and resistance mechanisms [7].

Polymyxins (colistin and polymyxin B), together with tigecycline and gentamicin, are among the few agents...
that retain activity against KPC-KP, and are key components of the combination antimicrobial regimens that are recommended for treatment of these pathogens [8,9]. Therefore, the emergence of resistance to these last line drugs among KPC-KP is important to monitor.

Implementation of European Survey on Carbapenemase-producing Enterobacteriaceae in Italy
EuSCAPE is funded by the European Centre for Disease Prevention and Control (ECDC) and coordinated by the Department of Medical Microbiology of the University Medical Center Groningen in the Netherlands. This initiative aims to foster active surveillance of CPE through improving the diagnostic capacity of microbiological laboratories in Europe [10]. A crucial part of EuSCAPE consisted of a structured survey that between November 2013 and April 2014 involved hospital laboratories from 35 countries across Europe. In each participating country the National Expert Laboratory (NEL) collected and characterised clinical isolates of suspected carbapenem non-susceptible K. pneumoniae or Escherichia coli obtained from a sentinel network of peripheral laboratories (PLs). Each PL was asked to collect the first 10 consecutive non- replicate isolates of suspected carbapenem non-susceptible K. pneumoniae or E. coli obtained from clinical samples (blood, lower respiratory tract secretions, urine, puncture fluids and wound secretions) and to provide also relevant demographic and clinical data (age, sex, location of patient in hospital, previous hospital admission in the last six months, previous stay or travel abroad within the last six months).

In Italy, a total of 21 PLs that served 45 hospitals or outpatient clinics distributed across the country participated in the survey. PLs identified suspected carbapenem non-susceptible K. pneumoniae or E. coli by automated systems Vitek 2 (bioMérieux, Marcy l’Etoile, France) or Phoenix (Becton Dickinson Diagnostic Systems, Sparks, MD, USA). Subsequently these isolates were sent to the NEL in Rome, who in collaboration with the NEL in Siena, performed confirmation and further characterisation. NELs confirmed species identification by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Vitek MS, bioMérieux), and carried out susceptibility testing against carbapenems and other antimicrobial agents by reference broth microdilution [11] using commercial microtitre plates (Alere Technologies, GmbH, Jena, Germany) and manually prepared plates for colistin testing. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints [12]. The presence of carbapenemase genes of the \( \text{bla}_{KPC} \), \( \text{bla}_{VIM} \), \( \text{bla}_{OXA-48} \), and \( \text{bla}_{NDM} \) types was investigated by polymerase chain reaction (PCR) using the protocol recommended by EuSCAPE (available upon request from the EuSCAPE Coordinator, Prof. Hajo Grundmann).

Results of the survey
A total of 197 suspected carbapenem non-susceptible K. pneumoniae or E. coli isolates were collected by the PLs in the study period. Of these, 187 K. pneumoniae and four E. coli were confirmed as non-susceptible to at least one carbapenem antibiotic (imipenem, meropenem or ertapenem). The \( \text{bla}_{KPC} \) determinant was found to be the most prevalent among carbapenem non-susceptible isolates, being detected in 178 K. pneumoniae and in three E. coli, while other carbapenemase genes were infrequently found (Table).

KPC-KP were obtained from urine (67 isolates), blood (61 isolates), lower respiratory tract (21 isolates), wound secretions (10 isolates), and other specimens (19 isolates). Patients with KPC-KP had a median age of 72 years (range: 16–94 years); 106 (60%) were males and 72 (40%) were females. Of these patients, 41(23%) had KPC-KP isolates detected while in intensive care unit (ICU), 127 (71%) were found while in a medical or surgical ward, and 10 (6%) were outpatients or patients seen at the emergency department. Another hospital admission in the previous six months was reported for 96 (64%) of patients for whom the information was available (n=150). Travelling abroad during the last six months was reported for only 3 (3%) of the patients for whom the information was available (n=111). Thus, 97% (108/111) of KPC-KP infections are endemic cases.

### Carbapenemase determinants detected in the confirmed carbapenem non-susceptible isolates collected as part of the EuSCAPE survey, Italy, November 2013–April 2014 (n=191)

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of isolates per type of carbapenemase</th>
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<tr>
<td></td>
<td>( \text{bla}_{KPC} )</td>
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<tr>
<td>K. pneumoniae</td>
<td>178(1)</td>
</tr>
<tr>
<td>E. coli</td>
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</table>

EuSCAPE: European Survey on Carbapenemase-producing Enterobacteriaceae.

K. pneumoniae carbapenemase-producing K. pneumoniae (KPC-KP) were reported from all peripheral laboratories.

- Detected from all the 21 peripheral laboratories.
- The four carbapenemase-producing E. coli isolates were from different peripheral laboratories.
Antimicrobial susceptibility data for the 178 KPC-KP isolates revealed that 76 (43%) were resistant to colistin, 11 (6%) resistant or intermediate to tigecycline, 29 (16%) resistant or intermediate to gentamicin, and 146 (82%) resistant or intermediate to trimethoprim-sulfamethoxazole (SXT). Two isolates (1%) were resistant or intermediate to all four antibiotics. Colistin-resistant KPC-KP isolates were detected from all PLs, although at variable percentages (Figure).

Discussion and conclusions

Although most recent data from April 2014 to date are not available at this time, the results of this survey confirmed the widespread endemicity of KPC-KP in Italian healthcare facilities, and their predominant role among CPE. Infections with KPC-KP affect mostly older patients hospitalised in medical or surgical wards with a known history of previous hospital admission in the country. The results of this present study also reveal a concerning percentage of resistance to colistin, which is a matter of major concern given the dearth of treatment options against CPE.

In Italy, the emergence of colistin-resistant KPC-KP has been reported since 2010 [13] and, in the first Italian nationwide cross-sectional survey on CRE, carried out in mid-2011, the overall percentage of colistin resistance among KPC-KP was found to be 22.4%, with colistin-resistant isolates reported from 13 of 25 participating hospital laboratories [7]. In the EuSCAPE study, the colistin resistance percentage found among KPC-KP was almost double, and colistin-resistant KPC-KP isolates were detected from all 21 PLs in the study. We did not have information to derive the total number of affected hospitals among the 45 served by the 21 PLs, however the PLs were distributed all across the country. A similar situation of nationwide dissemination of colistin-resistant KPC-KP has not yet been reported in other settings of high KPC-KP endemicity [14].

According to data available from the European Surveillance of Antimicrobial Consumption Network (ESAC-NET) database [15], consumption of polymyxins in the hospital sector in Italy increased from 0.0017 to 0.0194 Defined Daily Dose (DDD) per 1,000 inhabitants per day in the period from 2007 to 2012. This 10-fold increase reflects the increasing dissemination of multidrug-resistant Gram-negative infections for which colistin remains one of the few therapeutic options and most likely contributed to selection of colistin-resistant strains among KPC-KP.

To control the spread of KPC-KP in Italy, in February 2013 the Ministry of Health issued a circular letter [16] asking the Italian regions to report all cases of bloodstream infections due to CPE of the species *K. pneumoniae* or *E. coli* and recommending control measures to limit the spread in healthcare settings. These control measures consist of: (i) active screening of selected patient groups including patients who have been in contact with CPE-colonised or infected patients, and patients coming from countries with high CPE endemicity and, if feasible, patients admitted to ICU or other high-risk wards and patients with a history of previous hospitalisation; (ii) isolation or cohorting of infected/colonised patients, separate cohort nursing care, and implementation of contact precautions, according to the recommendations issued at national and international level [17-20].

These measures require huge efforts and resources in an endemic situation like the one highlighted in this study, since patients with KPC-KP infection or colonisation are not confined to ICUs, but can be found in normal hospital wards. It seems therefore urgent to develop and implement a national plan for the prevention and control of CPE infections in Italy that includes
an extensive surveillance system and more comprehensive guidelines on infection control measures. Sufficient resources should be allocated to contain the further dissemination of CPE in healthcare institutions.

Acknowledgements

We thank Alessandra Carattoli for helpful discussion and support to this study.

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ed in part by a grant from the Italian Ministry of Health (CCM 2013 “Sorveglianza di laboratorio di infezioni batteriche da patogeni antibiotico-resistenti sottoposti a sorveglianza eu-
ropea”). NEL in Siena was partially supported by a research grant from EvoTAR (no. HEALTH-F3-2011-282004) to G.M.R.

Conflict of interest

MM, MR, AGF, HG and AP have nothing to declare; GMR has received research grants from Pfizer, Astra-Zeneca, Cubist, Angelini, Becton-Dickinson, bioMérieux, Biotest, VenatoRx, has served as consultant for Pfizer, Astra-Zeneca, Cubist, Angelini, Menarini, Achaogen, Rempura, Dureta, Medivir, Biotest, and has served in the Speaker’s Bureau for Pfizer, Astra-Zeneca, Novartis, Angelini, Curetis, Biotest and Basilea. TG has served in the Speaker’s Bureau for bioMérieux.

Author contributions

MM, TG, AP and GMR contributed to the design of the study, to draft and finalise the manuscript; TG, SP, FA performed the phenotypic characterisation of the isolates; MM, MR, AGF performed the detection of the carbapenemases genes by PCR; MM, MR, AGF, TG, SP, FA entered and analysed data; HG planned and coordinated the EuSCAPE study and revised the final manuscript; PLs provided isolates and clinical and demographic data of patients.

Members of the Network EuSCAPE-Italy


References


We present preliminary results of influenza vaccine effectiveness (VE) in New Zealand using a case test-negative design for 28 April to 31 August 2014. VE was estimated for age and time of admission among all ages against severe acute respiratory illness hospital presentation due to laboratory-confirmed influenza was 54% (95% CI: 19 to 74) and specifically against A(H1N1)pdm09 was 65% (95% CI: 33 to 81). For influenza-concordant primary care visits, VE was 67% (95% CI: 48 to 79) overall and 73% (95% CI: 50 to 85) against A(H1N1)pdm09.

Introduction
The SHIVERS (Southern Hemisphere Influenza and Vaccine Effectiveness, Research and Surveillance) study [1] has allowed estimation of vaccine effectiveness (VE) against influenza illness requiring hospitalisation since 2012 and against influenza illness requiring primary care consultation (sentinel general practices) since 2013. The study captures an ethnically diverse urban population of approximately 838,000 people in Auckland, New Zealand. Patients in the 16 sentinel general practices are part of the population served by the four participating hospitals. VE estimates for 2012 from the hospital arm of the study [2] and from both hospital and community arms in 2013 [3] have been reported previously. Here we report the 2014 influenza season interim estimates of VE against laboratory-confirmed influenza general practice (primary care) visits and hospitalisations in Auckland, New Zealand.

In New Zealand, seasonal trivalent inactivated influenza vaccine is offered annually free of charge to all adults aged 65 years and over, pregnant women and all individuals over six months of age with chronic medical conditions that are likely to increase the severity of the infection. Influenza vaccines are also available on the private market for all other individuals over six months of age. The influenza season usually occurs between March and September and the vaccine is available from late February.

The influenza strains in the southern hemisphere vaccine in 2014 were A/California/7/2009 (H1N1)-like virus, A/Texas/50/2012 (H3N2)-like virus and B/Massachusetts/2/2012-like virus (B/Yamagata lineage) as recommended by the World Health Organization for trivalent influenza vaccines [4].

Methods
Using the case test-negative design to estimate VE as previously described [3], we estimated the effectiveness of seasonal trivalent inactivated influenza vaccine against laboratory-confirmed influenza in patients hospitalised with severe acute respiratory infections (SARI) and in patients presenting to a sentinel general practice with an influenza-like illness (ILI) during the 2014 influenza season. Ethics approval was obtained from the Northern A Health and Disability Ethics Committee (NTX/11/102 AM02).

Patients with SARI or ILI were defined as requiring hospitalisation (SARI) or attending a general practice (ILI) with a history of fever or measured temperature ≥38 °C, cough and onset within the past 7 days.
Hospitalised patients were recruited from individuals aged six months and older who were admitted to one of the four public hospitals covering all the population in the study catchment area in south, central and east Auckland. Community cases were identified from 16 sentinel general practices with 103,884 enrolled patients selected to be broadly representative of the population.

Data collection began on 28 April 2014. Analysis was restricted to the influenza season, which defined as being from the start of the first two consecutive weeks with two or more influenza cases (2 June 2014). The interim data collection was until 31 August 2014, based on the requirements to complete the analysis in time for the World Health Organization strain selection meeting in September.

Hospitalised patients were identified following screening by research nurses of all patients admitted with respiratory illness. Patients who gave verbal consent completed a case report form and provided a nasopharyngeal swab or aspirate for influenza virus testing.

All ILI patients presenting to one of the sentinel general practices were screened by the general practitioner or practice nurse, and data for all consenting patients were entered on an electronic form in the practice management system. A nasopharyngeal or throat swab was collected for influenza virus testing.

A confirmed case of influenza was defined as a patient with SARI or ILI with a positive laboratory result for any influenza virus detected by real-time reverse transcription polymerase chain reaction (rRT-PCR). Nasopharyngeal and throat swabs were tested using the United States Centers for Disease Control and Prevention (CDC) rRT-PCR protocol [5] or the AusDiagnostic PCR protocol [6]. The two assays performed very similarly [3]. rRT-PCR assays detected influenza virus types A and B and subtyped. A convenience sample was characterised antigenically using established methods [7].

For ILI cases, vaccination status was based on the presence or absence of documentation in the general practice electronic records of receiving one or more doses of the 2014 influenza vaccine, depending on age who were admitted to one or more doses of the 2014 seasonal influenza vaccine.

Patients excluded were infants less than 6 months of age who are not recommended to be vaccinated, those vaccinated less than 14 days before admission or presentation and those with symptom onset more than seven days before admission or presentation. For patients with multiple episodes, the first influenza virus-positive episode was used for the analysis or the first illness episode if there was no influenza virus-positive episode.

For all patients, covariates included age, sex, ethnicity, current smoking status and chronic medical conditions. Further data collected on SARI patients included a patient- or caregiver-reported measure of dependence (classified as the requirement for assistance with normal activities or full dependency on nursing care), long-term use of oxygen, low income (using a small neighbourhood measure reflecting eight dimensions of deprivation [8]), a clinical judgement of obesity and a standard self-rated health item scored dichotomously as fair or poor versus good, very good or excellent overall health [9].

VE is presented for all influenza viruses and A(H1N1)pdm09. For the SARI dataset, less than 1% (3/319) of data were missing for any variable. The ILI dataset had no missing values. Interim VE estimates were calculated from all participants enrolled between 28 April and 31 August 2014. Standard logistic regression was used to compare the odds of vaccination among influenza-positive versus influenza-negative participants for both ILI and SARI, with VE estimated as 100% × (1 – odds ratio). VE was also calculated adjusting for age and the week of the admission or presentation. As a sensitivity analysis for the SARI data, a more comprehensive adjustment was also carried out, similar to the previously reported analysis in 2013 [3]. For this adjustment, we used 2013 data to model the propensity to be vaccinated based on all potential confounders. The VE was then calculated adjusted for each individual’s propensity to be vaccinated.

**Results**

The number of ILI and SARI patients in this study are shown by influenza virus status in Figure 1.

A total of 1,272 SARI patients were eligible: all were recruited and swabbed for influenza. A total of 1,226 ILI patients were recruited, of whom 1,221 were swabbed (99.6%). A total of 519 SARI and 919 ILI patients were included in the analysis, of whom 148 (29%) and 384 (42%) were influenza virus positive, respectively (Figure 2).

Of the 532 influenza cases detected in both SARI and ILI patients, 466 (88%) were type A, with 339 (64%) A(H1N1)pdm09, 32 (6%) A(H3N2) and 95 (18%) not subtyped (Table 1).

There were 66 (12%) type B detections. Among the 66 influenza B viruses, 48 were Yamagata lineage, one was Victoria lineage, and lineage was not determined in 17. Of the 48 Yamagata lineage, 25 were antigenically typed as B/Massachusetts/2/2012 00-like viruses and 23 were not antigenically typed. The one B/Victoria lineage virus was antigenically typed as B/Brisbane/60/2008-like virus.
Vaccine effectiveness

Of the 148 SARI patients who tested influenza virus positive, 35 (24%) were vaccinated, compared with 113 (30%) of the 371 who tested negative. Of the 384 ILI patients who tested influenza virus positive, 37 (10%) were vaccinated, compared with 116 (22%) of the 535 who tested negative (Figure 2).

The proportion vaccinated did not change throughout the season. For influenza-confirmed SARI, the crude VE for one or more vaccine doses against all circulating influenza virus strains was 34% (95% confidence interval (CI): −3 to 57) (Table 2).

After adjustment for age and week of admission, the estimated VE was 54% (95% CI: 19 to 74). The adjusted VE for the prevailing circulating subtype, influenza A(H1N1)pdm09, was 65% (95% CI: 33 to 81). VE was not calculated for other subtypes, or for individuals 6 months to 17 years of age because of sparse data. Adjusted VE against all influenza hospitalisation in the 18–49-year age group was 46% (95% CI: 3 to 63) and in the 65 and over age group, 58% (95% CI: −36 to 87). SARI influenza-positive cases were significantly more like to be young (under five years of age) or old (65 years and older) and smokers than were SARI influenza-negative patients. There was no significant difference by chronic disease, sex, income, pregnancy or self-reported health status. In the SARI sensitivity analysis adjusted for the propensity to be vaccinated, the VE for all ages was 50% (95% CI: 19 to 69).

For influenza-confirmed ILI cases, the crude VE was 61% (95% CI: 43 to 74). After adjustment for age and week of presentation, the estimated VE was 67% (95% CI: 48 to 79). The adjusted VE for the prevailing circulating subtype, influenza A(H1N1)pdm09, was 73% (95% CI: 50 to 85). VE was not calculated for younger people or those aged 65 years and over because of sparse data. For the 18–49-year age group, the adjusted VE was 66% (95% CI: 30 to 84) and in the 50–64 year-olds, it was 57% (95% CI: −1 to 82).

Discussion

The SHIVERS study allows timely estimation of the protective effect of seasonal influenza vaccine in the southern hemisphere season. These preliminary results suggest that the 2014 vaccine was 54% effective in preventing hospitalisation for influenza and 67% effective against presentations to sentinel general practices. The 2014 season has been dominated to date by the influenza A(H1N1)pdm09 virus. VE was similar across adult age groups, although numbers were too small for accurate estimates in children and elderly people.

The New Zealand seasonal experience is very similar to interim VE estimates reported from Canada and the United States for the 2013/14 influenza season, when the dominant circulating virus was also A(H1N1)pdm09: the VE point estimate was 59% for preventing hospitalisation [10] and 74% for preventing medically attended influenza [11] in Canada, while in the United States, the interim VE was 61% against medically attended infections.
**Figure 2**
Flowchart of all selected, recruited and tested patients with influenza-like illness and severe acute respiratory infection for interim influenza vaccine effectiveness analysis, New Zealand, 28 April–31 August 2014

**Table 1**
Vaccinated and unvaccinated influenza cases by virus type and subtype among hospital (n=519) and general practice participants (n=919), New Zealand, 2 June–31 August 2014

<table>
<thead>
<tr>
<th>Influenza virus type</th>
<th>Hospitalised with severe acute respiratory infection</th>
<th>General practice visits for influenza-like illness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number vaccinated (%)</td>
<td>Number unvaccinated (%)</td>
</tr>
<tr>
<td>All</td>
<td>35 (100)</td>
<td>113 (100)</td>
</tr>
<tr>
<td>Any A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30 (86)</td>
<td>108 (96)</td>
</tr>
<tr>
<td>A(H1N1)pdm09</td>
<td>22 (63)</td>
<td>97 (86)</td>
</tr>
<tr>
<td>A(H3N2)</td>
<td>7 (20)</td>
<td>7 (6)</td>
</tr>
<tr>
<td>All B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5 (14)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>B/Victoria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B/Yamagata lineage</td>
<td>1 (3)</td>
<td>2 (2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Not all cases of influenza A and B were subtyped. The number of subtypes does not add up to the number of all influenza A viruses identified.

<sup>b</sup> B/Victoria = B/Victoria lineage-B/Brisbane/60/2008-like.

ILI: influenza-like illness; SARI: severe acute respiratory infections.
influenza [12]. In contrast, the interim VE point estimate from Spain was 44% against all influenza strains, and even lower (33%) for the dominant circulating virus, A(H1N1)pdm09 [13].

Our interim report has several limitations. Similar to other interim VE reports [11], we relied on self-reported vaccination status for hospitalised patients. Not all laboratory results were available as of 31 August 2014 (70 ILI, 356 SARI). In addition, the analysis is adjusted for only two potential confounders (age and week of admission or presentation), although a propensity-adjusted sensitivity analysis for SARI patients produced a similar VE estimate. For this interim estimate, we were unable to estimate VE for young children with two doses of vaccine. We expect to be able to examine this and produce stratified VE estimates by age in our final season report.

This is the third year we have reported the effectiveness of trivalent seasonal influenza vaccine in the New Zealand setting. We have shown the continued predominance of circulating influenza A(H1N1)pdm09 virus and a continued moderate vaccine effectiveness against this strain, similar in magnitude to the North American estimates for the 2013/14 season. The 2014/15 northern hemisphere seasonal vaccine will contain the same components as the 2014 southern hemisphere vaccine [14]. These results may thus add useful information to consider in preparing for the upcoming northern hemisphere influenza season and in selecting strains for the next southern hemisphere season.

### Table 2
Estimated influenza vaccine effectiveness, by participant age group and by influenza virus type and subtype: crude plus age- and time-adjusted models, New Zealand; 2 June–31 August 2014

<table>
<thead>
<tr>
<th>Influenza type by age group</th>
<th>Influenza positive</th>
<th>Influenza negative</th>
<th>Vaccine effectiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number vaccinated</td>
<td>Total</td>
<td>%</td>
</tr>
<tr>
<td>SARI Overall</td>
<td>35</td>
<td>148</td>
<td>24</td>
</tr>
<tr>
<td>6 months–17 years</td>
<td>4</td>
<td>42</td>
<td>10</td>
</tr>
<tr>
<td>18–49</td>
<td>9</td>
<td>58</td>
<td>16</td>
</tr>
<tr>
<td>50–64</td>
<td>10</td>
<td>29</td>
<td>34</td>
</tr>
<tr>
<td>≥65</td>
<td>12</td>
<td>19</td>
<td>63</td>
</tr>
<tr>
<td>A(H1N1)pdm09</td>
<td>22</td>
<td>119</td>
<td>18</td>
</tr>
<tr>
<td>IRI Overall</td>
<td>37</td>
<td>384</td>
<td>10</td>
</tr>
<tr>
<td>6 months–17 years</td>
<td>2</td>
<td>143</td>
<td>1</td>
</tr>
<tr>
<td>18–49</td>
<td>12</td>
<td>168</td>
<td>7</td>
</tr>
<tr>
<td>50–64</td>
<td>12</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>≥65</td>
<td>11</td>
<td>13</td>
<td>85</td>
</tr>
<tr>
<td>A(H1N1)pdm09 all</td>
<td>14</td>
<td>220</td>
<td>6</td>
</tr>
<tr>
<td>A(H1N1)pdm09 ≥65 years</td>
<td>1</td>
<td>2</td>
<td>50</td>
</tr>
</tbody>
</table>

CI: confidence interval; ILI: influenza-like illness; NA: not applicable, as there were insufficient data to report VE estimates, SARI: severe acute respiratory infections.

* Adjusted for six age groups: 6 months–5 years, 6–17, 18–44, 45–64, 65–79 and ≥80 years and week in the season.

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**Acknowledgements**

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The 16 participating sentinel general practices from Auckland Primary Health Organisation, East Tamaki Health Care and ProCare.

Conflict of interest
None declared.

Authors’ contributions
Nikki Turner: principal investigator, involved in study design, implementation, analysis, manuscript development. Nevil Pierse: involved in study design, methodological design, data analysis, interpretation and manuscript development. Q Sue Huang: principal investigator for the larger SHIVERS study, involved in study design, implementation and manuscript development. Sarah Radke: involved in study design, data collection and analysis and manuscript development. Ange Bissielo: involved in data collection and analysis. Mark Thompson: involved in study design, interpretation and manuscript development. Heath Kelly: involved in study design, methodological analysis, data analysis and interpretation, manuscript development and editing.

References


An increase of invasive meningococcal disease caused by Neisseria meningitidis serogroup Y has been noted in Sweden since 2005, and to a lower extent throughout Europe. The present study describes the epidemiology of invasive N. meningitidis isolates in Sweden in the period between 2010 and 2012, with a focus on serogroup Y. We also aimed to find an optimal molecular typing scheme for both surveillance and outbreak investigations. All invasive N. meningitidis isolates in Sweden during the study period (n=208) were genetically characterised. Serogroup Y predominated with 22/57, 31/61 and 44/90 of all invasive isolates (incidence 0.23, 0.33 and 0.46 per 100,000 population) in 2010, 2011 and 2012 respectively. In each of these years, 15/22, 22/31 and 19/44 of serogroup Y isolates were genetically clonal (Y: P1.5–2,10–1,36–2: F4–1: ST-23(cc23), ‘porB’ allele 3–36, fHbp allele 25 and penA allele 22). Our findings further support those of others that currently recommended FetA typing could be replaced by FHbp. Moreover, in line with a previous study that we conducted, the current results indicate that highly variable multilocus variable-number tandem repeat analysis (HV-MLVA) can be used as a first-hand rapid method for small outbreak investigations.

Introduction

Neisseria meningitidis (the meningococcus) is a Gram-negative diplococcus carried asymptomatically in the pharynx by approximately 10% of the population [1]. It is also a potentially devastating pathogen causing meningitis and septicaemia. Invasive meningococcal disease (IMD) occurs mainly in sporadic cases but also as outbreaks and epidemics. Meningococcal populations are genetically and antigenically highly diverse [2] and vary greatly globally and over time, but the majority of IMD is caused by a limited number of clonal complexes, known as hyper-virulent lineages [3]. Therefore, detailed characterisation of circulating meningococcal strains is important in terms of vaccination policy decisions, outbreak management, as well as monitoring antibiotic susceptibility and vaccine coverage.

The polysaccharide capsule surrounding the bacterium defines the meningococcal serogroup. The capsule is an important virulence factor and IMD is mainly restricted to encapsulated meningococci belonging to serogroups A, B, C, W, X and Y. The capsule is also a polysaccharide vaccine component in available conjugate vaccines for serogroups A, C, W and Y [4]. The serogroup distribution is highly regional [5]. In Europe, the main circulating strains belong to serogroups B and C [6]. As previously described, serogroup Y has increased in Sweden from 0.04 per 100,000 population in 2005 to 0.23 per 100,000 population in 2010 [7]. An emergence of serogroup Y has also been noted in some other European countries, however, the highest relative proportions are found in Scandinavia [8,9].

In addition to serogroup designation, it is currently recommended by the European Meningococcal Disease Society (EMGM) that meningococcal strains are designated by variable regions (VR) in the Porin A (PorA) and the Ferric enterobactin transport protein A (FetA) proteins as well as multilocus sequence typing (MLST) sequence type (ST) and clonal complex (CC) [10]. PorA and FetA are two surface antigens, which are recommended for rapid investigation of disease outbreaks. MLST, based on seven housekeeping genes, is ideal for studying population biology and evolution of the organism on a national and international level. For enhanced resolution, genotyping of a third surface antigen, Porin B (PorB), may also be performed [11]. Finally, further characterisation can be achieved with the penA gene encoding the penicillin-binding protein 2 (used in surveillance of penicillin susceptibility) [12] and fHbp encoding the serogroup B vaccine component Factor H binding protein (fHbp) [13,14].

Another molecular method that has been proposed for an alternative typing paradigm, mainly suited for investigating localised outbreaks, is multilocus variable-number tandem repeat analysis (MLVA) [15-18]. MLVA is a polymerase chain reaction (PCR)-based technique, which uses the variability in the numbers of short tandem repeats to create DNA fingerprints used...
in epidemiological studies. The highly variable MLVA (HV-MLVA) developed by Schouls et al. [18] has shown high discriminatory capacity for serogroup C isolates and has been considered suitable for outbreak identification [19].

The aims of the present study were to describe the current epidemiology of invasive \textit{N. meningitidis} isolates including the dominating serogroup Y in Sweden, and to find an optimal molecular typing scheme with appropriate resolution power for both surveillance and outbreak investigations.

**Methods**

**Bacterial isolates and phenotypic characterisation**

In Sweden, all invasive cases of meningococcal disease according to the European Union case definition are mandatorily reported by clinicians to the Swedish Institute for Infectious Disease Control (SMI) [20]. The corresponding isolates are sent to the Public Health Agency of Sweden, where they are routinely cultured on chocolate agar at 37°C with 5% CO₂ overnight and subsequently serogrouped by co-agglutination [21]. Further genosubtyping (PorA typing) is then conducted as previously described [22] and antibiotic susceptibility determined using the Epsilometer (E)test method (bioMérieux, Marcy l’Etoile, France). Basic epidemiological data (age, sex, area of residence, clinical site of isolation and date of sample collection) are gathered for all isolates from cases. This study included all invasive \textit{N. meningitidis} isolates in Sweden between 2010 and 2012. The serogroup B strain MC58 [23] was included in all analyses as a reference.

**Nucleic acid extraction**

The DNA used for sequence-based typing methods and MLVA was extracted with a NorDiag Bullet instrument (DiaSorin, Dublin, Ireland). For the automatic extraction, 20 colonies from each cultured organism were suspended in 2 ml NaCl (0.85%) and 100 µl of this solution were subsequently processed with the NorDiag Bullet with the Bullet BUGS’n BEADS kit according to the manufacturer’s recommendation (DiaSorin). All DNA preparations were stored at 4°C prior to the PCR.

**Figure 1**

Incidence of invasive meningococcal disease caused by \textit{Neisseria meningitidis} serogroups B, C, W and Y in Sweden, 2000–2012 (n=642)

**Figure 2**

Distribution of the genetically defined predominant \textit{Neisseria meningitidis} clone YI with different sulfamethoxazole susceptibilities, the second and third most common serogroup Y clones (YII and YIII) and all other invasive \textit{N. meningitidis} serogroup Y isolates in Sweden, 2000–2012 (n=163)

Data for this Figure originate both from this study and a previous one [7].
Polymerase chain reaction and DNA sequencing

The MLST genes: abcZ, adk, aroE, fumC, gdh, pdhC and pgm together with fetA, fHbp, porB and penA were amplified and sequenced as previously described [7,19]. In short, the PCR was performed using a Rotor-Gene Q real-time PCR system (Qiagen, Hilden, Germany) and detected with SYBR green I. The nucleotide sequences were determined by capillary electrophoresis using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator v3.1 (Applied Biosystems, Warrington, UK). The sequence alignments for the MLST genes were assembled using the Bionumerics software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium, http://www.applied-maths.com/bionumerics) and all other genes were assembled using the ChromasPro software version 1.33 (Technelysium Pty Ltd, Brisbane, Australia, http://technelysium.com.au/). The different sequences were assigned allele numbers using the N. meningitidis sequence query database [24].

Multilocus variable-number tandem repeat analysis

The HV-MLVA with four highly variable variable-number tandem repeat (VNTR) loci (VNTR4–4, VNTR9–2, VNTR4–2, and VNTR4–3) was performed as previously described by Törös et al. [19] with the primers from Schouls et al. [18]. In short, the PCR was performed on an Applied Biosystems 9700 or 2720 PCR machine and the fragments were separated on an ABI PRISM 3130xl Genetic Analyzer using the GeneScan LIZ 500 size standard and GeneScan module with filter set G5 (Applied Biosystems). The sizing of the fragments was performed with the GeneMapper software v4.0 (Applied Biosystems). All isolates were run in duplicates in separate runs.

Data analysis

The ability of each method to discriminate between strains was evaluated on the basis of their discrimination index, using Simpson's index of diversity (ID) [25]. The ID determines the probability that two randomly picked strains are allocated to different types. Confidence intervals (CI) of 95% were calculated [26]. Cluster analysis of the MLST data was performed using a categorical coefficient and displayed in a minimum spanning tree (MST) created with the Bionumerics software version 7.1, with the priority rule of first linking types which have the highest number of single-locus variants. For the HV-MLVA, a dendrogram was generated using a categorical coefficient and unweighted pair group method with arithmetic average (UPGMA).

Results

Epidemiology

A total of 208 invasive N. meningitidis isolates were characterised during the study period, including 57 in 2010, 61 in 2011 and 90 in 2012. The isolates originated from clinical specimens of cerebrospinal fluid (n=44),
The current clonal pattern among serogroup Y isolates is further illustrated in Figure 3 and 4 where the PorA types P1.5–2,10–1,36–2 and P1.5–1,2–2,36–2, FetA VR F3–1 and 5–8, fHbp allele 25 and ST-23 are the most frequent. Furthermore, 29/57 (51%) of all serogroup C isolates have PorA type P1.5,2,36–2, FetA VR F3–3, fHbp allele 22 and ST-11 (Figures 3 and 4).

The age distribution of patients with IMD during 2010 to 2012 is shown in Figure 5. Serogroup B was most common among children (median age: 19 years; inter quartile range (IQR): 7–33), serogroup C among young adults (median age: 20 years; IQR: 17–59) and serogroup A among an older age group compared to the other serogroup Y isolates. The mortality rate among patients with IMD caused by serogroup Y from 2010 to 2012 was 6/97 (6%; 95% CI: 1.4–11%) compared to the total mortality rate due to all serogroups combined during the same time period which was 18/208 (9%; 95% CI: 5–12%). The IMD mortality rate in Sweden during 2000 to 2009 was 67/569 (12%; 95% CI: 9–14%).

**Resolving power of molecular typing methods used for surveillance**

The discriminative ability of a MLST and the porA, porB, fetA, fHbp and penA genes shows that as single targets PorA VR 1, 2, and 3 had the highest Simpson’s ID (0.849; 95% CI: 0.811–0.886) and serogroup had the lowest (0.664; 95% CI: 0.628–0.700). Among combinations of four, on the basis of serogroup, porA and MLST, this combination including fetA had the highest
ID (0.956; 95% CI: 0.940–0.972) and serogroup, porA and MLST including fHbp had the lowest (0.950; 95% CI: 0.933–0.967).

Outbreak investigations

A spatiotemporal association was defined as isolates of the same serogroup, collected in the same central county within a timeframe of one month. Of the 208 N. meningitidis invasive isolates from 2010 to 2012, 35 isolates were spatiotemporally associated in 16 different clusters and represented potential outbreaks. There were 13 clusters of two isolates and three clusters of three isolates. Eight of the spatiotemporal clusters (cluster nr 1–8 in Figure 6) did not share a common PorA type within the clusters and the cases had not been in direct contact with each other and therefore there were no further outbreak investigations. In spatiotemporal clusters nr 10–13, the isolates were identical within each cluster regarding serogroup and all 12 target genes (Figure 6), but no connection between cases was found and the isolates were separated by HV-MLVA. Within each of the three spatiotemporal serogroup B clusters 14–16 (Figure 6) the isolates were identical regarding all 12 sequenced genes (the isolates in cluster 14 all had one insertion in aroE allele 9 but were still regarded as belonging to ST-41). In addition, the isolates were clustered together in the HV-MLVA when MLVA types did not differ in more than one VNTR locus (single-locus variant, SLV). However, spatiotemporal cluster 14 and 16 were the only spatiotemporal clusters which had confirmed connections between cases. The HV-MLVA results from all 208 isolates showed another seven HV-MLVA clusters comprising fifteen isolates in total (if SLVs were allowed), of which four did have identical genetic profiles, but none of them did share a spatiotemporal link and were therefore not included in Figure 6.

Discussion

This study aimed to describe the epidemiology of invasive N. meningitidis isolates in Sweden between 2010 and 2012, specifically the dominating serogroup Y, and to identify an optimal molecular typing scheme with appropriate resolution power for both surveillance and outbreak investigations in low-endemic areas. Although whole genome sequencing is becoming more cost beneficial than traditional Sanger sequencing per isolate, optimal molecular typing schemes will still be important when fast results are needed, and for smaller laboratories that lack the need of a next generation sequencer. All isolates causing IMD in Sweden during 2010, 2011 and 2012 were characterised by capsular group, MLST, sequencing of the porA, fetA, porB, penA, fHbp genes and a MLVA using four highly variable loci.

The epidemiology in Sweden has changed most notably with an increase of IMD between 2010 and 2012. IMD caused by serogroup C has declined slightly from 2010 to 2011 and 2012, and serogroup B somewhat increased (Figure 1). The genetic characterisation of circulating N. meningitidis causing IMD shows that serogroup Y was the most prevalent in Sweden, and the previously genetically described predominant clone YI [7] was still dominating among serogroup Y strains. However, the overall IMD incidence is still low and although a vaccine against serogroup Y is available, it is probably not
**Figure 6**
Dendrogram of invasive *Neisseria meningitidis* isolates generated from a highly variable multilocus variable-number tandem repeat analysis, Sweden, 2010–2012 (n=35 isolates)

Spatiotemporal cluster

<table>
<thead>
<tr>
<th>SG</th>
<th>PorA VR1, VR2, VR3</th>
<th>FetAVR</th>
<th>ST</th>
<th>'porB</th>
<th>fHbp</th>
<th>penA</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>P1.17-1,2-3,37</td>
<td>F1-34</td>
<td>1127</td>
<td>3-37</td>
<td>14</td>
<td>391</td>
</tr>
<tr>
<td>B</td>
<td>P1.17-1,2-3,37</td>
<td>F1-34</td>
<td>1127</td>
<td>3-37</td>
<td>14</td>
<td>391</td>
</tr>
<tr>
<td>B</td>
<td>P1.7,16,35</td>
<td>F3-3</td>
<td>32</td>
<td>3-24</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Y</td>
<td>P1.5-1,2-19,36-2</td>
<td>F5-8</td>
<td>10211</td>
<td>3-36</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>Y</td>
<td>P1.5-1,2-2,36-2</td>
<td>F5-8</td>
<td>23</td>
<td>2-55</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
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FetA: Ferric enterobactin transport protein A; PorA: Porin A; SG: serogroup; ST: sequence type; VR: variable region.

A similarity line has been drawn at 75% (maximum one single-locus variant). Only spatiotemporally associated isolates (Sweden, 2010–2012) are included and have been designated a spatiotemporal cluster number 1–16. Additional information about serogroup and allele or VR from sequencing the *porA*, *fetA*, *'porB* (partial fragment), *fHbp* and *penA* genes and the ST from the multilocus sequence typing is provided for each strain.
justifiable to change the vaccine policy in Sweden which currently does not include any vaccines against IMD in the general vaccination programme. Moreover, a shift of sulfamethoxazole susceptibility has occurred sometime around 2010 where isolates otherwise identical to the predominant serogroup Y clone instead are susceptible to sulfamethoxazole. Sequencing of the folP gene in a representative collection of the serogroup Y isolates in this study has not shown any of the mutations previously associated with sulphonamide resistance (data not shown) [27,28]. Investigating larger parts of the genome, including differences in expression, can possibly elucidate the mechanisms responsible for this sulfamethoxazole susceptibility shift. Like serogroup Y, serogroup C also presented a fairly clonal pattern whereas serogroup B isolates were rather genetically heterogeneous (Figure 3 and 4).

The age distribution of patients with IMD in Sweden during 2010 to 2012 regarding serogroups B and C is similar to the age incidence pattern during the period from 1995 to 2009 where serogroup B is most common among patients under 10 years of age and serogroup C is most common in the age group including 10 to 19 year-olds (data not shown). The mean age among patients with IMD caused by serogroup Y in 2010 to 2012, 58.9 years, has somewhat increased compared to the mean age of 54.5 years for this serogroup in 2000 to 2009 (data not shown). A considerably lower average age of serogroup Y patients in 2011 has been reported in Denmark (26 years), France (20 years), Italy (26.9 years), Portugal (15.5 years) and Spain (31 years) [9]. Further studies need to be performed to give a clearer picture as to whether the virulence or transmissibility is increased in the predominant serogroup Y clone. Moreover, the mortality rate for IMD has somewhat decreased during 2010 to 2012 compared to the mortality rate during 2000 to 2009, however this was not statistically significant. The mortality rate among serogroup Y cases has also decreased slightly between the periods 2000 to 2010 and 2010 to 2012, however the difference was again not statistically significant. The decrease may be partly due to the recent small decrease in median age among patients with IMD caused by meningococci belonging to this serogroup.

The resolving power of a molecular typing method is recommended to have a discrimination level of at least 0.90 [25]. Serogroup:porA:fetA typing has previously shown a value of 0.963 [29] which is similar to the discrimination index achieved in this study (0.952; 95% CI: 0.935–0.969) with the same typing targets (data not shown). Although serogroup:porA:fetA:ST had the highest index of diversity achieved in this study, our results show that replacing the fetA gene with fHbp only reduces the discrimination ability by 0.006 (no statistically significant difference). Concurrently, Lucidarme et al. [30] investigated the comparability between fetA and fHbp in terms of diversity, after it had been recommended in England and Wales to additionally incorporate fHbp for routine genotypic surveillance. Their study (on 613 invasive isolates) actually showed that fHbp had significantly (non-overlapping 95% CIs) better resolving power than fetA. These findings indicate that the level of discrimination gained from fHbp is partially complementary to that of fetA. This could strengthen the argument that considering labour and cost, and with regard of the new serogroup B vaccines, it would be beneficial to substitute the current routine marker fetA with fHbp.

In terms of outbreak investigations, HV-MLVA detected three small clusters with spatiotemporal connections and identical genetic profiles (spatiotemporal clusters 14–16 in Figure 6), which further supports that these strains were truly involved in small outbreaks. Although no connection could be confirmed between cases in spatiotemporal cluster 15, the cases were both of similar age and from the same county and thus the cases could still have been related.

Fifteen isolates in the present study were clustered in seven HV-MLVA clusters without having a spatiotemporal connection (data not shown). However, without a spatiotemporal connection, these would normally never have been subjected to a HV-MLVA analysis. Moreover, the results of the HV-MLVA suggest that, after identifying the capsular group and receiving clinical data, it could have been sufficient to perform only the HV-MLVA to get indications of potential outbreaks. In an outbreak situation where time is of high importance, not having to await the results of PorA typing or whole genome sequencing for the subsequent HV-MLVA analysis would be beneficial.

We have previously compared HV-MLVA to rep-PCR with the DiversiLab system and DNA sequencing on invasive serogroup C isolates which showed that HV-MLVA helped strengthen all of the spatiotemporal linkages [19]. The use of MLVA to trace transmission has been deemed questionable due to the low stability of VNTRs. Transmission-dependent variation of tandem repeats in meningococci has been investigated by Elias et al. [31] in a study using four highly variable VNTR loci together with another eight standard MLVA loci. The observed overall variation was considerably smaller than predicted and the method was considered most useful for outbreaks containing few transmissions. Considering this, HV-MLVA could be valuable in low-endemic areas such as Sweden where outbreaks are fairly rare and connected cases usually only consists of no more than three contacts, as shown by our results. A fast detailed typing of spatiotemporally linked cases which can separate outbreaks from sporadic cases is important to inform public health measures to control IMD, such as deciding whether or not to offer prophylaxis in the form of antibiotics or vaccines.

In summary, serogroup Y was found to be the most prevalent serogroup in Sweden, and the previously genetically described predominant clone Y1 [7] (Y: P1.5–2,10–1,36–2: F4–1: ST-23(cc23), ‘porB allele 3–36,
fHbp allele 25 and penA allele 22) is still dominating. However, a sulfamethoxazole susceptibility subvariant of the clone appears to have emerged in 2010, and in 2011 represented 11/22 (50%) of the genetically defined predominant clone Y1 isolates. Our study supports previous studies that suggests that the FetA typing could be replaced by fHbp in the recommended designation (serogroup: \texttt{porA}\texttt{fetA}\texttt{ST}(CC)), which may be more suitable in the current vaccine era. Furthermore, this study including the additional serogroups A, B, E, W and Y, strengthens previous results on serogroup C isolates [19] suggesting that HV-MLVA is a first-hand rapid method for investigating outbreaks with few transmission events, where isolates have an identical serogroup and a common spatiotemporal connection.

Acknowledgements

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Conflict of interest

None declared.

Author’s contributions

Bianca Törös, Sara Thulin Hedberg and Paula Mölling were mainly responsible for the design and supervision of the study. Bianca Törös and Susanne Jacobsson performed the laboratory work. Bianca Törös analysed the results and all authors were involved in the discussion of the results. Bianca Törös drafted the paper and Sara Thulin Hedberg, Susanne Jacobsson, Hans Fredlund, Per Olcén and Paula Mölling revised the paper.

References


Proven transmission of *Chlamydia psittaci* between humans has been described on only one occasion previously. We describe an outbreak which occurred in Sweden in early 2013, where the epidemiological and serological investigation suggests that one patient, severely ill with psittacosis after exposure to wild bird droppings, transmitted the disease to ten others: Two family members, one hospital roommate and seven hospital caregivers. Three cases also provided respiratory samples that could be analysed by PCR. All the obtained *C. psittaci* sequences were indistinguishable and clustered within genotype A. The finding has implications for the management of severely ill patients with atypical pneumonia, because these patients may be more contagious than was previously thought. In order to prevent nosocomial person-to-person transmission of *C. psittaci*, stricter hygiene measures may need to be applied.

**Introduction**

Psittacosis is an infectious disease caused by *Chlamydia psittaci*, a strict intracellular bacterium. Typical symptoms include abrupt onset of fever, rigors, headache, myalgia, malaise, cough which usually is unproductive and atypical pneumonia [1-2]. Birds are the natural host for the bacterium but other animals including humans can get infected. Humans can get infected after contact with birds by inhaling dried contaminated bird secretions, dried-out droppings or dust from feathers [3]. The incubation period is approximately one to four weeks. Most infected people only experience mild influenza-like disease but severe illness can occur. The disease, which is notifiable by law in Sweden, is not common, with only five to ten cases reported yearly in the years preceding 2013 [4].

Cases are usually sporadic without epidemiological links to a common source. Between January and March 2013, there was an unusual increase in psittacosis cases in southern Sweden, when a total of 17 sporadic cases of psittacosis were reported, distributed across four counties. The annual number of cases in these counties had ranged from one to six during the preceding years. The primary case in this report was one of the sporadic cases. Investigations revealed that the main risk factor for the sporadic cases was exposure to wild birds and their droppings, as previously reported by Rehn et al. The increase in cases was suggested to have been due to weather factors that increased the secretion from affected birds, an unusual epizootic among wild birds, or a more transmissible strain [5].

Person-to-person transmission of *C. psittaci* has previously not been considered as an important pathway for transmission. It has only been described in two suspected episodes in the literature. In a report from 1977, a patient suffering from pneumonia believed to have been caused by *C. psittaci* transmitted the disease to his son, a neighbour, another patient and to eight hospital staff [6]. However, at the time of this study, the existing serological tests could not discriminate between *C. psittaci* and *C. pneumoniae*. In light of this and the fact that *C. pneumoniae* is known to spread readily between humans, it is questionable whether the outbreak was caused by *C. psittaci*. This issue has also been discussed by the United States Centers for Disease Control and Prevention [7]. There is, however, one recent documented outbreak with person-to-person transmission of psittacosis [8] that occurred in Scotland in 2012. In the outbreak, the primary case had pneumonia and transmitted the infection to five others. Four of these were family contacts and one a healthcare worker.

**Outbreak description**

On 23 January 2013, the communicable disease control unit in Kronoberg County, Sweden, was notified of a patient hospitalised with severe psittacosis. After two weeks, more cases of psittacosis were reported, all with an obvious epidemiological link to the primary case. An investigation was started in order to look into
the possibility and magnitude of human-to-human transmission. The primary case, a 73 year-old man was admitted to hospital on 13 January with a three-day history of chills and fever. X-ray imaging showed signs of pneumonia and the patient received intravenous cefotaxime treatment. Despite antibiotic treatment, his condition worsened during the next couple of days. His body temperature rose to above 40°C and his oxygen saturation fell from 95% to 80%. After three days he was transferred to the intensive care unit (ICU) and given moxifloxacin as additional treatment. A bronchoscopy was performed in the ICU and samples from bronchoalveolar lavage was sent for microbiological analysis. Test results came back positive for C. psittaci by polymerase chain reaction (PCR) but negative for Legionella pneumophila, Mycoplasma pneumoniae, influenza virus, respiratory syncytial virus and general bacterial culture. After only one day in ICU he had to be transferred to a university hospital for extracorporeal membrane oxygenation (ECMO) treatment where he was treated for 26 days, after which time he was moved back to the local ICU where he died a month later. On 25 January, an assistant nurse, who had been taking care of the primary case in the ICU on 18 January, fell ill in what she believed was influenza. After four days with high fever she was admitted to hospital with atypical pneumonia and was diagnosed with psittacosis by PCR. The same day, 25 January, a doctor who had also worked in the ICU on 18 January fell ill with similar symptoms as the assistant nurse. At that time he was off duty, and suspecting psittacosis, started to treat himself with doxycycline without taking any tests. When he came back to work on 11 February he still had a high C-reactive protein level of 230 mg/l (normal < 5 mg/l). Initial serological investigation was negative but on repeated sampling he showed evidence of past infection. The doctor who performed the bronchoscopy also self-treated with antibiotics as soon as he learned of the diagnosis and did not develop any symptoms. On 28 January an 89 year-old man was diagnosed with pneumonia at the same hospital after falling ill with fever and chills two days earlier. He was admitted and tested positive for psittacosis by PCR. This man had influenza. However, they were all subsequently diagnosed with pneumonia and three of them were admitted to hospital. When tested, they were negative for influenza, but three showed an acute serological response to C. psittaci, one showed signs of infection in follow-up and one was negative in all testing. Methods Epidemiological investigation All reported cases of psittacosis in Kronoberg County were interviewed about risk factors and exposure history. Staff working at the hospital were informed of the outbreak. A confirmed case was defined as a person who had been exposed to the primary case while he was symptomatic and subsequently, within the incubation period for psittacosis, presented with symptoms compatible with a clinical diagnosis of psittacosis, and where no other more likely risk exposures were present. In addition, laboratory confirmation of the diagnosis should have been established. Laboratory confirmation was considered fulfilled if C. psittaci was detected in respiratory secretions by PCR, or if a raised IgM antibody titre was detected or an elevation of IgG in two consecutive samples was shown. A probable case was defined as a person fulfilling the criteria of a confirmed case but lacking other laboratory proof of infection than a C. psittaci IgG titre. A possible case was defined as a person fulfilling the criteria of a confirmed case but with no laboratory evidence of C. psittaci infection. Incubation periods for the cases were investigated. Laboratory investigation C. psittaci was identified in respiratory samples by amplification of an 84-base pair (bp) fragment of the outer membrane protein A gene (ompA) according to Heddemaa et al. [9]. The assay was run as a duplex real-time PCR including screening for Legionella species.
and an internal amplification control. In order to determine the genotype of *C. psittaci*, all PCR-positive samples were further investigated by amplification and sequence analysis of a 560 bp fragment of *ompA* covering variable domain I and II.

IgG and IgM antibodies specific to *C. psittaci* were shown by microimmunofluorescence performed at a laboratory accredited for this test since the 1990s [10]. The serum samples were simultaneously tested for antibodies against *C. pneumoniae*, *C. trachomatis* and *C. psittaci*. Threshold titre for positive test was for IgG 1/64 and for IgM 1/16. Parrot faecal samples were analysed for *C. psittaci* using the MagAttract Viral RNA M48 extraction kit (Qiagen, Hilden, Germany) and real-time PCR detection of the 23S gene, as previously described [11].

**Environmental investigation**
The possibility of recovering samples from the primary case’s bird feeder was investigated. Faecal samples were taken from an ICU nurse’s boyfriend’s parrot.

**Results**

**Epidemiological investigation**
Interviews with the primary case and relatives regarding potential risk factors for psittacosis revealed that the primary case had cleaned a garden bird feeder indoors two weeks before signs of disease. No other connection with domestic or wild birds or their droppings could be identified. He did not live close to a poultry farm or other bird holding. The primary case’s son helped to feed the birds when his father was hospitalised. When the father was diagnosed, his son removed the bird feeder and destroyed it by burning. This took place 12 days before the son himself fell ill. The primary case’s wife did not have contact with the birds or the bird feeder.

None of other cases had any history of bird exposure before falling ill except for one of the ICU nurses whose boyfriend had a parrot that she had helped to feed. They did not live close to poultry farms or similar.

In total, in addition to the primary case, six confirmed, three probable and one possible secondary case of psittacosis were identified. Three of these additional cases were male and the median age was 54 years, (range 33–89 years). Case details are summarised in the Table and Figure. Six of the secondary cases were hospitalised. No further transmission from the secondary cases was discovered.

The incubation period ranged from 7 to 20 days in affected cases (mean 12.4) when including all cases. First exposure for the wife and son could not be defined as they had multiple contacts with the primary case.

**Laboratory investigation**
The results of the microbiological and serological testing are summarised in the Table. The owner of the parrot was sampled but showed no serological response to psittacosis.

Three cases provided respiratory samples that could be analysed by PCR. All the obtained *C. psittaci* sequences were indistinguishable and clustered within genotype A.
Environmental investigation

Unfortunately no samples could be taken from the bird feeder in the primary case’s garden as it had been destroyed. *C. psittaci* could not be detected in bird droppings from the parrot.

Control measures

As soon as transmission of the pathogen between patients was suspected, the staff working in the ICU at the time of the incident were informed and asked to seek care should they develop symptoms. The hospital staff on the ward where the patient had initially been treated were informed on 31 January. Instructions were given that all patients with atypical pneumonia should be treated in single ward rooms. The hospital staff were instructed to use filtering face piece (FFP3) masks during procedures with high risk of aerosol-creating procedures such as respiratory training.

Discussion and conclusion

The primary case in this investigation is likely to have fallen ill from contact with wild birds as one of the many sporadic cases explained by this risk factor at the time [5]. Person-to-person transmission of psittacosis is likely to be rare, but this study clearly supports the previous limited evidence that it may occur. In this outbreak we identified three PCR-confirmed psittacosis cases and seven with less solid evidence of infection, i.e. serological indication only for *C. psittaci* infection. We presume that all these 10 cases were caused by the primary case as the parrot tested negative for the disease and the boyfriend did not have any serological response of psittacosis. Irrespective of the total number, the finding of human-to-human transmission is of significance as it shows that the Scottish incident [8] is not unique and this may have consequences for the management of psittacosis cases.

A few of the cases did not respond with high titres in the serological tests and some only with IgG response. This is not unique and this may have consequences for the management of psittacosis cases. Serological aetiological diagnosis of pneumonia has its limitations. For that reason the patients were classified into confirmed, probable and possible cases. To set an aetiological diagnosis, both an acute and serological indication with less solid evidence of psittacosis cases. The previous limited evidence is likely to be rare, but this study clearly supports the previous limited evidence that it may occur.

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**Control measures**

As soon as transmission of the pathogen between patients was suspected, the staff working in the ICU at the time of the incident were informed and asked to seek care should they develop symptoms. The hospital staff on the ward where the patient had initially been treated were informed on 31 January. Instructions were given that all patients with atypical pneumonia should be treated in single ward rooms. The hospital staff were instructed to use filtering face piece (FFP3) masks during procedures with high risk of aerosol-creating procedures such as respiratory training.
phase serum sample and a convalescent serum sample some weeks after the acute infection is often needed unless an IgM test is positive in the acute phase. For that reason the diagnosis may be delayed. Further, cross-reacting antibodies between *C. psittaci* and *C. pneumoniae* have been under discussion [12]. Often a late convalescent serum can be helpful to confirm the aetiology.

As we see it, there are two possibilities for why person-to-person transmission took place in this event. The primary case could have been especially contagious or he could have had a *C. psittaci* strain that was especially transmissible. It is well known that strains with the same ompA gene can differ in their virulence [13]. However, as we did not detect onward transmission from the secondary cases and as the limited genetic analysis did not show any abnormalities from other strains, we believe the first theory. In further support of this hypothesis, although it is likely that our primary case and the other sporadic cases in Sweden at the time were infected with the same strain from a wild bird source, there were no reports of onward transmission from the other sporadic cases notified at the time in Sweden. However the contacts of the other cases may not have been followed up as closely. The genotyping of a subset of the sporadic cases believed to have been infected by wild birds between January and March 2013 showed the same type A subtype as our primary and secondary cases who were positive by PCR. All had gen-otype A, which is mainly associated with parrots and other psittacine birds but which has also been found in passerine birds [14]. It is the genotype causing most human cases worldwide [15]. But to completely rule out the possibility of a more pathogenic strain being the reason for the increased transmissibility in this outbreak, whole genome analysis is required. This could not be performed due to lack of an isolate. We believe that the primary case was more contagious because he was very ill and therefore excreted more bacteria. In support of our theory of increased risk of transmission from severely ill patients, the data on incubation peri-
ods for infected cases shows a possible dose response association. Those who were highly exposed, like the nurse and the doctor who treated the primary case in the ICU, and the patient sharing a room with the case, had a shorter incubation time (7, 7 and 10 days respec-

tively) than the cases who were only exposed to the patient while caring for him on the ward and who had an average of 15 days before symptoms started (range 11–20 days) (Figure). However, due to the low number of cases in the outbreak, more observational studies like this one are needed to show whether this is cor-

rect. Although the ICU nurse who attended at the bronchoscop-ecopy fell ill, we believe that the shorter incubation period had more to do with the patient having become more severely ill and thus being treated in ICU than the bronchoscopy procedure itself, as the doctor who fell ill was not present at the procedure and only examined the patient.

It seems probable that our preventative measures did not prevent any further transmission since all of the secondary cases were related to the primary case. He had already been transmitted to ECMO-treatment in a university hospital when the staff were informed and stricter hygienic measures regarding treatment of patients with atypical pneumonia were implemented. It is likely, however, that the measures may have short-

ed the duration of illness of some of the secondary cases as they are likely to have received treatment earlier than they would otherwise have done.

**Public health implications**

Our previous report of the unusual increase of psittacosis in Sweden this year concluded that psittacosis is likely to be a more common disease in Sweden than previously thought, as our study suggested that it may be overlooked by clinicians and not tested for in cases of atypical pneumonia by laboratories unless specifically requested. The fact that we have now shown that nosocomial transmission may occur from seriously ill patients increases the importance of diagnosing cases of atypical pneumonia correctly, as it has implications for the management of patients with pneumo-

nia. In order to prevent nosocomial transmission from patients with psittacosis, enhanced protection may be needed when caring for severely ill patients with atypical pneumonia, for example, using airway protec-
tion with facemasks and treating the cases in isolation. Staff and others exposed to a psittacosis patient should also be informed of the symptoms so that they seek care should they fall ill.

**Authors’ contributions**

A Wallensten drafted, finalised and submitted the manu-
script. H Fredlund was responsible for the serological labo-

ratory investigation during the outbreak and for writing the laboratory investigation part of the manuscript which he also helped revise. A Runehagen managed the outbreak, contributed with all epidemiological information regarding the outbreak and helped to draft and revise the manuscript.

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