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# Ongoing outbreak of invasive and non-invasive disease due to group A *Streptococcus* (GAS) type *emm66* among homeless and people who inject drugs in England and Wales, January to December 2016

N Bundle<sup>1,2,3</sup>, L Bubba<sup>4,5</sup>, J Coelho<sup>6</sup>, R Kwiatkowska<sup>2,7,9</sup>, R Cloke<sup>8</sup>, S King<sup>9</sup>, J Rajan-Iyer<sup>8</sup>, M Courtney-Pillinger<sup>8</sup>, CR Beck<sup>2</sup>, V Hope<sup>10,12</sup>, T Lamagni<sup>11</sup>, CS Brown<sup>6</sup>, D Jermacane<sup>2,3</sup>, R Glass<sup>12</sup>, M Desai<sup>12</sup>, M Gobin<sup>2</sup>, S Balasegaram<sup>2</sup>, C Anderson<sup>2</sup>

1. United Kingdom Field Epidemiology Training Programme, Public Health England, United Kingdom
2. Field Epidemiology Services, National Infection Service, Public Health England, London and Bristol, United Kingdom
3. European Programme for Intervention Epidemiology Training (EPIET), European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden
4. Reference Department Microbiology Services Division, National Infection Service, Public Health England, London, United Kingdom
5. European Programme for Public Health Microbiology Training (EUPHEM), European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden
6. Respiratory and Vaccine Preventable Bacteria Reference Unit, National Infection Service, Public Health England, London, United Kingdom
7. United Kingdom Public Health Specialty Training Programme
8. Public Health England South East, Horsham, United Kingdom
9. Public Health England South West, Bristol, United Kingdom
10. Public Health Institute, Liverpool John Moores University, Liverpool, United Kingdom
11. Healthcare-Associated Infection & Antimicrobial Resistance Department, National Infection Service, Public Health England, London, United Kingdom
12. HIV and STI Department, National Infection Service, Public Health England, London, United Kingdom

**Correspondence:** Nicholas Bundle (nick.bundle@phe.gov.uk)

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**We report an outbreak of invasive and non-invasive disease due to an unusual type of *Streptococcus pyogenes* (group A *Streptococcus*, *emm66*) among a vulnerable, largely homeless population in southern England and Wales, detected in September 2016. Twenty-seven confirmed cases were subsequently identified between 5 January and 29 December 2016; 20 injected drugs and six reported problematic alcohol use. To date, we have ruled out drug-related vehicles of infection and identified few common risk factors.**

On 26 September 2016, a cluster of invasive disease caused by *Streptococcus pyogenes* (group A *Streptococcus*, GAS) was detected among people who inject drugs (PWID) or who were street homeless in a town in the south of England. A local outbreak control team (OCT) was set up to investigate this cluster which included infections due to *emm66*, a GAS type rarely identified by the Public Health England (PHE) Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU) in previous years. Additional cases of both invasive (iGAS) and non-invasive disease due to GAS type *emm66* were retrospectively identified in the RVPBRU database and a review of local health

protection team (HPT) case records revealed the majority of them to have occurred among PWID, those homeless, or reporting problematic alcohol use. A national OCT was convened on 14 October 2016 with representation from local and national health protection, epidemiology and microbiology services. We describe the ongoing outbreak of invasive and non-invasive disease caused by GAS *emm66* as at 12 January 2017.

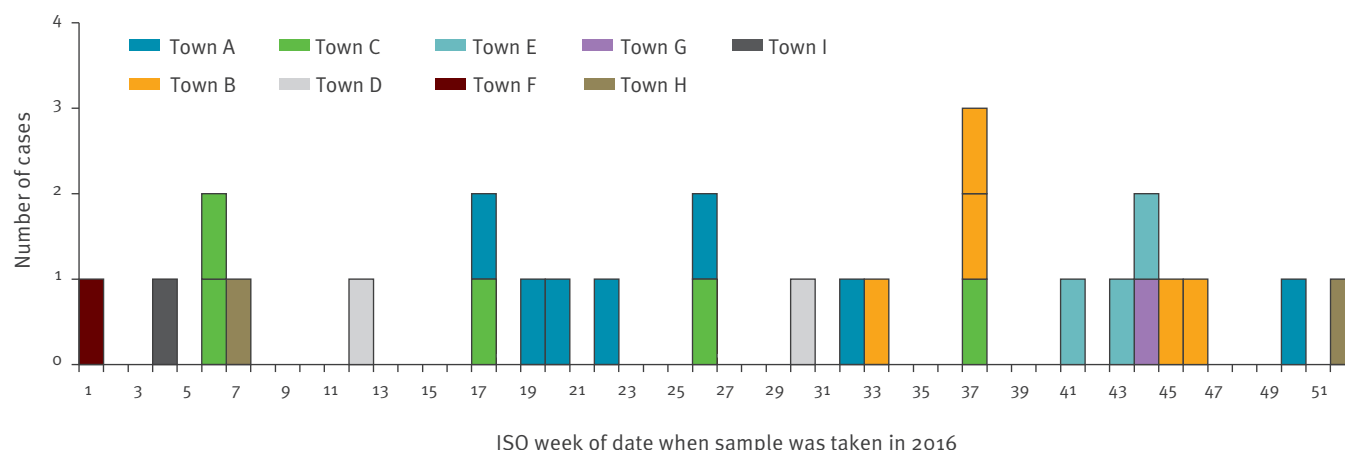
## Epidemiological investigation and microbiological characterisation

The outbreak case definition is individuals with confirmed GAS type *emm66* infection (invasive and non-invasive) in England and Wales with a sample date from January 2016, who are, or are epidemiologically linked to someone who is, homeless, PWID or reporting problematic alcohol use.

Cases were identified from notifications made to HPTs in England and Wales [1,2] and from typed isolates from RVPBRU. Invasive disease was defined through the isolation of GAS from normally sterile sites.

**FIGURE 1**

Outbreak cases of group A *Streptococcus* type *emm66* infection, by week of sample date and town of residence, England and Wales, 2016 (n=27)



ISO: International Organization for Standardization.

We gathered information on lifestyle risk factors, including alcohol use and vehicles of infection related to drug use or homelessness, potential venues and modes of transmission, from a hypothesis-generating questionnaire. Questions covered accommodation, social contacts and drug use, including injection practices, in the seven days before illness and in the past year. We used validated questions, where possible, from the United Kingdom (UK)'s Unlinked Anonymised Monitoring Survey (UAM) of PWID [3] so as to provide comparator data. Questionnaires were completed by local HPTs and homeless/drug outreach services. We also summarised information obtained from outreach services, case records, local investigations and laboratory surveillance.

Initial testing for GAS usually occurs at local hospital laboratories that forward most isolates from invasive infections to RVPBRU for further characterisation. In this outbreak, laboratories were also encouraged to forward isolates from non-invasive infections in people who might meet the case definition. GAS types were determined by the *emm* sequence typing method [4], which compares the *emm* sequences obtained by PCR and Sanger sequencing to those available in the *emm* database using the BLAST algorithm [5].

### Description of the outbreak

RVPBRU identified 30 *emm66* infections in 2016, of which 27 (iGAS: 20; non-invasive GAS: 7) met the outbreak case definition with samples taken between 5 January and 29 December 2016 (Figure 1).

As at 12 January 2017, 12 questionnaires were returned; ten fully completed from case interviews and two partially completed from case information and interview with a homeless outreach worker. Demographic and

clinical characteristics of cases are summarised in Table 1.

The cases were predominantly clustered across a 280 km span of southern England and Wales, bounded by Towns B and C to the east and Town H to the west. Nine towns in total had cases. Towns A, C, D, E, F, G and H are located along, or near to, a major road and rail transport corridor that connects London and South Wales. Town B is linked to this corridor via Town C. Town I is situated in the north of England. (Figure 2).

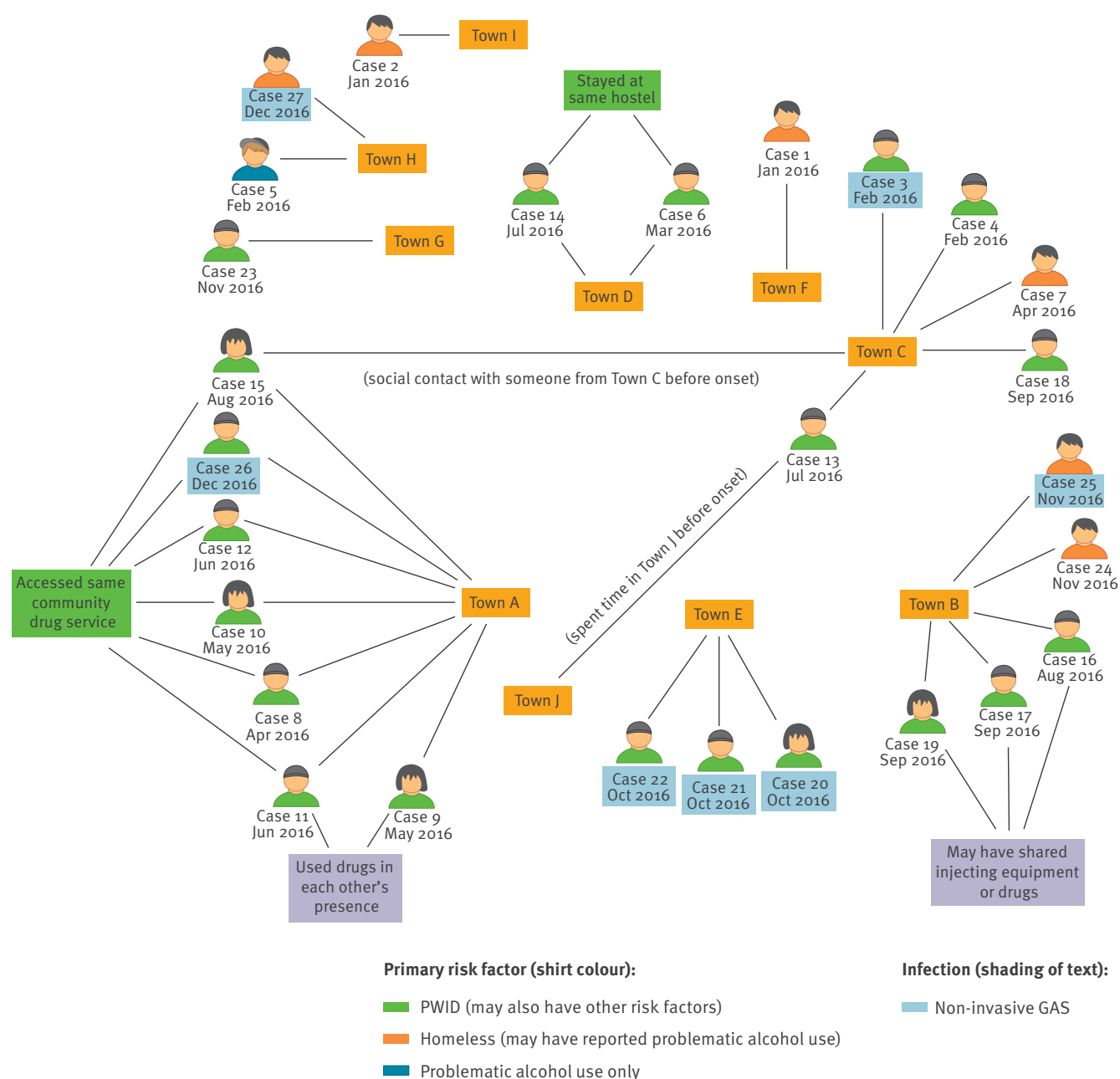
In Town A, all seven cases were linked through drug use or access to the same community drug service and six of these cases were also clustered in time (April–August 2016). All cases in Town B were street homeless, with three cases linked in time (August–September) and through anecdotal reports of sharing needles or drugs. All cases in Town C appeared unconnected. A common homeless hostel linked cases in Town D and information is pending about Town E. There is no known staff travel between hostels in different towns. There was social contact by one case between Towns A and C whose onset was among the latest in each of these locations. Another Town C case reported spending time, before onset of illness, in Town J, located 40 km from Town A.

Among the ten cases interviewed, the median delay between the sample and interview date was 104.5 days (range 3–263). Six of these interviewees were retrospectively identified cases and a much shorter median delay of 8.5 days (range 3–27) was seen among the four prospectively identified cases. (Table 2).

All ten cases reported drug use in the seven days before illness, with the eight PWIDs mainly using

**FIGURE 2**

Network map summarising links between cases by town, place and social contact, outbreak of group A *Streptococcus* type *emm66*, England and Wales, 5 January–29 December 2016 (n=27)



GAS: group A *Streptococcus*; PWID: people who inject drugs.

heroin and crack. Some reported sharing of spoons/mixing containers and filters, but not needles. Two PWIDs linked to different towns (A and D) reported having changed dealers in the seven days before illness onset. Otherwise, there was no notable change in reported injecting behaviour or other drug use compared with that during the year prior to questionnaire administration.

## Control measures

We cascaded a health alert on 2 November 2016 to HPTs, microbiology services and local authorities

working with affected populations to highlight the need for early detection of infection, swabbing of PWID for non-invasive GAS, referral of isolates to RVPBRU for typing, emphasising safe and hygienic injection practices when communicating with PWIDs and ensuring their easy access to needle and syringe programmes (NSP).

In towns with multiple cases, local OCTs reviewed the policies and practices of affected NSP and homeless hostels around injecting, infection control and environmental cleaning, comparing them against national

**TABLE 1**

Features of outbreak group A *Streptococcus* type *emm66* cases, England and Wales, 5 January–29 December 2016 (n = 27)

Total cases	n
<b>Demographics and risk factors<sup>a</sup></b>	
Male	22
Median age (range) in years	38 (29–56)
Homeless at time of illness onset	20
Street homeless at time of illness onset	13
People who inject drugs	20
Problematic alcohol use	6
<b>Initial clinical presentation</b>	
Abscess	7
Injection site infection	6
Septic arthritis	3
Bacteraemia	3
Muscle or deep tissue infection	3
Cellulitis	2
Unspecified soft tissue infection	1
Necrotising fasciitis	1
Pneumonia	1
<b>Diagnosis, outcomes and co-infection<sup>a</sup></b>	
Invasive GAS infection	20
Non-invasive GAS infection (including three severe infections)	7
Hospitalisation	21
Amputation	1
Died due to GAS infection	1
Previously tested positive for hepatitis C	15
Previously tested positive for hepatitis B	1

GAS: group A *Streptococcus*.

<sup>a</sup> More than one answer could be chosen.

guidance [1,2] and published evidence [6]. Teaching sessions were organised for frontline homeless service providers to raise awareness of GAS infection and infection control measures. Targeted communications to raise awareness of early symptoms and to encourage prompt healthcare attendance in the event of skin problems at injection sites were disseminated via general practitioners, local authorities, pharmacies and included in the equipment packs of one NSP.

## Discussion

Large outbreaks of GAS type *emm66* have not previously been described. In this outbreak although the cases occurred disproportionately among PWID, transmission appears unrelated to drug usage. Illness occurred over an 11-month period, cases were representative of the wider UK PWID population in terms of sex, age and hepatitis C prevalence [3] and we identified no notable changes in drug using practice in the period before illness. The age and sex distribution of cases, low mortality (only one case died) and predominance of abscesses and injection site infections were

also broadly consistent with the pattern seen among iGAS cases in PWID in England in the early 2000s [7,8].

We have identified potential transmission clusters within three of the affected towns but only limited epidemiological links between cases in different towns. Travel along the major transport routes connecting the towns remains a plausible hypothesis for the disease propagation observed. Whole genome sequencing (WGS) of outbreak and historic *emm66* isolates held by RVPBRU may have potential to identify links between cases, improve the specificity of our case definition and establish whether the outbreak strain has any genes suggestive of increased virulence. However, the role of WGS in an outbreak of such a rare GAS type, with limited availability of historical isolates for comparison, is the subject of ongoing discussion.

Difficulties in interviewing the affected population, especially for cases identified retrospectively, and accurately establishing networks of contacts pose challenges for investigation and control. Close coordination between local HPTs and frontline drug/homeless outreach services has been essential for accessing the affected population and implementing control measures to date.

The proportion of iGAS infections reported to RVPBRU attributed to PWID (other risk factors are not recorded) has increased annually since 2013 from 0.2% to 1.7% in 2016. A previous rise, dominated by GAS type *emm83*, was recorded in the early 2000s, with PWID accounting for 20% of all iGAS cases in England and Wales at its peak in 2003 [8,9]. The requirement for iGAS isolate submission has not changed during 2013–16 and it is unclear whether the increase represents a true change in disease burden among PWID, increased awareness of injection site infections and/or access to healthcare, or if it is an artefact of increased PWID reporting on referral forms. Prior to the increase in GAS type *emm66* in 2016, the most common type seen among PWID with iGAS was *emm94*, with six cases in the entire period 2010–15 and two cases in 2016. GAS type *emm66* is very uncommon in high income settings such as Europe and North America [10–12]. It comprised 1.3% of iGAS isolates submitted to RVPBRU for typing in 2016; an increase from 0.16% of the isolates during 2010–15. There is very limited mention of type *emm66* in the literature beyond a single case in Hungary in 2004–05 [13] and sequencing of an isolate from within a cluster of 13 cases in France in 2009–13 [14]. The rapid increase in the number of type *emm66* iGAS cases detected in England and Wales in 2016 and the high proportion of PWID among cases leave us confident that this is a genuine outbreak. So far, despite the wide geography, the outbreak has been limited to this vulnerable marginalised group and *emm66* may now be the dominant circulating type within that population. Unfortunately, there are no data on background carriage rates of *emm* types within different groups.



**TABLE 2**

Summary of responses from interviews with group A *Streptococcus* type *emm66* outbreak cases, England and Wales, 2016 (n = 10)

Positive responses	n
<b>Country of origin, accommodation and contacts in the seven days before illness onset<sup>a</sup></b>	
Born in the UK	9
Stayed in a house or flat (own, partner's or friend's)	6
Stayed outside (street, park or abandoned building)	5
Stayed in a squat	3
Stayed in a hostel	1
Accessed community drug or health services	6
Knew someone suffering from the same illness, infection or sore throat at a similar time	5
Visited or spent time with someone from another part of the UK	3
<b>Drug use and behaviour in the seven days before illness onset<sup>a</sup></b>	
<b>Any drug use</b>	10
<b>Injecting drug use<sup>a</sup></b>	8
Heroin	8
Crack	6
Used spoons/mixing containers or filters previously used by someone else	4
Changed drug dealer	2
<b>Other drug use<sup>a</sup></b>	8
Smoked crack	7
Smoked, chased or snorted heroin	6
Smoked or swallowed cannabis	3
Snorted cocaine	2
Swallowed non-prescribed benzodiazepines	2

UK: United Kingdom.

<sup>a</sup> More than one answer could be chosen.

There is no indication of a drug-related vehicle of infection and we can conclude that there have been no common risk factors identified to date other than those listed in the case definition. These are all associated with increased vulnerability to iGAS and may result in protracted incidence as previously described in a Canadian GAS type *emm59* outbreak [15]. We must therefore maintain increased awareness of the early signs of GAS infection among the affected population and those that serve them. As our investigations continue, we expect lessons from this outbreak to emerge around transmission routes and effectiveness of control measures that will have relevance to other countries facing GAS outbreaks in vulnerable, under-served populations.

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The Blast-*emm* database is hosted by Centres for Disease Control and Prevention (<https://www2a.cdc.gov/ncidod/bio-tech/strepblast.asp>)

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## Conflict of interest

None declared.

## Authors' contributions

All authors contributed to data collection and interpretation. Nick Bundle undertook the data analysis, drafted and revised the manuscript based on all authors' contributions.

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# Infectious Zika virus in vaginal secretions from an HIV-infected woman, France, August 2016

P Penot<sup>1,2</sup>, S Brichler<sup>3</sup>, J Guilleminot<sup>4</sup>, C Lascoux-Combe<sup>2</sup>, O Taulera<sup>5</sup>, E Gordien<sup>3</sup>, I Leparc-Goffart<sup>6,7,8</sup>, J Molina<sup>2,8</sup>

1. Internal Medicine and Infectious Diseases Department, Andre Gregoire Hospital, Montreuil, France

2. Infectious and Tropical Diseases Department, Saint Louis Hospital, Paris, France

3. Clinical Microbiology Department, Avicenne Hospital, Bobigny, France

4. Family Planning Clinic, Saint Louis Hospital, Paris, France

5. Internal Medicine Department, Saint Louis hospital, Paris, France

6. Institut de Recherche Biomédicale des Armées, National Reference Laboratory for arboviruses, Marseille, France

7. UMR 'Emergence des Pathologies Virales' (EPV Aix Marseille university, IRD French Institute of Research for Development, INSERM French National Institute of Health and Medical Research, EHESP French School of Public Health), Marseille, France

8. These authors contributed equally to this work

**Correspondence:** Pauline Penot (penotpauline@gmail.com)

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**A woman with controlled HIV infection developed in late August 2016 a pruritic rash with fever and conjunctival hyperaemia after a trip to the French Caribbean islands. On day 3 after symptom onset, Zika virus RNA was detected in plasma, urine and vaginal samples with respective viral loads of 3.8, 6.1 and 5.3 log copies/mL. Notably, we demonstrated the presence of infectious Zika virus particles in the vaginal samples by isolation in cell culture.**

A female patient returned to France from the Caribbean islands with acute Zika virus infection. After obtaining informed consent, we collected vaginal swabs for both RNA detection and virus culture in order to investigate the infectivity of vaginal secretions.

## Case presentation

A French woman in her 40s, with a controlled HIV-1 infection, travelled to French Caribbean islands (Martinique for seven days then Guadeloupe for three weeks) in summer 2016. On the day of return to France, she felt asthenic with myalgia. Two days later, a pruritic rash appeared on her face, chest, back and arms, with abdominal pain and diarrhoea. She consulted her general practitioner, who referred her to our department on the same day.

Her medical history was limited to a virologically suppressed HIV-1 infection with CD4+ T-cell count over 500/mm<sup>3</sup> under antiretroviral therapy (emtricitabine/tenofovir and nevirapine). Physical examination showed a widespread itching maculopapular exanthema, conjunctival hyperaemia and mild fever (38.1°C). Intense asthenia and diffuse myalgia were still ongoing.

As the symptoms were consistent with acute Zika virus infection, the patient consented to have a vaginal swab, after being informed that there would be no direct benefit from knowing the test result. In the absence of a standardised collection protocol, vaginal secretions were collected by direct swab (vaginal sample 1) and after instilling 5 mL of saline solution between the cervix and the posterior vaginal wall (vaginal sample 2). At her request, her scheduled cervical Pap smear was performed at the same time.

Standard laboratory tests showed moderate lymphocytopenia with 1,120 cells/mm<sup>3</sup> (norm: > 1,200/mm<sup>3</sup>) and C-reactive protein 8 mg/L (norm: < 5 mg/L). RT-PCR assays were negative for dengue and chikungunya viruses in plasma (Fast-Track Diagnostics, Luxembourg) and a test for NS1 antigen for dengue virus was also negative (Bio-Rad, Marne la Coquette, France). Detection of Zika virus RNA by RT-PCR (Altona Diagnostics, Hamburg, Germany) was positive in plasma, urine and vaginal secretions, three days after onset of symptoms. The viral RNA load was 3.8 log copies/mL in plasma, 6.1 in urine, 5.3 in vaginal sample 1 and 3.9 in vaginal sample 2. The use of a saline solution instillation for the second sampling possibly led to a dilution of the sample. The two vaginal samples were inoculated on Vero and C6/36 cells, and Zika virus was isolated from both, demonstrating the presence of infective Zika virus. All methods were performed as detailed in the supplementary data of a previous publication [1]. According to French guidelines, no serological analysis was performed for dengue and Zika viruses.



The patient's fever subsided on day 3, and the rash on day 6 after symptom onset. A transient bilateral knee pain occurred on day 4.

The patient consented to a second genital swab 10 days after the onset of symptoms: no viral RNA was detected in vaginal secretions and in cervical mucus. No serum collection was performed during the second visit. At that time, she had recovered from all symptoms except a mild persistent asthenia. Her husband did not present symptoms evocative of Zika virus infection during or after the journey. The patient stated that their sexual intercourses were always protected by condom use.

## Background

Initially, Zika virus was thought to be exclusively transmitted through mosquitoes, but sexual transmission was subsequently reported. Transmission from men to their female or male sexual partners has been well and often demonstrated and has been observed up to 44 days after symptom onset [2-5], whereas only one case report suggests a possible woman-to-man transmission, which was concurrent with the acute infection symptoms in the female partner [6]. Three teams have reported the presence of Zika virus RNA in female genital secretions up to 14 days after symptoms onset, but Zika virus infectivity was not proven [7-9]. None of the women with symptomatic Zika infection involved in an assisted reproductive technology programme on Guadeloupe had detectable Zika virus in their genital tract beyond the second week of follow up, despite regular monitoring by Zika RT-PCR for up to 3 months [7].

## Discussion

This seldom reported and transient presence of Zika virus in the female genital tract contrasts with an extensive literature about its persistence in semen, where RNA has been detected up to six months after return from endemic areas [4,10-14]. Moreover, Zika virus from semen has been isolated in cell culture in four patients, up to 69 days after onset of symptoms, but had never been isolated from the human female genital secretions before [1,15-17].

The short time period during which we could detect Zika virus in our patient's genital tract is consistent with our prior failure to detect the virus by RT-PCR from vaginal swabs in two other patients, seven and eight days after the onset of symptoms [18]. Although viral load was higher in our patient's vaginal secretions than in her serum, it remained lower than what could be measured in some semen samples [1,15,16]. Our patient had been living with virologically HIV suppressed infection for years and had an almost normal lymphocyte cell count. It is unlikely that her HIV-infection influenced the evolution of her Zika virus infection, especially as the clinical presentation and evolution were similar to those observed in HIV-negative patients.

Recently, a mouse model of Zika virus infection by vaginal exposure demonstrated that Zika virus replicated

within the genital mucosa and could lead to a fetal infection [19], but no data has been available up to now on the infectiousness of a vaginally situated virus in humans. Our findings suggest a short period of infectivity of women with acute Zika virus infection through their genital secretions. This short duration of virus shedding in genital secretion may explain why to date only one case of female to male transmission has been reported. Yet, it remains unknown whether Zika virus can establish a reservoir in the female genital tract and infect follicles and/or ovules.

Recent evidence of extended presence of the virus in semen and of possible transmission from women to men has led to an update of the United States Centers for Disease Control and Prevention (CDC)'s guidance on the prevention of sexual transmission of Zika virus [20]. Current French guidelines and the CDC recommend a deferral period of at least 2 months before women returning from an area with circulating Zika virus can access medically assisted reproductive technology programmes. When this delay cannot be adhered to (e.g. fertility preservation before chemotherapy), testing vaginal samples by RT-PCR should be recommended.

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## Conflict of interest

None declared.

## Authors' contributions

Pauline Penot, Olivier Taulera, Caroline Lascoux-Combe took care of the patient; Pauline Penot and Jean Guilleminot sampled the genital swabs; Ségolène Brichler performed the PCR; Isabelle Leparc-Goffart isolated the virus; Pauline Penot wrote the first draft of the manuscript. All authors reviewed and approved the final manuscript.

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# Large measles epidemic in the Netherlands, May 2013 to March 2014: changing epidemiology

T Woudenberg<sup>1,2</sup>, RS van Binnendijk<sup>1</sup>, EAM Sanders<sup>1,2</sup>, J Wallinga<sup>1,3</sup>, HE de Melker<sup>1</sup>, WLM Ruijs<sup>1</sup>, SJM Hahné<sup>1</sup>

1. National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

2. University Medical Center Utrecht, Utrecht, the Netherlands

3. Leiden University Medical Center, Leiden, the Netherlands

Correspondence: Tom Woudenberg (tom.woudenberg@rivm.nl)

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Since the early 1990s, the Netherlands has experienced several large measles epidemics, in 1992–94, 1999–2000 and in 2013–14. These outbreaks mainly affected orthodox Protestants, a geographically clustered population with overall lower measles-mumps-rubella first dose (MMR-1) vaccination coverage (60%) than the rest of the country (>95%). In the 2013–14 epidemic described here, which occurred between 27 May 2013 and 12 March 2014, 2,700 cases were reported. Several control measures were implemented including MMR vaccination for 6–14-month-olds and recommendations to reduce the risk in healthcare workers. The vast majority of reported cases were unvaccinated (94%,  $n=2,539$ ), mostly for religious reasons (84%,  $n=2,135$ ). The median age in the epidemic was 10 years, 4 years older than in the previous epidemic in 1999–2000. A likely explanation is that the inter-epidemic interval before the 2013–2014 epidemic was longer than the interval before the 1999–2000 epidemic. The size of the unvaccinated orthodox Protestant community is insufficient to allow endemic transmission of measles in the Netherlands. However, large epidemics are expected in the future, which is likely to interfere with measles elimination in the Netherlands and elsewhere.

## Introduction

Measles is a highly contagious infectious disease caused by the measles virus. It can lead to serious illness, life-long complications and death [1]. Measles vaccination programmes have contributed to a steep decline in the number of infections and deaths, but in 2014 measles still caused an estimated 114,900 deaths worldwide, mostly in low-income countries [2]. Case fatality is reported to be up to 6% in developing countries and is especially high in infants and young children [3].

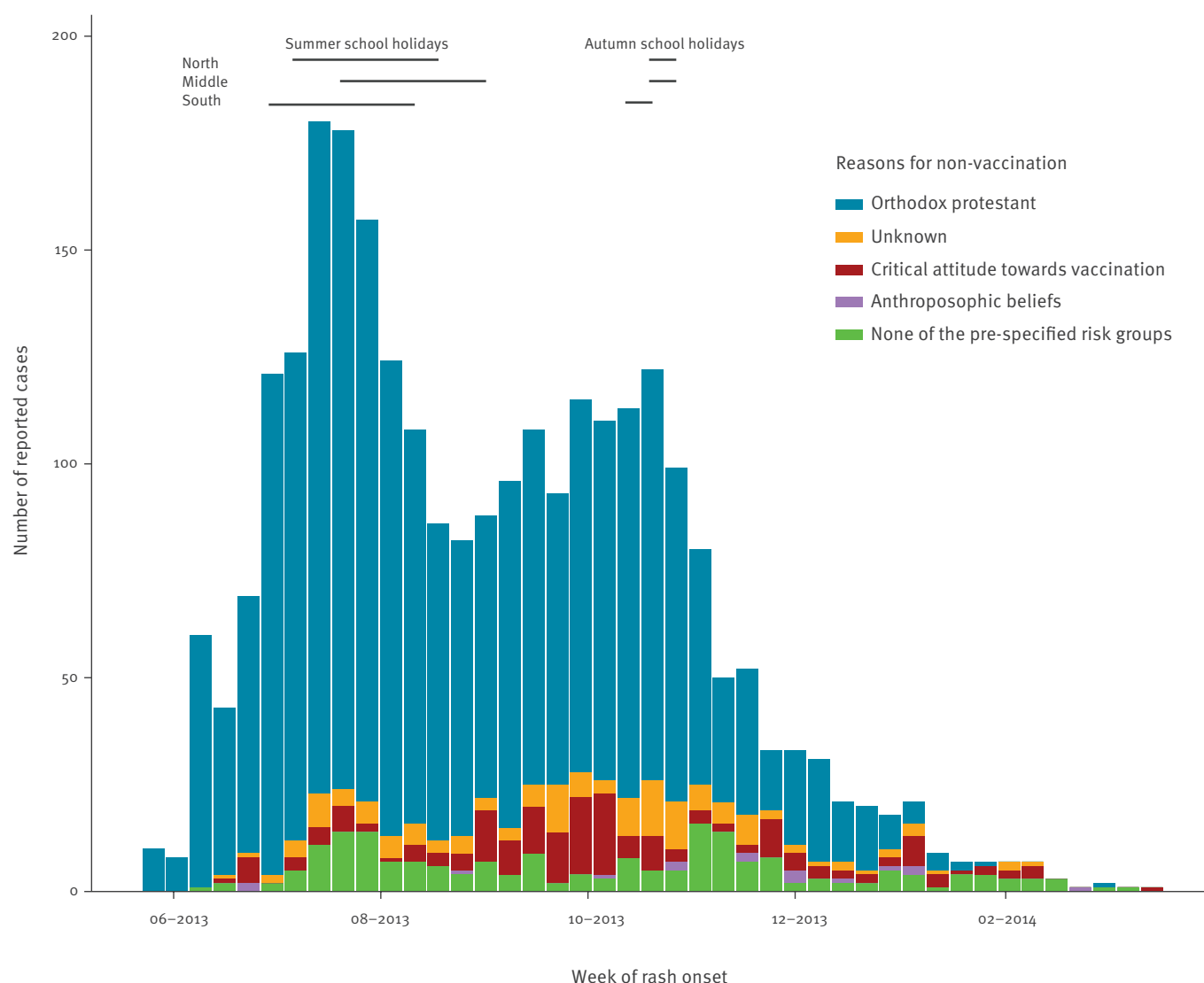
In the Netherlands, a single-dose measles vaccination programme was introduced within the national

immunisation programme (NIP) in 1976 for all infants at 14 months of age. Since 1987, a two-dose programme using measles-mumps-rubella (MMR) vaccine has been offered at 14 months and 9 years of age. Vaccine coverage of the first dose of MMR vaccination has been above 95% for 20 years [4]. Coverage for two doses at the age of 10 years has been around 93% for 10 years. Introduction of measles vaccination in the Dutch NIP resulted in a large decrease in the number of reported cases [5]. However, epidemics still occur due to socio-geographically clustered individuals who refrain from vaccination. A large measles epidemic occurred in 1999–2000 with 3,292 reported cases, most of whom were unvaccinated (94%) and belonged to the orthodox Protestant community (83%) [6]. Between 2001 and 2012 the incidence of measles was lower than the five cases per million set as a target by the World Health Organisation (WHO) in 2010 [7], except for 2008 when the incidence was seven per million, due to an outbreak in individuals with anthroposophic beliefs [8].

The orthodox Protestant population comprises around 1% of the total population in the Netherlands [9]. Vaccine coverage in these communities is around 60% on average, but varies widely between churches, with coverage ranging from less than 30% among members of the most orthodox churches to vaccination rates comparable to the rest of the Netherlands in the least traditional churches [10]. In general, orthodox Protestants form close-knit communities. The majority of them, ca 75%, live geographically clustered in the region known as the Bible belt. In this region, stretching from the south-west to the north-east of the country, 29 municipalities have MMR vaccination coverage of less than 90% [11]. Children in these communities often attend orthodox Protestant primary and secondary schools. Some of these schools are known to have an MMR-1 and diphtheria-tetanus-pertussis vaccination coverage below 15% [12]. A serological survey carried out in 2006–2007 confirmed a high risk of a large

**FIGURE 1**

Reported measles cases by risk group and week of rash onset, the Netherlands, reported between 27 May 2013 and 12 March 2014 (n = 2,700)



If the first day of rash onset was not available, the date of the onset of the prodromal phase + 3 days was used [17]. Horizontal lines indicate the timing of school holidays, which differ by region.

measles epidemic in these communities [13]. The seroprevalence was especially low in children 1–4 years of age (36%) and 5–9 years of age (63%).

The most recent epidemic started in May 2013 when two unvaccinated children attending an orthodox Protestant school were reported to have measles [14]. In response to the subsequent outbreak, on 17 June 2013 a national outbreak management team (OMT) advised early MMR vaccination for infants aged 6–14 months living in municipalities with MMR-1 vaccination coverage <90% [15]. Infants of this age are too young to have been vaccinated in the regular schedule, but have lost their maternal antibodies against measles [16] and are at the highest risk of complications [17]. Parents of eligible infants were contacted directly and invited to this additional MMR vaccination (MMR-0 for 6–11

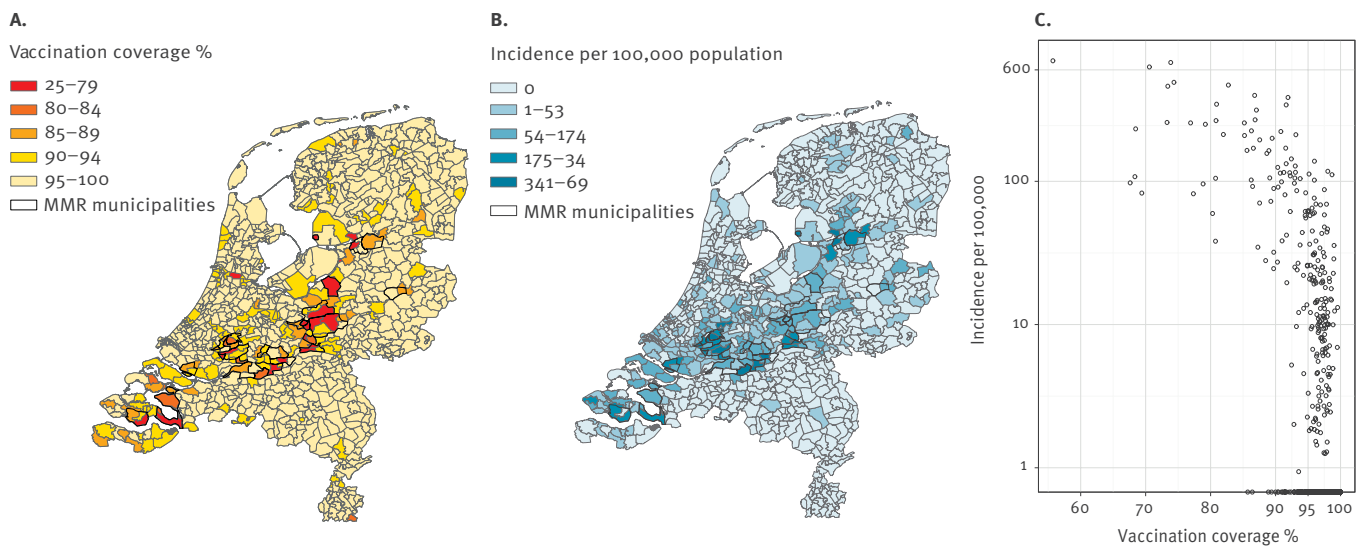
month-olds) or early (MMR-1 for 12–14 month-olds). This intervention was implemented between July 2013 and February 2014. In total, 5,800 infants out of 10,097 (57%) received an early MMR vaccination before 14 months of age.

Furthermore, the OMT advised communication via the media that children and teenagers up to 19 years of age were entitled to receive a free catch-up MMR vaccination. This was also communicated through a newspaper and family magazines widely read by orthodox Protestants, even though previous research showed low acceptance of catch-up vaccination among this group [18].

The OMT also advised assessment of the immune status of healthcare workers (HCW) and provision of

**FIGURE 2**

A) MMR-1 vaccination coverage combined for birth cohorts 2011/2010/2009 at the age of 2 years, by three-digit postal code. B) Measles incidence from reported cases from May 2013 until March 2014 ( $n = 2,689$ ), by three-digit postal code in the Netherlands. C) Scatterplot (log-scale) of three-digit postal code areas vaccination coverage and reported measles incidence.



Data on vaccination coverage by postal code area were obtained from the national vaccination register. MMR municipalities are those 29 municipalities where the early MMR vaccination campaign was conducted. In Vaals municipality in the far south-east of the Netherlands, a considerable number of infants receive their vaccinations in Germany and are therefore not included in the Dutch vaccination register. Eleven cases lacked location data and could not be included for measles incidence.

additional MMR vaccination when required [15]. HCW who were born before 1965 or had been vaccinated twice were considered to be protected, and all other HCW were advised to complete their MMR vaccination schedule. Letters were posted to all academic and community hospitals explicitly requesting them to bring this advice to the attention of the infection control committee.

Here we describe the epidemiology of the 2013–2014 measles epidemic in the Netherlands and compare it with the previous epidemic in 1999–2000.

## Methods

### Notification of measles

Measles is a mandatory notifiable disease in the Netherlands. Physicians and laboratories are required to report cases to Municipal Health Services (MHS). Directors of schools and day care centres are required to report rash clusters in their institutions to MHS. For every reported case, a MHS physician or nurse must complete a standardised questionnaire. The questionnaire covers, among others things, demographic characteristics, disease onset dates, hospitalisation, possible source, presence of complications, probable place of infection, vaccination status and reasons for non-vaccination. A possible source of infection is defined as contact with another reported case 7 to 21 days before the onset of the rash. Reasons for non-vaccination are pre-specified in the questionnaire and cases can be categorised into one of the following risk

groups: orthodox Protestant, individual with anthroposophic beliefs, individual with a critical attitude towards vaccination, unknown or none of the pre-specified risk groups. The National Institute of Public Health and the Environment (RIVM) maintains an electronic web-based register for notifications by the MHS.

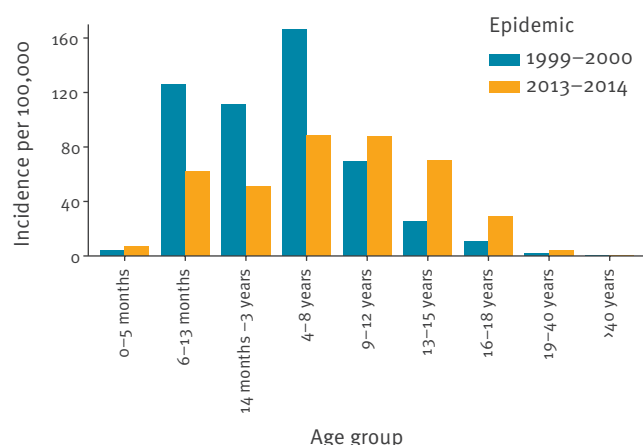
### Case definition

Clinical measles is defined as fever and a maculopapular rash accompanied by at least one of the following three symptoms: cough, coryza or conjunctivitis. Cases of measles are defined as clinical measles in a person with laboratory-confirmed measles virus infection and/or an epidemiological link to a laboratory-confirmed case. A case is epidemiologically linked if the individual had contact with a laboratory-confirmed case in the 3 weeks before onset of disease. Laboratory confirmation is based on positive measles-specific IgM serology and/or detection of measles virus RNA by PCR in a throat swab, oral fluid or urine specimen [19]. Physicians were advised to rapidly diagnose individuals presenting with severe illness, which was mostly done by testing for measles-specific IgM. In other cases, the use of less invasive sampling of oral fluid was recommended, which comprised 60% of the specimens forwarded to the national laboratory for PCR testing; the remainder were throat swabs or urine specimens. The majority of PCR-positive specimens were selected for genotyping using primers amplifying the N-terminal 450-nt fragment of the measles nucleocapsid gene, according to WHO-approved sequencing methods for genotyping as previously described [20]. In case of successful and



**FIGURE 3**

Incidence of reported cases by age group for the 1999–2000 epidemic ( $n = 3,170$ ) and the 2013–2014 epidemic ( $n = 2,700$ ), the Netherlands



Age was calculated by subtracting date of birth from date of rash onset. For individuals older than 2 years, only the year of birth was known. For the purposes of the study, these cases were assumed to have been born on 30 June in the reported year of birth. For cases younger than 2 years, the month but not the day of birth was known, and so these cases were assumed to have been born on the 15th day of the reported month of birth.

complete sequencing results, genotypes were generated and representative sequences were reported to the WHO Measles Nucleotide Surveillance (MeaNS) database.

From mid-July 2013 onwards, MHS located in the Bible belt were advised by the RIVM to limit the use of laboratory diagnostics of measles to cases with complications, vaccinated cases, cases in newly affected schools, villages or risk groups and cases that were reported by general practitioners without an epidemiological link.

Our analyses included all cases reported between 27 May 2013 and 12 March 2014, respectively the first and last date with a case laboratory-confirmed with the predominant outbreak strain. Imported cases and cases with a genotype or strain other than the outbreak strain were excluded. Cases that were epidemiologically linked to excluded cases were also excluded.

Population data was retrieved from Statistics Netherlands. Vaccine coverage by municipality and postal code area was available from the national vaccination register. Proportions were compared using the chi-squared test or Fisher's exact test. The age distributions of both epidemics were compared using the Kolmogorov-Smirnov test. To test differences in medians we used Mood's median test. All analyses were performed using R software, version 3.1.0. Maps were created with ArcGIS version 10.2.2.

## Results

### Outbreak description

Overall, 2,766 measles cases were reported between 27 May 2013 and 12 March 2014. Molecular typing of the outbreak strain showed a genotype D8 measles virus (strain MVs/Alblasserdam.NLD/22.13, WHO/MeaNS Id 50730, GenBank Id KMo66606), with a sequence indistinguishable from the strain that was first identified in the United Kingdom (UK) in 2012 (MVs/Taunton.GBR/27.12, WHO/MEANS Id 23447, GenBank Id JX984461). Two per cent ( $n=66$ ) of the cases were excluded because they had a different genotype ( $n=11$ ) or were imported ( $n=25$ ). Epidemiologically linked to these different genotypes and importations were 20 and 10 cases, respectively. Of the 11 different genotypes found, 10 were genotype B3 and one genotype H1. We included the remaining 2,700 cases in our analyses.

The first two cases were reported on 27 May 2013 in two unvaccinated children attending the same orthodox Protestant primary school. These children had not travelled abroad and the source of their measles infection was unknown. The epidemic peaked in the second week of July 2013 with 180 reported cases, with a subsequent rapid decline during school holidays in July and August 2013 (Figure 1). Coinciding with the new school year, from September 2013 onwards, reported cases increased until another peak of 122 cases occurred in the third week of October. Subsequently, the number of cases per week declined. The last case was reported on 12 March 2014.

The vast majority of reported cases were unvaccinated (94%,  $n=2,539$ ) (Table), mostly based on religious grounds (84% of unvaccinated cases,  $n=2,135$ ). Others who refrained from vaccination were people who had anthroposophic beliefs (1%,  $n=16$ ), had a critical attitude towards vaccination (7%,  $n=172$ ) or had other reasons to refrain from vaccination (4%,  $n=108$ ). Of vaccinated cases ( $n=141$ ), 89% ( $n=125$ ) had been vaccinated once, 11% had been vaccinated twice ( $n=15$ ), and one individual had been vaccinated three times (0.1%) (Table). Sixty-eight per cent ( $n=85$ ) of the 125 once-vaccinated cases were between 14 months and 8 years of age, and of those, 49% ( $n=61$ ) were between 4 and 8 years of age. The majority of the 16 twice-vaccinated cases were older than 18 years of age ( $n=13$ ).

The epidemic mainly affected low-vaccination-coverage areas. Nearly half of reported cases (49%) occurred in the 29 municipalities with vaccination coverage below 90% (range 60–90%). In total, 41% of 408 municipalities ( $n=169$ ) reported at least one case. Within municipalities, there was a considerable heterogeneity in vaccination coverage and incidence by postal code area (Figure 2A and 2B).



**TABLE**

Reported measles cases by vaccination status (n = 2,700), hospitalisation (n = 2,677) and complications (n = 2,581) during a measles epidemic, the Netherlands, May 2013 – March 2014

No. (%) <sup>a</sup> reported cases by age group								p value <sup>b</sup>
	0–13 months	14–48 months	4–8 years	9–17 years	18–40 years	>40 years	Total	
<b>Vaccination status</b>	<b>n=78</b>	<b>n=260</b>	<b>n=824</b>	<b>n=1,268</b>	<b>n=226</b>	<b>n=44</b>	<b>n=2,700</b>	
Unvaccinated	75 (96)	236 (91)	760 (93)	1246 (99)	183 (81)	39 (89)	2,539 (94)	NA
Once	3 (4)	24 (9)	61 (7)	16 (1)	20 (9)	1 (2)	125 (5)	NA
Twice or more	0 (0)	0 (0)	0 (0)	3 (0)	13 (6)	0 (0)	16 (1)	NA
Unknown	0 (0)	0 (0)	3 (0)	3 (0)	10 (4)	4 (9)	20 (1)	NA
<b>Complication<sup>c</sup></b>	<b>(n=75)</b>	<b>(n=247)</b>	<b>(n=787)</b>	<b>(n=1,208)</b>	<b>(n=221)</b>	<b>(n=43)</b>	<b>(n=2,581)</b>	
All complications	12 (16)	41 (17)	108 (14)	111 (9)	17 (8)	7 (16)	296 (11)	<0.01
Pneumonia <sup>d</sup>	8 (11)	23 (9)	54 (7)	61 (5)	12 (5)	3 (7)	161 (6)	0.07
Otitis media <sup>d</sup>	4 (5)	16 (6)	48 (6)	41 (3)	4 (2)	0 (0)	113 (4)	<0.01
Encephalitis <sup>d</sup>	0 (0)	0 (0)	1 (0)	1 (0)	0 (0)	0 (0)	2 (0)	NS
Dehydration/diarrhoea <sup>d</sup>	0 (0)	4 (2)	8 (1)	12 (1)	3 (1)	3 (7)	30 (1)	0.07
Other <sup>de</sup>	0 (0)	0 (0)	3 (0)	2 (0)	1 (0)	1 (2)	7 (0)	NS
<b>Hospitalisation status<sup>f</sup></b>	<b>(n=77)</b>	<b>(n=257)</b>	<b>(n=819)</b>	<b>(n=1,254)</b>	<b>(n=226)</b>	<b>(n=44)</b>	<b>(n=2,677)</b>	
Hospitalised	8 (10)	24 (9)	51 (6)	55 (4)	32 (14)	11 (25)	181 (7)	<0.01

NA: not applicable; NS: not significant.

<sup>a</sup> Proportion of the number of reported cases by age group.

<sup>b</sup> Fisher's exact test or chi-squared test.

<sup>c</sup> Information on complications was unknown for 119 cases.

<sup>d</sup> Individual cases could have multiple complications. For example, five cases had both otitis media and pneumonia

<sup>e</sup> Other complications comprised bilateral striatal necrosis (n=1), hepatitis (n=1), keratitis (n=1), stomatitis (n=1), tonsillitis (n=2), and transverse myelitis (n=1). Respiratory infections other than pneumonia were not included under 'other complications'.

<sup>f</sup> Information on hospitalisation was unknown for 23 cases.

The incidence of reported cases by postal code area increased with a lower MMR-1 vaccination coverage (Figure 2C; Spearman's correlation coefficient: -0.42).

The median age of reported cases was 10 years (range 0–68 years). Most reported cases were between 4 and 17 years of age (n=2,092, 77%) (Table). Three per cent of the cases (n=78) were under 14 months of age. Of these 78, three had been vaccinated once before onset of disease. Six cases were below 6 months of age (0.2%). Highest incidence rates were found in 4–8 year-olds and 9–12 year-olds (89 and 88 cases per 100,000, respectively) (Figure 3). Males and females were equally affected (1,355 of 2,684 cases where sex was known were female (50%).

### Laboratory results

About a third of reported cases (n=888, 33%) were laboratory-confirmed; all other cases were reported based on an epidemiological link. Most laboratory-confirmed measles cases (84%, n=749) were confirmed using PCR testing of oral fluid or urine specimens. Another 13% (n=116 cases) were confirmed by detection of measles-specific IgM antibodies in serum. In 2% of the cases (n=16), both IgM and PCR test results were reported. For 1% of the cases (n=7), the diagnostic test was not reported. Of the 749 PCR confirmed cases, 73% (n=548) were sent to the national laboratory for

sequencing. In 7% (n=39) the sequence could not be identified, in 93% (n=509), the sequence was identified as the D8 measles virus (MVs/Alblasserdam, NLD/22.13).

### Complications and hospitalisation

For 11% of the cases (n=296) one or more complications were notified (Table). The occurrence of complications was unknown for 4% of the cases (n=119). More than half of the cases with complications had pneumonia (54%) and about one third had otitis media (38%). The risk of complications was highest in cases below 4 years or above 40 years of age (both 16%). Otitis media was especially prevalent in children aged between 14 months and 3 years (6%). Pneumonia occurred most frequently in cases younger than 4 years of age (10%). Two reported cases were hospitalised with encephalitis: a 17-year-old girl and an 8-year-old boy. The girl had severe underlying medical conditions and died due to encephalitis and pneumonia.

Overall, seven per cent of the cases (n=181) were hospitalised, most commonly for pneumonia (48%, n=86) or dehydration/diarrhoea (15%, n=27). For one per cent (n=23), we do not know whether or not cases were hospitalised. Seven cases required intensive care admission for pneumonia (n=5), encephalitis (n=1) or both (n=1). The median duration of stay in the hospital

due to measles was 4 days (interquartile range 3–5 days). Adults with measles were at higher risk of hospitalisation than children (Table).

### Healthcare workers

In total, 19 HCW were reported to have acquired measles at work. Two of these were born before 1965 and were unvaccinated. Eight of the HCW with measles were born between 1965 and 1975, of whom only one was vaccinated (one dose). Of the four HCW born in 1975, 1976 and 1977 (these cohorts were offered only one vaccination during their childhood), three had been vaccinated once and one was unvaccinated. Five HCW were born after 1978, of whom two were unvaccinated and three had been vaccinated at least twice. Most infected HCW were working in a general practice ( $n=8$ ) and three HCW acquired measles while working in a hospital. There were no reports of infected HCW transmitting measles to patients or other HCW, nor reports from patients infected while hospitalised.

### Comparison with the 1999–2000 epidemic

The 2013–2014 epidemic was comparable with the 1999–2000 epidemic in that it took place in the same low-vaccination-coverage areas and affected mostly the unvaccinated orthodox Protestant population. The age distribution of the epidemics, however, differed markedly (Figure 3). First, the median age in the 1999–2000 epidemic was 6 years [6], compared with 10 years in the recent epidemic ( $p$  value  $< 0.01$ ).

Second, the incidence by age group of the two epidemics differed ( $p < 0.01$ ). Older age groups (9 years and older) had a higher incidence in 2013–2014 than in 1999–2000, while the incidence in age groups below 9 years of age were halved in 2013–2014 compared with 1999–2000. Among infants aged 6–13 months, who were offered an early MMR vaccination in 2013–2014 but not in 1999–2000, the incidence in 2013–2014 was 62 per 100,000. This is significantly lower than the incidence of 126 per 100,000 reported in this age group in 1999–2000 ( $p < 0.05$ ). In contrast, the incidence in infants below 6 months of age was higher in 2013–2014 than in the 1999–2000 epidemic (7 and 4 per 100,000, respectively) ( $p = 0.529$ ).

### Discussion

Despite an MMR-1 vaccination coverage above 95% for the past 20 years in the Netherlands, a large measles epidemic of 2,700 reported cases, including cases with severe illness and one death, occurred in 2013–2014 among sociogeographically clustered orthodox Protestant communities with low vaccination coverage. The total costs of this epidemic were recently estimated at EUR 3.9 million [21].

In comparison with the previous epidemic in this group in 1999–2000, older age groups were more affected. There was a striking decline in reported cases during the summer holidays, which could be due to reduced transmission of measles and/or reduced reporting. The

change of guidelines communicated by the RIVM to the MHS in mid-July 2013 to reduce the workload may also have influenced reporting.

The vast majority of reported cases were among unvaccinated orthodox Protestant individuals. The number of cases in other risk groups remained relatively low, which suggests limited contact with orthodox Protestants and more protection from herd immunity. Of the 141 vaccinated cases, most were in children between 4 and 8 years of age who had been vaccinated only once. Bringing the second MMR dose forward from 9-year-olds to 4-year-olds can reduce the susceptibility in this age group [22].

A limitation of our study is that it was based on reported cases only. After the 1999–2000 epidemic it was estimated that only 7% of all individuals with measles were reported [23]. Another study carried out a survey after the epidemic and identified 164 measles cases, of which only 9% ( $n=15$ ) had been reported during the 1999–2000 epidemic [24]. We found similar completeness of reporting of measles infections in this measles epidemic (data not shown). Based on this, the estimated number of individuals with measles infection in the 2013–2014 epidemic is ca 30,000. The use of non-invasive samples such as saliva and urine for measles diagnosis contributed to a higher proportion of infections being laboratory-confirmed or epidemiologically linked to a confirmed infection, and hence to a more complete reporting.

Eleven per cent of all reported cases had one or more complications. Similar to other epidemics [6,25–27], complications and hospitalisations were more likely to occur in young children and adults [17]. Cases with complications and/or hospitalisations were probably more likely to be reported than cases without complications, thus the true rate of complications and hospitalisations among all measles infections during this epidemic is likely to be lower than the 11% and 7% we found in reported cases, respectively.

A rare complication of measles, subacute sclerosing panencephalitis (SSPE), occurs months to years after measles infection. Recently, a case of SSPE was reported in a Dutch 17-year-old who died 4 months after diagnosis [28]. He had acquired measles in the Netherlands during the epidemic of 1999–2000 at the age of four years. SSPE is a very rare fatal complication of measles: estimates of SSPE incidence are ca 0.4–1.1 cases of SSPE per 10,000 cases of measles [29]. Assuming that 30,000 individuals acquired measles virus infection in the 2013–2014 epidemic, up to three cases of SSPE can be expected in the next two decades.

High measles vaccination coverage among HCW has been associated with decreased healthcare-associated measles virus infections among patients and personnel [30]. During this measles epidemic, 16 of 19 HCW with

measles were incompletely vaccinated although they were eligible to complete their MMR vaccination schedule according to the advice of the OMT. An assessment of barriers to implementation of the recommendations is ongoing.

Compared with the previous epidemic in orthodox Protestants, we found a higher median age in the 2013–2014 epidemic and higher incidence rates in age groups above 8 years of age. This is likely due to the longer inter-epidemic interval before the 2013–2014 epidemic compared with the interval before the 1999–2000 epidemic [31]. The epidemic preceding the 1999–2000 epidemic was in 1992–1994, whereas the epidemic preceding the 2013–2014 epidemic was in 1999–2000. As a result, the susceptible population, consisting of individuals born since the previous epidemic, had a wider age range in 2013 than in 1999.

The cause of the lower incidence in children below nine years in the 2013–2014 epidemic compared with the 1999–2000 epidemic may be due to an increase in vaccination coverage among children under 9 years old in orthodox Protestant communities. Evidence for this was found in the serological surveys performed in 2006–2007 and 1995–1996, in which a higher proportion of diphtheria protection was found in the most recent survey [32]. Second, vaccination uptake in orthodox Protestants seems to be increasing generation on generation, as found in 2013 by assessing vaccination status of orthodox Protestants from the age of 18 to 40 years, their parents and their children (data not shown). Increasing vaccination coverage within these communities may also explain the longer inter-epidemic period [31] and, at least partly, the higher median age. The distribution of cases comprises a smaller proportion of young cases compared with the previous epidemic.

The lower incidence among infants 6–13 months of age could reflect the administration of early MMR vaccination. However, results are difficult to interpret given that the incidence was also relatively low in the adjacent older age groups. The incidence in infants aged less than 6 months was higher in 2013–2014 than in the 1999–2000 epidemic. This is likely to be related to the lower level of maternal antibodies in children born to vaccinated mothers compared with children born to unvaccinated mothers [16]. Measles vaccination began in the Netherlands in 1976. Therefore, in 2000, the proportion of infants born to vaccinated mothers was probably lower than in 2013.

The source of the first measles cases from this outbreak is unknown. According to the MEANS database, the Taunton sequence was first identified in Wales, UK, in the second half of 2012, and subsequently in many other cities in the UK throughout 2012 and the first half of 2013. At the time when the first Dutch case was identified with the Taunton sequence in May 2013, ca 900 identical sequences had been reported to MEANS, not only from the UK but several other countries within

the WHO European Region (e.g. France, Ireland, the Russian Federation). Therefore, a particular source country is hard to identify [33,34]. The epidemic in the Netherlands, however, was indicated as the origin of outbreaks in Belgium [35] and Canada [36,37]. From Canada, onward transmission continued into the United States [38]. The likely spread to Belgium led to an outbreak in a day care centre with 33 reported cases. In Canada an outbreak took place in Alberta with 43 reported cases and another in British Columbia with 444 reported cases. Social ties exist between orthodox Protestants in the Netherlands and Canada and the spread of infections such as poliomyelitis, measles, mumps, and rubella to Canada has been reported before [39].

Improved vaccination coverage among orthodox Protestants is essential to prevent future outbreaks. It is therefore one of the prioritised interventions in the Netherlands' national measles elimination plan [40]. Since orthodox Protestants base their vaccination decisions largely on religious arguments [41], specific information materials were developed focusing on religious arguments for and against vaccination. These brochures aim to facilitate decision making about vaccination among orthodox Protestants and were distributed during the epidemic [42]. An evaluation of their acceptability and impact is currently ongoing.

Vaccination coverage seems to be increasing within the orthodox Protestant community. An improvement in vaccination coverage will be reflected in a different epidemiology of future epidemics. In the current epidemic, a longer inter-epidemic period resulted in older age groups affected in comparison to the previous epidemic.

The number of individuals refraining from vaccination is insufficient to sustain endemic measles transmission in the Netherlands. Nevertheless, this situation does pose a risk to public health in the Netherlands and contributes to the worldwide spread of measles, thus forming an impediment to the elimination of measles in Europe and elsewhere.

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## Conflict of interest

None declared.

## Authors' contributions

HM, RB, SH, WR and TW analysed and interpreted the data. The overall paper outline was developed by SH, TW and WR. ES, HM, JW, RB, SH, TW and WR critically revised the manuscript. All authors approved the final version.

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# Development of oseltamivir and zanamivir resistance in influenza A(H1N1)pdm09 virus, Denmark, 2014

R Trebbien<sup>1</sup>, SS Pedersen<sup>2</sup>, K Vorborg<sup>1</sup>, KT Franck<sup>3,4</sup>, TK Fischer<sup>1</sup>

1. National Influenza Center, Virological Surveillance and Research, Department of Microbiological Diagnostics and Virology, Statens Serum Institut, Denmark
2. Department of Infectious Diseases, Odense University hospital, Odense, Denmark
3. Department of Microbiological Diagnostics and Virology, Statens Serum Institut, Denmark
4. Department of Clinical Microbiology, Copenhagen University Hospital, Herlev, Denmark

Correspondence: Ramona Trebbien (ratr@ssi.dk)

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Antiviral treatment of immunocompromised patients with prolonged influenza virus infection can lead to multidrug resistance. This study reveals the selection of antiviral resistance mutations in influenza A(H1N1)pdm09 virus in an immunocompromised patient during a 6-month period. The patient was treated with two courses of oseltamivir (5 days and 2 months, respectively), with the first course starting at symptom onset, and subsequently zanamivir (2 months and 10 days, respectively). Respiratory samples were investigated by Sanger and next generation sequencing (NGS) and, for NGS data, low-frequency-variant-detection analysis was performed. Neuraminidase-inhibition tests were conducted for samples isolated in Madin-Darby canine kidney cells. In a sample collected 15 days after the end of the first treatment with oseltamivir (Day 20 post-symptom onset), oseltamivir resistance was detected (mutation H275Y with 60.3% frequency by NGS). Day 149 when the patient had almost completed the second zanamivir treatment, mixes of the following resistance mutations were detected; H275Y(65.1%), I223R(9.2%), and E119G(89.6%), accompanied by additional mutations, showing a more complex viral population in the long-term treated patient. Two samples obtained on Day 151 from bronchoalveolar lavage (BAL) and nasopharyngeal swab, respectively, showed different mutation profiles, with a higher frequency of antiviral resistance mutations in BAL. The results emphasise the importance of timely antiviral resistance testing both for treatment of individual patients as well as for preventive measures to control the development and transmission of antiviral resistant viruses.

## Introduction

Influenza virus is the cause of annual seasonal epidemics worldwide, and leads to high morbidity in the population. Severe disease and deadly outcome due to influenza virus are recognised in the defined risk groups, in particular elderly persons >65 years of age

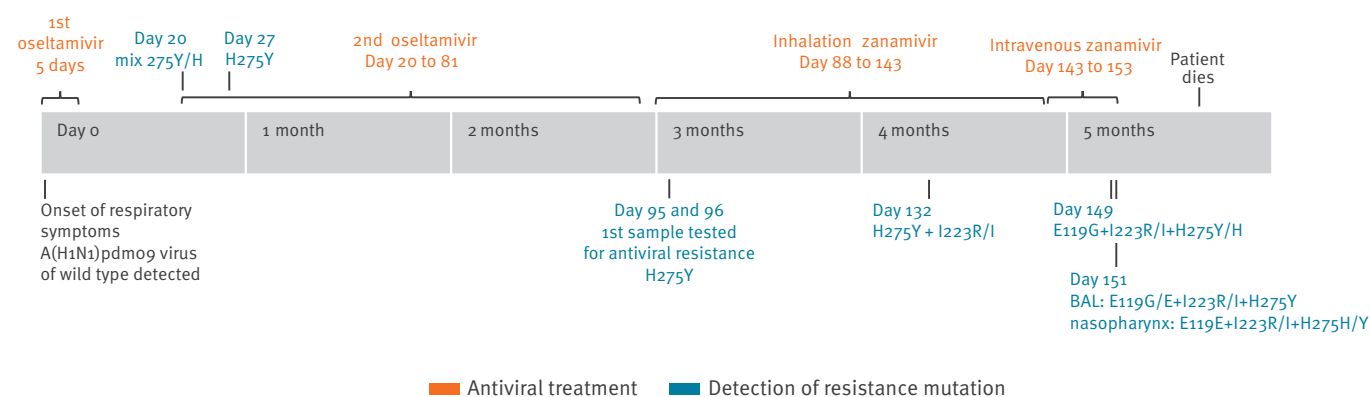
and immunocompromised patients. Influenza is normally an acute self-limiting disease with a duration of 5 to 7 days, however, in immunocompromised patients prolonged infections lasting several months have been reported [1-4]. Prevention of severe influenza disease is mainly based on immunisations with split vaccines which are produced annually to accommodate the changing antigenicity of seasonal epidemic viruses [5,6]. The effect of vaccination in immunocompromised patients is questionable and this is why other modes of prevention and/or treatment often are considered for this risk group [7-12]. For treatment of influenza viruses only a few antiviral drugs are available; the neuraminidase (NA) inhibitors and the matrix-2 (M2)-ion channel inhibitors. The current circulating epidemic influenza viruses harbour natural resistance towards the M2-ion channel inhibitors therefore these are not an option for treatment [13-15]. The NA inhibitors bind to the NA surface protein and prevent it from facilitating the release of new virus particles from an infected cell [16]. In Denmark, two different NA inhibitors are approved and available for treatment of influenza: oseltamivir (Tamiflu) and zanamivir (Relenza). Oseltamivir is the drug of choice for treatment due to its easy oral administration whereas zanamivir (intravenous or inhalation) is often used when the effect of oseltamivir is limited, e.g. in case of development of resistance. In the NA gene of the H1N1 viruses a range of amino acid mutations are recognised to confer reduced inhibition by NA inhibitors [16-18]. Among these, two well characterised mutations are the H275Y mutation which results in viruses with highly reduced inhibition by oseltamivir and the I223R mutation which results in reduced inhibition by both oseltamivir and zanamivir [16,17].

Antiviral treatment of immunocompromised patients with prolonged influenza virus infection can lead to multidrug-resistant influenza quasispecies in the same patient [1]. We describe how the emergence of such



## FIGURE

Time line of the treatment course of an immunocompromised patient with influenza with oseltamivir and zanamivir and of development of antiviral resistance mutations, Denmark, 2014



BAL: bronchoalveolar lavage.

virus variants poses challenges in the combat of a severe influenza infection in a Danish patient treated with antivirals. The patient had sustained shedding of influenza A(H1N1)pdm09 virus for 6 months and was treated with oseltamivir and subsequently zanamivir. Antiviral resistance mutation profiles were evaluated using conventional Sanger sequencing and next generation sequencing (NGS).

## Methods

### Case and samples

The immunocompromised patient had chronic lymphocytic leukaemia (CLL) and was aged between fifty and sixty years. Influenza-vaccination status was unknown. Respiratory samples were obtained at frequent intervals during the course of infection. These included nasopharyngeal swabs, bronchoalveolar lavage (BAL), and expectorates, which were used for diagnostic of influenza virus and detection of antiviral resistance. The first sample was collected at the onset of respiratory symptoms. Since it tested positive for influenza A(H1N1)pdm09, oseltamivir treatment was immediately started. Approximately 3 months later, a first sample for antiviral resistance testing was submitted to the National Influenza Center, Denmark. Subsequent samples were then also investigated, as well as those collected before this time point, which were retrospectively analysed. The overall treatment consisted of two courses of oral oseltamivir, one course of inhalation therapy with zanamivir, and a compassionate-use programme with intra venous (i.v.) zanamivir (Figure).

### Virus detection

Total nucleic acid was extracted using 200 µL of sample material and the MagNA Pure LC Total Nucleic Acid Isolation Kit on the MagNapture 96/32 (Roche).

A multiplex real-time reverse transcription-PCR (RT-PCR) detecting the influenza A matrix (M) gene as well as H1N1pdm09 NA gene was performed using the MX3005P Stratagene platform according to the protocol designed by the National Influenza Center Denmark (Statens Serum Institut, Copenhagen, Denmark). The cycle threshold (Ct) values for the M-gene were used as a relative measure of viral load.

### Virus isolation

Isolation of influenza virus was performed in duplicates in Madin-Darby canine kidney (MDCK) cells using standard methods [19]. Because of challenges caused by poor constitution/lack of sample material and difficulties in cultivating the virus in ca 50% of samples, it was not possible to report viral load in PFU/mL or TCID<sub>50</sub>.

Due to loss of the zanamivir antiviral resistance mutations during cell-propagation, experimentation with addition of zanamivir and oseltamivir in different concentrations and combinations was performed in an attempt to rescue the mutated virus. Virus growth medium was supplemented with zanamivir and oseltamivir at the following concentrations: 1 µM, 0.1 µM, 0.01 µM, 0.001 µM. The two drugs were mixed or added alone to the virus growth medium in the different concentrations.

### Detection of the H275Y mutation using allele-specific real-time reverse transcription-PCR

For rapid initial screening of the oseltamivir resistance conferring single nt polymorphism (SNP) mutation H275Y, an allele specific real-time RT-PCR was applied to the samples, following a protocol designed by the National Influenza Center Denmark (Statens Serum Institut, Copenhagen, Denmark).

TABLE 1

Amino acid substitutions in the influenza A(H1N1)pdm09 virus neuraminidase, reported to be involved in antiviral resistance [16], which were found in an immunocompromised patient treated with oseltamivir and zanamivir, Denmark 2014

Sample number: cells for virus culture and antiviral added if any	Day	Origin of the sample	M-gene Ct-value	Reads NGS	Sequencing method	Amino acid position and type in the consensus sequence of reference virus A/California/07/2009; for comparison to the sequence of the virus infecting the patient										
						117 <sup>a</sup>	118 <sup>b</sup>	119 <sup>b</sup>	136 <sup>a</sup>	199 <sup>b</sup>	223 <sup>b</sup>	247 <sup>a</sup>	275 <sup>b</sup>	402 <sup>b</sup>		
						I	R	E	Q	D	I	S	H	Y		
1	0	Nasopha.	29.55	1,525,617	NGS (%)	*	*	*	*	*	*	*	*	*		
					S	*	*	*	*	*	*	*	*	*	*	
2	20	Nasopha.	27.47	914,914	NGS (%)	M (1.04)	*	*	*	*	R (3.4)	*	Y (60.3)	*		
					S	*	*	*	*	*	*	*	Y/H	*		
3	27	Nasopha.	31.5	4,483	NGS (%)	*	*	*	*	*	*	*	Y (>99)	*		
					S	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
4	95	BAL	25.88	n.d.	NGS (%)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
					S	*	*	*	*	*	*	*	Y	*		
4: 2MDCK Z		Cell culture	34.35	1,216,299	NGS (%)	*	*	G (24.3)	*	*	*	N (72.4)	Y (>99)	*		
					S	*	*	E/G	*	*	*	S/N	Y	n.d.		
4: 2MDCK Z/O		Cell culture	34.53	1,566,074	NGS (%)	*	*	G (30.4)	*	G (1.6)	*	N (42.6)	Y (>99)	*		
					S	*	*	E/G	*	*	*	S/N	Y	*		
5		96	BAL	26.53	n.d.	NGS (%)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
						S	*	*	*	*	*	*	S/N	Y	*	
5: 3MDCK			Cell culture	16.82	1,458,499	NGS (%)	*	*	*	*	*	*	*	Y (>99)	N (1.47)	
						S	*	*	*	*	*	*	*	Y	*	
5: 4MDCK Z			Cell culture	22.96	1,233,121	NGS (%)	*	*	*	*	*	*	*	Y (>99)	*	
						S	*	*	*	*	*	*	n.d.	Y	*	
5: 4MDCK Z/O			Cell culture	23.72	n.d.	NGS (%)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
						S	*	*	*	*	*	*	*	Y	*	
6	132		Expectorate	28.83	1,132,787	NGS (%)	*	*	*	*	*	R (53.4)	R (1.1)	Y (>99)	*	
						S	*	*	*	*	*	*	I/R	*	Y	*
6: 2MDCK		Cell culture	18.73	1,012,035	NGS (%)	*	*	*	*	*	*	*	Y (>99)	*		
					S	*	*	*	*	*	*	*	Y	*		
6: 2MDCK Z		Cell culture	32.01	2,955,354	NGS (%)	*	*	*	*	*	R (75.3)	*	Y (>99)	*		
					S	*	*	*	*	*	*	I/R	*	Y	*	
6: 2MDCK Z/O		Cell culture	34.13	641,179	NGS (%)	*	*	*	*	*	R (49.3)	I (1.9)	Y (>99)	*		
					S	*	*	*	*	*	*	I/R	*	Y	*	
6: 3MDCK		Cell culture	18.71	1,255,140	NGS (%)	*	*	*	*	*	*	*	*	Y (>99)	*	
					S	*	*	*	*	*	*	*	*	Y	*	
7	149	Expectorate	32.61	1,413,804	NGS (%)	L (7.04)/M (9.3)	*	G (89.6)	*	*	R (9.2)	N (6.7)	Y (65.1)	*		
					S	*	*	G/E	*	*	*	*	H/Y	*		
8	151	BAL	36.99	3,036,466	NGS (%)	*	*	G (35.9)	*	*	R (51.8)	*	Y (88.2)	*		
					S	*	*	E/G	*	*	I/R	*	Y	*		
8: 2MDCK		Cell culture	17.93	1,823,988	NGS (%)	*	*	*	*	*	*	*	Y (>99)	*		
					S	*	*	*	*	*	*	*	Y	*		
8: 3MDCK		Cell culture	15.94	1,274,826	NGS (%)	*	*	*	*	*	*	*	Y (>99)	*		
					S	*	*	*	*	*	*	*	Y	*		
9	151	Nasopha.	36.56	1,699,587	NGS (%)	*	M (1.1)	G (7.3)	K (2.5)	*	R (34.2)	N (6.2)	Y (74.9)	*		
					S	*	*	*	*	*	I/R	*	H/Y	*		

AA: amino acid; BAL: bronchoalveolar lavage; Ct-value: cycle threshold value obtained by real-time reverse transcription-PCR; M-gene: matrix gene; MDCK: Madin-Darby canine kidney cells; nasopha.: nasopharyngeal swab; n.d.: no data; NGS: next generation sequencing; S: Sanger sequencing; z: virus isolate cultivated with 1µM zanamivir; z/o: virus isolate cultivated with 1µM zanamivir and 0.1µM oseltamivir.

The samples are ordered and numbered in the chronological order of collection. When the virus was isolated in culture from a sample, the types of cells and the number of passages are indicated next to the sample number. The Table also depicts the type of sample material, which was initially collected. NGS and S-sequencing of samples or the respective virus isolates were performed to infer the AA sequence. When more than one AA type was inferred at a given sequence position by NGS, the frequency of each AA identified is provided (in the rows: NGS (%)). The \* indicates that the AA is identical to the reference virus A/California/07/2009(H1N1)pdm09 consensus sequence.

<sup>a</sup> Non-active site of the neuraminidase enzyme.

<sup>b</sup> Active site of the neuraminidase enzyme.

TABLE 2

Influenza A(H1N1)pdm09 virus neuraminidase amino acid substitutions proposed to be involved in viral fitness and related to antiviral resistance and cell adaptation, which were found in samples from an immunocompromised patient treated with oseltamivir and zanamivir, Denmark 2014

Sample	Day	Material	Reads NGS	Sequencing method	Amino acid position and type in the consensus sequence of reference virus A/California/07/2009; for comparison to the sequence of the virus infecting the patient											
					83	86	147	149	173	232	241	248	313	314	416	438
					V	A	G	I	R	A	I	D	Q	I	D	T
1	0	Nasopha.	1,525,617	NGS (%)	*	*	*	*	*	*	*	*	*	*	*	*
				S	*	*	*	*	*	*	*	*	*	*	*	*
2	20	Nasopha.	914,914	NGS (%)	*	*	*	*	*	*	*	*	*	*	N (20.4)	*
				S	*	*	*	*	*	*	*	*	*	*	*	*
3	27	Nasopha.	4,483	NGS (%)	*	*	*	*	*	*	*	*	*	*	*	*
				S	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4	95	BAL	n.d	NGS (%)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
				S	*	*	*	*	*	*	*	*	*	*	*	*
4: 2MDCK Z		Cell culture	1,216,299	NGS (%)	*	*	R (2)	*	*	*	V (7.7)	N (1.6)	*	*	*	*
				S	*	*	*	*	*	*	*	*	*	*	n.d.	n.d.
4: 2MDCK Z/O		Cell culture	1,566,074	NGS (%)	*	*	*	*	*	*	V (4.5)	N (1.9)	*	T (3.2)	*	A (4.5)
				S	*	*	*	*	*	*	*	*	*	*	*	*
5	96	BAL	n.d.	NGS (%)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
				S	*	*	*	*	*	*	D/(N)	*	*	*	*	*
5: 3MDCK		Cell culture	1,458,499	NGS (%)	*	*	*	*	*	*	V (10.6)	*	*	*	*	*
				S	*	*	*	*	*	*	*	*	*	*	*	*
5: 4MDCK Z		Cell culture	1,233,121	NGS (%)	*	*	*	*	*	*	V (4.8)	*	*	*	*	*
				S	*	*	*	*	*	*	*	*	*	*	*	*
5: 4MDCK Z/O		Cell culture	n.d.	NGS (%)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
				S	*	*	*	*	*	*	*	*	*	*	*	*
6	132	Expectorate	1,132,787	NGS (%)	I (44.9)	*	R (33.4)	T (6.6)	*	*	V (5.1)	*	K (3.5)	*	N (4.1)	A (1.8)
				S	V/I	*	G/(R)	*	*	*	*	*	*	*	*	*
6: 2MDCK		Cell culture	1,012,035	NGS (%)	*	T (71)	*	T (23)	K (30)	*	V (3.8)	*	K (70)	*	*	*
				S	*	T	*	*	R/K	*	*	*	K	*	*	*
6: 2MDCK Z		Cell culture	2,955,354	NGS (%)	I (14.8)	*	R (15.4)	T (4.1)	*	*	V (1.7)	*	K (4.1)	*	N (2.7)	*
				S	V/(I)	*	*	*	*	*	*	*	*	*	*	*
6: 2MDCK Z/O		Cell culture	641,179	NGS (%)	I (41.6)	*	R (40)	T (6.9)	*	*	V (4)	*	K (2.6)	*	N (1.1)	*
				S	V/I	*	G/R	*	*	*	*	*	*	*	*	*
6: 3MDCK		Cell culture	1,255,140	NGS (%)	*	T (80)	*	T (16)	K (21)	*	V (3.8)	*	K (79)	*	*	*
				S	*	T	*	*	R/K	*	*	*	K	*	*	*
7	149	Expectorate	1,413,804	NGS (%)	I (4.6)	*	R (2)	*	*	V (21.8)	V(3)	N (46)	R (2.8)	T (17.8)	*	A (56.9)
				S	*	*	*	*	*	*	*	D/N	*	*	*	T/A
8	151	BAL	3,036,466	NGS (%)	I (6.1)	*	R (21.3)	*	*	V (14)	V (8.1)	N (9.2)	*	T (5.6)	*	A (3)
				S	*	*	*	*	*	*	*	*	*	*	*	*
8: 2MDCK		Cell culture	1,823,988	NGS (%)	*	T (62)	*	T (32)	K (40)	*	V (3.1)	*	K (60)	*	*	*
				S	*	T	*	I/(T)	R/K	*	*	*	Q/K	*	*	*
8: 3MDCK		Cell culture	1,274,826	NGS (%)	*	T (74)	*	T (22)	K (29)	*	V (5.3)	*	K (71)	*	*	*
				S	*	T	*	*	R/K	*	*	*	K	*	*	*
9	151	Nasopha.	1,699,587	NGS (%)	*	*	*	*	*	V (15.3)	V (5.4)	N (44.9)	R (9.9)	T (8.8)	*	A (2.5)
				S	*	*	*	*	*	*	*	D/N	*	*	*	*
Proposed function <sup>a</sup>					f	c	f	u	c	f	u	f	c	f	u	f

AA: amino acid; BAL: bronchoalveolar lavage; Ct-value: cycle threshold value obtained by real-time reverse transcription-PCR; nasopha.: nasopharyngeal swab; n.d.: no data; MDCK: Madin-Darby canine kidney cells; NGS: next generation sequencing; S: Sanger sequencing; z: virus isolate cultivated with 1µM zanamivir; z/o: virus isolate cultivated with 1µM zanamivir and 0.1µM oseltamivir.

The samples are ordered and numbered in the chronological order of collection. When the virus was isolated in culture from a sample, the type of cells and the number of passages are indicated next to the sample number. The Table also depicts the type sample material, which was initially collected. NGS and Sanger sequencing of samples or the respective virus isolates were performed to infer the AA sequence. When more than one AA type was found at a given sequence position by NGS, the frequency at which each AA identified is provided (in the rows: NGS (%)). The \* indicates that the AA is identical to the reference virus A/California/07/2009(H1N1pdm09) consensus sequence.

<sup>a</sup> f: fitness related to antiviral resistance; c: cell adaptation due to cultivation; u: unknown.

**TABLE 3**

Results from neuraminidase inhibition assay of virus isolates, Denmark, 2014

Virus isolate	Amino acid substitution	Mean IC <sub>50</sub> (nM) for oseltamivir	Fold change to wild type for oseltamivir	Conclusion: oseltamivir	Mean IC <sub>50</sub> (nM) for zanamivir	Fold change to wild type for zanamivir	Conclusion: zanamivir
5: 3MDCK	H275Y	440	880	HRI	0.03	2.1	NI
6: 2MDCK	H275Y	245.8	491.6	HRI	0.03	2.13	NI
8: 3MDCK	H275Y	250.9	501.8	HRI	0.02	1.59	NI
A/California/07/2009	Wild type	0.5	Ref	NI	0.01	Ref	NI

HRI: highly reduced inhibition; IC<sub>50</sub>: 50% inhibitory concentration; MDCK: Madin-Darby canine kidney cells; NI: normal inhibition; ref: reference.

The mean IC<sub>50</sub> is indicated in nM for both oseltamivir and zanamivir as well as the fold change to wild type control virus A/California/07/2009(H1N1pdm09).

### Sequencing of the neuraminidase gene and minority variant analysis

Full-length Sanger sequencing of the NA gene was performed by RT-PCR using in-house primers and Big Dye chemistry on an ABI3500 capillary sequencer (Applied Biosystems) and was analysed using Bionumerics (Applied Maths, Belgium) and Molecular Evolutionary Genetics Analysis (MEGA)6 software.

NGS was performed using NexteraXT DNA sample preparation kit (Illumina) and subsequent sequencing on the MiSeq (Illumina).

Minority variant analysis was performed on NGS data using CLC Genomics Workbench version 8 (Qiagen, Germany).

### Neuraminidase inhibition assays

Oseltamivir and zanamivir inhibition of the NA activity of virus isolates was assessed using the NA-Fluor Influenza Neuraminidase Assay Kit from Applied Biosystems (Life technologies). The reference virus A/California/07/2009(H1N1pdm09) was used as wild type control. The mean 50% inhibitory concentration (IC) was calculated according to the kit protocol and as the fold-change to the wild-type control virus. World Health Organization (WHO) criteria for determination of inhibition by oseltamivir and zanamivir were used to evaluate the level of antiviral resistance [20]. Normal inhibition (NI) is <10 IC<sub>50</sub> fold change compared with wild-type virus, reduced inhibition (RI) 10–100, and highly reduced (HR) >100.

## Results

### Genotypic antiviral resistance testing results

The patient was treated with oseltamivir immediately after diagnosis. The sample from day 0, the same day the first oseltamivir treatment was initiated, was retrospectively analysed for antiviral resistance mutations. At this point there was no antiviral resistance mutations recognised (Table 1).

Despite the first oseltamivir treatment lasting 5 days, the patient continued to have symptoms and influenza A(H1N1)pdm09 positive samples. As a result, a second oseltamivir treatment was initiated at 20 days post-symptom onset (Day 20). Samples collected 15 days after termination of the 1st oseltamivir treatment (Day 20) and 7 days after initiation of the 2nd oseltamivir treatment (Day 27), were retrospectively investigated as well. Both contained virus with the H275Y mutation at a frequency of 60.3% (day 20) and >99% (day 27). Day 96, one week after initiation of inhalation therapy with zanamivir and three months after initiation of symptoms including two courses of treatment with oseltamivir another sample was collected and submitted for antiviral resistance testing to the National Influenza Center. The sample contained influenza A(H1N1)pdm09 virus with the H275Y mutation and Sanger sequencing revealed an additional S247N mutation (Figure, Table 1). Day 132, one and a half month after initiation of inhalation therapy with zanamivir a sample was investigated for further development of antiviral resistance mutations. At this time point the H275Y mutation was still recognised, however, a mixed population with the I223R/I mutation was also observed by Sanger sequencing. The I223R substitution was later confirmed by NGS with a frequency of 53.4% (Figure, Table 1).

As no clinical improvement of the patient was obtained, i.v. zanamivir treatment was carried out for 10 days. Samples from Day 149 and 151, six and eight days after initiation of i.v. zanamivir treatment, respectively, revealed a mixed population of virus with wild type and resistant-conferring residues at position 275 (H275Y/H) as well as at position 223 (I223R/I) using Sanger sequencing (Figure, Table 1). By NGS a more differentiated viral population was observed involving a range of mutations (Table 1 and 2). Interestingly, a discrepancy was discovered between two samples collected on day 151. In a sample obtained as BAL there was a higher frequency of the major resistance-inducing mutations (E119G: 35.9%, I223R: 51.8%, and H275Y: 88.2%) compared with a sample obtained as nasopharyngeal swab (E119G: 7.3%, I223R: 34.2% and H275Y: 74.9%). The nasopharyngeal swab on the other hand showed three

additional mutations related to antiviral resistance, however, at a low frequency (R118M: 1.1%, Q136K: 2.5%, and S247N: 6.2%).

### Genotypic antiviral resistance testing of cell propagated samples

It was possible to propagate virus from four of the samples in MDCK cells. During propagation it was observed by sequencing that the antiviral resistance mutation I223R found in the original sample materials was lost after cell propagation, while new mutations indicative of cell adaptation occurred at positions A86T, R173K, and Q313K (Table 1 and 2). In an attempt to preserve the antiviral resistance mutations, the growth medium was supplemented with zanamivir alone or zanamivir and oseltamivir. For two virus isolates propagated with antivirals in the growth medium it was possible to rescue viruses with the I223R mutation (Table 1).

### Phenotypic antiviral resistance testing results

Due to a low amount of sample material it was not possible to perform NA inhibition tests on any of the samples directly. Three virus isolates carrying only the H275Y mutation had mean IC<sub>50</sub>s against oseltamivir, which were ca 500–900 fold higher than the wild type H275 strain A/California/07/2009(H1N1pdm09) virus, thereby showing highly reduced inhibition (Table 3). Against zanamivir there was normal inhibition of the virus isolates carrying the H275Y only. Unfortunately the virus isolates carrying both the I223R/I and H275Y mutations did not display NA activity and the phenotypic NA inhibition by oseltamivir and zanamivir could not be determined for these isolates.

### Discussion

This study describes a case of zanamivir and oseltamivir resistant influenza A(H1N1)pdm09 virus investigated in details with the use of NGS. Oseltamivir is the drug of choice when treating severely ill patients infected with influenza virus. Studies have shown that resistance towards oseltamivir develops fast within one week of treatment [1]. This coheres with the fact that only one mutation (H275Y) is needed to induce resistance against oseltamivir in H1N1pdm09 virus [21]. Antiviral resistance of influenza viruses against zanamivir is more rarely reported than oseltamivir resistance. The reason for this is likely due to the more extensive use of oseltamivir compared with zanamivir. H1N1pdm09 viruses with both I223R and H275Y mutations are shown to have increased resistance against both oseltamivir and zanamivir [22]. LeGoff et al. 2012 [1] shows that I223R alone confers reduced susceptibility to both oseltamivir and zanamivir and is primarily selected by oseltamivir. In this study we observed that the I223I/R mutation was selected after the introduction of zanamivir treatment, whereas the H275Y mutation was induced rapidly after initiation of oseltamivir treatment.

In immunocompromised patients the frequency of developing antiviral resistance against oseltamivir

due to the H275Y mutation in connection to treatment can reach 13% [23] which is a substantially higher frequency compared with overall reporting of resistant H1N1pdm09 viruses which in the 2014/15 season was 0.4% [24]. Prolonged shedding of influenza virus in immunocompromised patients is well known and studies have provided evidence that the prolonged virus shedding can result in the emergence of additional mutations in the NA gene [1,25,26]. This indicates evolution of the viruses and the emergence of an increasingly more complex viral population in the antiviral-treated patient. This study contributes with further data to support this, as additional mutations were observed. The additional mutations discovered concerned amino acid substitutions both in the active and non-active site of the NA molecule, some of which have previously been described as involved in antiviral resistance, e.g. G147R and S247N [16,18]. In a recent published study by Takashita et al. [18] it was reported that H1N1pdm09 harbouring dual substitution at positions H275Y/G147R had a highly reduced inhibition by oseltamivir and peramivir, whereas, inhibition was within the normal range with zanamivir. The additional amino acid changing mutations not earlier described to induce antiviral resistance on their own, deserves further studies to clarify their potential effect on antiviral resistance. It could perhaps be an evolvement toward better fitness in the presence of the H275Y and I223R mutations under the selection of the antiviral drugs.

All samples were by default investigated by Sanger sequencing, however, due to the complex populations with mixed nt at important sites for antiviral resistance, NGS was performed to achieve deep sequencing and to provide the possibility to investigate minority variants. Interestingly, we found a larger proportion of minority variants by NGS. Furthermore, many of the mixed nt found by Sanger sequencing were confirmed by NGS which could additionally reveal the frequency of the different amino acid conferred by the nt variants. This underlines the limitations in interpretation of diverse viral populations/quasispecies using Sanger sequencing.

The present study emphasises the importance of rapid antiviral resistance testing during the course of a prolonged infection. The first sample submitted specifically for testing of antiviral resistance was obtained 96 days after the first influenza positive test. In the meantime the patient had been treated with two courses of oseltamivir and zanamivir treatment had been initiated. However, retrospective testing showed that the oseltamivir resistance mutation H275Y had been induced already after the first oseltamivir treatment, which means that the second course of oseltamivir treatment for 2 months had likely limited effect on the influenza virus infection. Whether an early antiviral resistance test revealing resistance towards oseltamivir and a subsequent rapid decision to shift to zanamivir could have improved the clearance of infection is however unknown. Studies indicate



that immunocompromised patients have a major risk for prolonged shedding of influenza virus, and this in combination with the questionable effect of antivirals against influenza virus administered later than 48 hours after symptom onset, makes it difficult to predict the outcome of treatment [4,27]. Development of antiviral resistance in influenza virus in hospitalised patients poses a concern for nosocomial transmission, and a further risk of spreading mutant viruses. In particular, the prolonged infections frequently observed in immunocompromised patients can foster adaptation of increased fitness for viruses harbouring the antiviral resistance mutations. Close monitoring of these patients and of the development of antiviral resistance is necessary, and preventative measures against viral transmission both in the hospital setting and in the community need to be implemented.

Due to the limited amount of sample material it was not possible to investigate the phenotypic antiviral resistance characteristics in the NA inhibition assay. It is difficult to assess the importance of mixed mutations and the direct effect on antiviral resistance, without the opportunity to test in a phenotypic assay. From the clinical point of view, the patient's condition did not improve with the administration of antivirals. Moreover laboratory testing revealed that the antiviral drugs had a limited effect against virus shedding, with emergence of resistance against both oseltamivir and zanamivir in the viral population infecting the patient. During cell propagation new mutations appeared which most likely can be attributed to cell adaptation, as the original samples did not contain the mutations but were represented in the virus isolates only. Cell adaptation of influenza viruses during propagation in MDCK cells is a normally observed phenomenon [28]. It is also problematic to perform phenotypic NA inhibition tests requiring virus isolation, when a sample contains a mixture of mutated resistant and wild type viruses. Indeed, during propagation there is a selection of viruses [26] and in this case the wild type virus was selected when propagation was performed without addition of antivirals to the growth medium. By addition of antivirals it was possible to rescue viruses with the combination of both I223R and H275Y mutations but none of the viruses displayed adequate NA activity to perform NA inhibition test. NA inhibition can be further assessed in a plaque reduction assay. However, due to limited amount of sample material left, this was not an option in our case. The lack of measurable NA activity could be due to the modifications of the NA protein caused by the induced mutations, even though it was possible for the virus to replicate in the cell cultures, or it could be due to remnants of antivirals in the cell growth medium interfering with the test.

An interesting finding in the study was the different antiviral resistance mutation profiles of two samples collected on the same day. The samples were obtained from a nasopharyngeal swab and a BAL, respectively, and the resistance mutation profiles differed, with the

BAL sample having a higher frequency of the antiviral resistance mutations E119G, I223R and H275Y, whereas the nasopharyngeal sample, had a low frequency of additional mutations not found in the BAL sample. This could indicate a difference in the viral populations replicating in the upper and lower respiratory tract, respectively. This finding could be of importance when considering the sampling site for antiviral resistance testing, as the antiviral resistance profile for treatment evaluation could be misleading depending on the sampling site. Further studies on the compartmentalisation of influenza virus in the infected respiratory tract are needed.

## Conclusion

The rapidly evolving antiviral resistance observed in this case, emphasises the importance of timely antiviral resistance testing during treatment of influenza virus infection in order to change treatment regime and avoid unnecessary administration of ineffective medicaments, as well as preventing spread of antiviral resistant viruses.

Surveillance of antiviral susceptibility and research in the development of antiviral resistance in influenza virus is important to prevent the spread of antiviral resistant viruses, both in the hospital setting with risk group patients, and on a larger scale in the general population. The study contributes to the expansion of knowledge regarding the complexity of treating immunocompromised patients with antivirals, and the ecology of the influenza A(H1N1)pdm09 viral population under the selective pressure of antivirals. The study furthermore suggests that compartmentalisation of antiviral resistant viruses in the respiratory tract is of importance for considering the sampling site for antiviral resistance testing.

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## Conflict of interest

None declared.

## Authors' contributions

Conceptualized the study: RT and TKF; drafted the manuscript: RT; performed laboratory investigations: KV and RT; performed data analyses and interpretation of data: RT; managed the patient: SSP; collected diagnostic and clinical data: KTF. All authors have been involved in revision of manuscript and have read and approved the final manuscript.

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