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**Rapid communication**

Expanding Usutu virus circulation in Italy: detection in the Lazio region, central Italy, 2017 to 2018

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Blood donation screening for West Nile virus (WNV) was mandatory in the Lazio region in 2017 and 2018 (June-November) according to the national surveillance plan. In these years, all five donations reactive in WNV nucleic acid amplification tests harboured instead Usutu virus (USUV). Clade ‘Europe 2’ was identified in four blood donations and a 2018 mosquito pool. The cocirculation of WNV and USUV in Lazio warrants increased laboratory support and awareness of possible virus misidentification.

In the summer and autumn seasons of 2017 and 2018, following serological identification of West Nile virus (WNV) infection in horses, mandatory WNV screening with nucleic acid amplification test (NAT) was established for blood donations in some provinces of the Lazio Region in central Italy: Viterbo (starting from 18 August 2017), Latina (12 September 2018) and Rome (10 October 2018). During this period, five blood donations were reactive in WNV NAT screening. In none of these samples could WNV positivity be established by further tests conducted at the Regional Reference Laboratory.

In the present study, we describe serological, detailed molecular and phylogenetic analyses undertaken to better characterise the WNV positivity observed during the screening.

**Processing of blood samples**

For the Lazio Region, centralised blood screening for WNV is performed at the Biological Qualification Center (CQB) for NAT blood screening at the Sandro Pertini Hospital in Rome, using NAT (Cobas WNV NAT screening test, Roche, Mannheim, Germany). Blood donations reactive in WNV NAT screening and confirmed twice by the same method at the CQB, are considered not suitable for the transfusion service and are discarded. Plasma aliquots of these donations are sent to the Regional Reference Laboratory for Arbovirosis (Laboratory of Virology, National Institute for Infectious Diseases L. Spallanzani, Rome) for confirmatory testing and further diagnostic investigation. Donors who provided WNV NAT-positive donations are recalled 2–3 weeks after donation, to complete serological investigation.

All five donors reactive in repeated WNV NAT were asymptomatic and fulfilled the eligibility criteria concerning WNV risk. According to Ministerial Decree of 2 November 2015, in order to guarantee self-sufficiency in blood components during the summer period, it is recommended to use the WNV NAT test as an alternative to the provision of 28 days suspension of donors who have spent at least one night in areas at risk of WNV infection [1]. The areas with a documented WNV circulation are established by an appropriate epidemiological surveillance system. The main demographical data of these donors are shown in the Table (Donors #1 to #5).

**Virological characterisation of WNV NAT-reactive samples**

Plasma samples were concentrated by ultracentrifugation and tested with a nested pan-flavivirus-RT-PCR targeting the NS5 gene (modified from [2], amplicon size: 210 nt), followed by amplicon sequencing. New sequences described in this report have been registered in GenBank under accession numbers MK015649,
The sequencing results indicated that all five blood donations harboured Usutu virus (USUV) and not WNV. These results were further confirmed with a USUV-specific real-time RT-PCR, modified from Nikolay et al. [3], and by an USUV-specific nested RT-PCR targeting NS5, followed by sequencing. The phylogenetic tree, built with both neighbour-joining and Bayesian maximum clade credibility approaches, indicated that the USUV strains detected in four of the five blood donors from the Lazio region belonged to the clade ‘Europe 2’, while the strain detected in donor #1 belonged to the clade ‘Europe 3’ (Figure 1 and 2).

Phylogenetic tree based on the partial NS5 gene (position 9004–9406 of HM569263), built using the neighbour-joining method. The evolutionary distances were computed using the Kimura 2-parameter method. The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.25). Each record consists of accession number, place and year of detection/isolation. Phylogenetic analysis was conducted in the MEGA7 software package.


Colour code of Usutu virus clades: red: Europe 1; blue: Europe 2; pink: Europe 3; green: Europe 4; yellow: Africa 3; black: Africa 2.

The serological investigation revealed that four donors did not have WNV- or USUV-specific antibodies at donation, while donor #1 had both IgG and IgM specific for WNV. In follow-up samples, obtained 2–3 weeks apart, USUV seroconversion was observed for donors #2 and #5, while the USUV-specific antibody titre exceeded the WNV-specific antibody titre in donor #1 (Table).
**Figure 1**
Phylogenetic analysis of Usutu virus strains, neighbour-joining method, central and northern Italy, 2017–2018 (n = 50)

Phylogenetic tree based on the partial NS5 gene (position 9004–9406 of HM569263), built using the neighbour-joining method. The evolutionary distances were computed using the Kimura 2-parameter method. The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.25). Each record consists of accession number, place and year of detection/isolation. Phylogenetic analysis was conducted in the MEGA7 software package.


Colour code of Usutu virus clades: red: Europe 1; blue: Europe 2; pink: Europe 3; green: Europe 4; yellow: Africa 3; black: Africa 2.
Vector investigation

According to the national integrated surveillance plan for WNV and USUV, the Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana performed the entomological investigation: adult mosquitoes were sampled at sites with known virus circulation (areas where horses positive for anti-WNV antibodies or blood donors reactive in WNV NAT had been identified) using traps of the model Italian Mosquito Trap (IMT; PeP, San Giuliano Milanese, Italy), BG Sentinel (BioGents, Regensburg, Germany) and Gravid (BioQuip Products, Rancho Dominguez, United States). The sampling protocol was defined depending on the number of WNV cases in the area and on the number of caught mosquitoes. After mosquito sorting and identification, those of the species C. pipiens were divided in pools of at most 100 specimens and analysed by RT-PCR for virus detection.

In the period between September and October 2018, 47 trapping exercises were performed at nine sites with known virus circulation in the provinces of Rome, Latina and Frosinone. A total of 2,443 specimens of C. pipiens were caught, divided into 38 pools and tested by RT-PCR for the presence of WNV and USUV RNA. Among the 38 tested pools, 14 were positive for USUV (minimum infectious rate: 1.3); they originated from two municipalities in the Latina province. Sequencing indicated that these viruses belonged to the ‘Europe 2’ clade and were very similar to those obtained from blood donors #2 to #5 (Figure 1).

During the same period, three horses with neurological signs and positive WNV serology were located in the province of Latina. One horse died, and WNV lineage 2 was detected by RT-PCR in its brain (cerebellum and medulla oblongata). These results were confirmed by the National Reference Centre for Foreign Animal Diseases (CESME), Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise.

Characterisation of Usutu virus strains detected in blood donors from northern Italy

We also performed molecular analysis on four USUV-positive blood donations identified in 2017 by the
Regional Reference Laboratory of the Lombardy Region (Molecular Virology Unit, Fondazione IRCCS Policlinico San Matteo Pavia) in northern Italy, where endemic cocirculation of different USUV strains was reported [4]. Their main demographical data are shown in the Table. We identified the ‘Europe 2’ clade in one donor (donor #7) and the ‘Europe 4’ clade in two donors (donors #6 and #8) (Figures 1 and 2). For the fourth donor, only the short pan-flavivirus RT-PCR amplicon could be sequenced; this sequence clustered with the ‘Europe 2’ clade.

Discussion

USUV is a mosquito-borne flavivirus, member of the Japanese encephalitis virus group, with six distinct clades so far recognised [5]. Its natural life cycle involves mosquitoes as vectors and birds as amplifying hosts. Humans and horses are dead-end hosts, as for WNV.

USUV was isolated in South Africa in 1959 and was first reported in Europe in 2001, causing mass mortality of Eurasian blackbird (Turdus merula); however, a retrospective study showed that it has been present in Italy at least since 1996 [6]. Subsequently, USUV spread to other country, including Hungary (2003–2006), Switzerland (2006), Spain (2003–2006), Germany (2010), Czech Republic (2014), Belgium, France and the Netherlands (2016), where the virus was detected or isolated from mosquitoes, birds and bats [7-10].

The first human USUV infection was reported in the Central African Republic in 1981 [11]; the first neuro-invasive human infection in Europe was reported in Italy in an immunocompromised host in 2009 [12]; several cases of disease in humans were described thereafter [13-15].

Recently, USUV circulation has been increasing in several Europe regions, overlapping with areas endemic for WNV that exploits the same main mosquito vector [16]. Molecular and serological evidence of USUV circulation has been reported in northern Italy, involving the ‘Europe 2’ and the ‘Europe 4’ clades [4] and infecting birds, mosquitoes and humans; human infections have been both asymptomatic and symptomatic [13].

An annual surveillance plan is issued by the Italian Ministry of Health with the aim of monitoring WNV and USUV circulation and triggering appropriate public health measures. The purpose is the limitation of virus spread, control of the vector population and elimination of transmission through blood, blood components, tissues and organ donation during the period of increased vector activity in the summer and autumn season (June–November) [1]. In 2017 and 2018, respectively two and three blood donations from the Lazio region were positive in WNV NAT screening. Molecular characterisation revealed that the WNV NAT positivity was indeed due to the presence of USUV RNA. Intensified entomological surveillance for arboviruses detected 14 mosquito pools that were positive for USUV RNA.

Partial molecular characterisation indicated close genetic relationships between human and mosquito USUV strains detected in Lazio region; all belonged to the ‘Europe 2’ clade. The only USUV strain of clade ‘Europe 3’, detected in donor #1, was presumably acquired in Switzerland, according to the travel history of the donor; in addition, this donor’s place of residence, Argentina, accounted for the serological pattern observed at donation, that was consistent with previous exposure to other related flaviviruses.

The presence of WNV lineage 2 detected by molecular assay in the brain of a symptomatic horse in the province of Latina is evidence of the cocirculation of WNV and USUV in this area. Data from blood donors in Lombardy confirmed the cocirculation, in northern Italy, of different USUV strains (belonging to clades ‘Europe 2’ and ‘Europe 4’). This finding is in line with previous reports [13] and indicates autochthonous circulation after multiple importation events.

This is the first report showing circulation of USUV virus in the Lazio region, both in mosquito vectors and in human hosts, while so far, USUV has not been detected in other animal hosts (neither equids nor birds).

This study corroborates the hypothesis that USUV can cause clinically asymptomatic infection in humans [17]; this, in turn, may be of concern regarding the safety of the blood supply in areas where the virus is active. Although there is no evidence of human-to-human transmission of USUV in the transfusion (or transplant) setting, asymptomatic infected donors may donate USUV-infectious blood, which may cause severe disease in immunocompromised patients [18].

Public health authorities, blood transfusion services and clinicians should be aware of the expanding risk of USUV infection in humans, especially during the summer, and advanced laboratory support is required to address the problem of possible virus misidentification by screening tests.

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Conflict of interest
None declared.

Authors’ contributions
Conceptualisation: FC, MRC; Methodology: FC, FR; Software: FC, FM; Formal analysis: FC, FR; Investigation: SM, CC, TC, FR, EP, FC, IR, CDL, FR, LB, EL; Resources: IR, CDL, FR, CC; Writing – original draft: FC; Writing – review and editing: MRC, FC, FR, SM; Supervision: MRC; Funding acquisition: FB, MRC, GI, AS.

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We report a seasonal increase of enterovirus D68 (EV-D68) cases in France, with 54 cases detected between 19 August and 14 November 2018. Molecular typing revealed that 20 of 32 of the isolates belonged to clade D1, only sporadically detected before in France. Median age of D1-cases was 42 years, 10 developed severe respiratory signs and one had neurological complications. The 2018-D1 viruses showed a genetic divergence of 3.34% with D1 viruses identified previously.

Since August 2018, a seasonal wave of enterovirus D68 (EV-D68) cases has been reported in France. Here, we present the molecular and clinical characteristics of the EV-D68 cases with a special focus on D1-associated cases and on severe cases.

Enterovirus D68 detection and typing

A total of 61 EV-D68 infections were reported in France from 1 January–18 December 2018, as part of the enterovirus infections national surveillance that comprises of a network of 35 hospital laboratories (Figure 1). Systematic screening for EV-D68 of all enterovirus/rhinovirus-positive respiratory samples was performed in three university hospitals (Lyon, Nantes, and Clermont-Ferrand). In other hospital laboratories, EV-D68 testing was performed occasionally for cases presenting with severe respiratory or neurological illness. EV-D68 could also be identified through routine genotyping of EV-positive samples.

Of 61 EV-D68 cases, 54 (88.5%) were detected from 19 August–14 November with a peak in cases (n = 10) in week 40 (15–21 October) (Figure 1A). We carried out molecular typing for 32 of 61 EV-D68-positive samples by Sanger sequencing of the complete viral protein 1 (VP1) sequence (n = 25), partial VP1 sequence (n = 6), or VP4-VP2 sequences (n = 1) as previously described [1,2]. Phylogenetic analysis found that the majority of EV-D68 isolates belonged to clade D1 (20/32), while the remaining belonged to clade B3 (12/32). Co-circulation of D1- and B3-viruses was detected throughout the 2018 epidemic (Figure 1A) and all over France (Figure 1B).

Clinical characteristics of enterovirus D68 clade D1- or B3 -positive patients

Among the 20 D1-positive cases (median age: 42 years; range: 0.1–79 years), the majority of infections (n = 14) were diagnosed in adults, with only six cases identified in children aged 5 years or below. Ten patients presented with severe respiratory signs including severe asthma (n = 2), acute respiratory distress (n = 4) and acute cardiorespiratory decompensation (n = 4). Among these severe respiratory cases, nine had underlying comorbidities (Table). Two patients in their early 70s with underlying malignancies died from cardio-respiratory failure.

One patient in their late 60s developed neurological signs shortly after influenza-like prodromal symptoms. They presented with an acute aphasia associated with upper and lower right limb weakness and facial paralysis, compatible with cranial nerve impairment. Cerebrospinal fluid analysis was compatible with aseptic meningitis (white blood cells (WBC): 34 per mm$^3$;
Norm: five or less WBC per mm$^3$; 95% of lymphocytes)
but extensive microbiological investigations, including
enterovirus PCR testing, were negative. Neurological
symptoms improved within 10 days. Brain MRI did
not show rhombencephalitis. No medullar MRI was
performed.

Of the 12 B3-associated cases the median age was 4.5
years (range: 0.2–65 years). The clinical spectrum was
dominated by respiratory symptoms primarily occur-
ing in children aged 5 years or below (n = 7). Eight
B3-associated cases had an underlying disease and six
cases developed a severe infection, including one case
of rhombencephalitis in a child aged 5 years (Table).

Molecular investigations
To characterise the global dynamics of EV-D68 clade
D1 strains over time, we performed a phylogenetic
analysis of the full VP1 sequences generated in this
study from patients from three different administra-
tive regions of France (GenBank accession numbers:
MK121710-MK121730) in comparison to all published
worldwide VP1 sequences (global dataset: 810
sequences available in GenBank at 11 Oct 2018) [3]
(Figure 2A). This dataset showed a strong temporal
signal (Figure 2B) and the time to most recent com-
mon ancestor (TMRCA) of clade D1 was estimated to
date back to 2010 (95% highest posterior density,
2008–2011) according to Bayesian inference. The first
clade D1 viruses were identified in 2011 (East Asia),
but since then and until 2018, they have been rarely
detected (Asia, 2011-2016, 33 sequences (Hong-Kong:
n=14, China: n=12, Taiwan: n=5, Japan: n=2); North
America, 2013-2014, 4 sequences (Canada: n=2; US:
n=2); Europe, 2012-2014, 34 sequences (Germany:
n=20, France: n=9, Italy: n=1, Spain: n=2, Denmark:
n=2) based on all whole genome sequences (n=508)
and VP1 sequences (n=810) available in GenBank at 11
Oct 2018).

In France, clade B2 and B3 largely predominated in
2014 and 2016, while clade D1 viruses were detected
for the first time in 2012 (one isolate) and represented
only 9 of 201 (4.5%) of the EV-D68 detected in France
in 2014. The 2018 D1 isolates showed a nt divergence
in the VP1 gene of 3.34% compared with the D1 strains
previously characterised (Figure 2A).

According to the Bayesian skyline analysis, the 2018
outbreak remains relatively modest (within the lim-
its of sequencing data publicly available) and has not
reached the levels of the 2014 or 2016 episodes (Figure
2C). In addition to conventional molecular typing, the
whole genome of thirteen EV-D68 were sequenced

Figure 1
(A) Temporal and (B) spatial distribution of enterovirus D68 infections, France, 2018 (n=61)

Panel B: Pie charts show the frequency of EV-D68 genotypes. The radius is proportional to number of cases.
using a validated metagenomic next-generation sequencing assay [4], including D1 strains from 2012 (n = 1), 2014 (n = 4) and 2018 (n = 2), as well as B3 isolates from 2016 (n = 3) and 2018 (n = 3) (GenBank accession numbers: MK105976-MK105989). No evidence of inter-clade recombination event involving the clade D1 was found using standard recombination detection package [5].

**Discussion and conclusion**

Since the global emergence of EV-D68 in 2014, this virus has been associated with a wide range of infections including neurological and severe respiratory diseases [6]. As EV-D68 circulation is driven by a biennial pattern in European countries, an upsurge of EV-D68 cases was expected in 2018 [3]. An increase of EV-D68 detection was detected in France during autumn 2018 and was recently reported in Wales [7]. Detection of EV-D68 was reported in other European countries as well (Sofie Midgley, European Centre for Disease Prevention and Control National Microbiology Focal Points meeting, October 2018). The burden and circulation of EV-D68 in France in 2018 is likely under-represented in this study, as cases were identified through hospital-based surveillance and only 3/35 laboratories performed systematic screening of EV-D68.

We found that EV-D68 clade D1 was the predominant circulating genotype, partially replacing the B3 genotype that was predominant in 2016 [3]. Viruses from clade D1 were initially detected in East Asia in 2011 but since then, only a few detections have been reported worldwide [8]. A continuous emergence and replacement of EV-D68 clades have been observed since 2010, in particular, a low circulation of the B3 lineage was noticed before its global spread in 2016 [3,9-11]. We also found that D1 viruses were mainly detected in adult cases, while B3 viruses were mainly detected in children. This age-effect depending on the EV-D68 circulating genotype had already been observed in previous studies, which found that viruses from clade D1 (previously classified as clade A2) predominantly infected adults [1,12].

In addition to severe respiratory illness, neurological complications were observed in one patient with a D1 EV-D68 infection, suggesting a neurotropic potential of this genotype. EV-D68 neurotropism was recently demonstrated in a neuroblastoma-derived neuronal cell line model, indicating that only contemporary strains of EV-D68 (including US/KY/14–18953 strain belonging to the clade D1) have acquired neurovirulence over time [13]. EV-D68 neurotropism could partly explain the spike of acute flaccid myelitis cases observed in the summer and early Autumn of 2014, 2016 and 2018 in the US [10,14]. Our data confirm that EV-D68 can be associated with severe respiratory disease in immunocompromised individuals particularly in patients with haematological malignancies [15].

Finally, using a metagenomic approach we provide the first European full-length genomes of clade D1 viruses, which contributes to improve our knowledge regarding EV-D68 genetic diversity. Whole genome sequence analysis did not find evidence of interclade recombination events [8,16] that could have led to the emergence of the D1 genotype.

The data presented here underline the value of extensive molecular and clinical investigations to fully describe the complex spectrum of EV-D68 infections. A global real-time surveillance of EV-D68 should be maintained and emphasised to rapidly alert epidemiologists, microbiologists and clinicians.

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Number of D1-cases (n = 20)</th>
<th>Number of B3-cases (n = 12)</th>
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</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
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<tr>
<td>Male</td>
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</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>5</td>
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<td><strong>Age group (years)</strong></td>
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<td>6</td>
<td>7</td>
</tr>
<tr>
<td>5–17</td>
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<td>3</td>
</tr>
<tr>
<td>18–64</td>
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</tr>
<tr>
<td>&gt; 65</td>
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<td><strong>Underlying co-morbidities</strong></td>
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<tr>
<td>Deaths</td>
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<td>0</td>
</tr>
</tbody>
</table>

BMI: body mass index.

*Haematological malignancies includes: lymphoma, acute leukaemia, multiple myeloma and essential thrombocythaemia.

*One case association with a *S.pneumoniae* respiratory infection and a myopericarditis.

*One case association with acute pancreatitis.*
**Figure 2**

Evolutionary history of enterovirus D68, 1962–2018

**A. Maximum-likelihood tree based on the global dataset of EV-D68 VP1 sequences**

**B. Global dataset root-to-tip distance**

**C. French EV-D68 effective population size fluctuation**

EV-D68: enterovirus D68.

Panel A: Maximum-likelihood tree of the global dataset of EV-D68 VP1 sequences based on the general-time-reversible (GTR) model of DNA sequence evolution. The arrows indicate strains involved in the 2018 outbreak (highlighted in orange). The scale shows the length of branch representing 0.04 nt substitutions per site.

Panel B: Root-to-tip genetic distances are plotted against sampling dates. The figure illustrates a positive correlation of divergence with sampling date, and, hence, a significant increase of nt sequence variation over the sampling time interval.

Panel C: Bayesian skyline plot showing the effective population size fluctuations of EV-D68 in the French cohort. The black line represents the median estimate of the estimated effective population size. The shade represents the upper and the lower estimates of 95% confidence interval.
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Conflict of interest

None declared.

Authors’ contributions

AB, LJ, IS and BL designed the study. TW performed the phylogenetic analyses. AB and MS performed the sample preparations and sequencing. IS, MC-B, ML, KS and KB-P contributed to data collection and interpretation. AB, IS and LJ drafted and revised the manuscript based on all authors’ contributions. All authors approved the final version of the manuscript.

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Assessing baloxavir susceptibility of influenza viruses circulating in the United States during the 2016/17 and 2017/18 seasons

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Citation style for this article:

The anti-influenza therapeutic baloxavir targets cap-dependent endonuclease activity of polymerase acidic (PA) protein. We monitored baloxavir susceptibility in the United States with next generation sequencing analysis supplemented by phenotypic one-cycle infection assay. Analysis of PA sequences of 6,891 influenza A and B viruses collected during 2016/17 and 2017/18 seasons showed amino acid substitutions: I38L (two A(H1N1)pdm09 viruses), E23G (two A(H1N1)pdm09 viruses) and I38M (one A(H3N2) virus); conferring 4–10-fold reduced susceptibility to baloxavir.

On 24 October 2018, the United States (US) Food and Drug Administration (FDA) approved a new anti-influenza therapeutic, baloxavir marboxil, following its approval in Japan earlier in the same year [1,2]. This antiviral is prescribed as a single tablet for treating acute uncomplicated influenza A and B infections in patients 12 years and older [3]. In clinical trials, treatment-emergent amino acid substitutions (AAS) in the polymerase acidic (PA) protein causing reduced susceptibility to baloxavir have been reported at the rate of 2-20%, depending on age and other factors [4-7].

Early detection of emerging resistance is essential for timely modification of public health policies and recommendations on the use of antiviral therapeutics. Therefore, our aim was to determine baloxavir susceptibility of seasonal viruses before the drug entered the US market and establish a methodology to conduct baloxavir surveillance.

Amino acid substitutions associated with reduced susceptibility to baloxavir
Baloxavir marboxil is metabolised to baloxavir acid, a potent inhibitor of the cap-dependent endonuclease of the PA protein. It halts viral mRNA synthesis, thereby stopping the early stages of virus replication [8]. Treatment-emergent reduced susceptibility was associated with AAS at residue 38 in the PA [4-7]. Viruses engineered to contain one of the three principal AAS (I38T, I38M or I38F) displayed 2–57-fold reduction in susceptibility, with I38T conferring the highest fold change [5]. The effects of these substitutions on drug susceptibility were type- and subtype-specific [5]. In addition, other AAS within the PA endonuclease active site were detected and investigated. Currently, there is no guidance issued by the Expert Working Group on Antiviral Susceptibility for the World Health Organisation (WHO) Global Influenza Surveillance and Response System (GISRS) regarding a threshold (fold change) for reporting, therefore an arbitrary threshold of > three-fold half maximal effective concentration of a drug (EC₅₀) was used to list PA AAS conferring reduced susceptibility to baloxavir (Table 1).

Influenza surveillance based on codon-complete next generation sequencing
In accordance with Centers for Disease Control and Prevention (CDC) guidance, public health laboratories (PHLs) submit up to two viruses of each subtype (type A) and lineage (type B) to national surveillance, twice a month. Since the implementation of the Sequence First Initiative, all submitted viruses have been subjected to codon-complete next generation sequencing (NGS) of viral genomes [9] (Table 2). In addition, NGS data were obtained for 235 viruses submitted as
part of enhanced antiviral surveillance in the 2017/18 season. All sequences were made public through the Global Initiative on Sharing All Influenza Data (GISAID). To ensure accuracy, the sequences were curated to remove duplicate sequences for individual viruses (Table 2). A large subset of clinical specimens was used for virus isolation to conduct antigenic, antiviral and other analyses.

### Table 1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Influenza Season</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2016/17</td>
<td>2017/18</td>
</tr>
<tr>
<td>A(H3N2)</td>
<td>1,594</td>
<td>1,570</td>
</tr>
<tr>
<td>A(H1N1)pdm09</td>
<td>383</td>
<td>967</td>
</tr>
<tr>
<td>B/Victoria</td>
<td>459</td>
<td>304</td>
</tr>
<tr>
<td>B/Yamagata</td>
<td>660</td>
<td>954</td>
</tr>
<tr>
<td>Total</td>
<td>3,096</td>
<td>3,795</td>
</tr>
</tbody>
</table>

References

1. Included in the analysis were 235 viruses, 185 A(H3N2) and 50 A(H1N1)pdm09, collected from 12 US states for enhanced antiviral surveillance.
2. The total number of influenza viruses with polymerase acidic sequences downloaded from Global Initiative on Sharing All Influenza Data database; duplicate sequences were not counted. Season 2016/17 dates are 1 October 2016–30 September 2017; season 2017/18 dates are 1 October 2017–30 September 2018.

### Table 2

<table>
<thead>
<tr>
<th>Virus</th>
<th>Influenza Season</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>954</td>
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<tr>
<td>Total</td>
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<td>3,795</td>
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</table>

<table>
<thead>
<tr>
<th>Virus of concern</th>
<th>Influenza Season</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>A(H3N2)</td>
<td>1,594</td>
<td>1,570</td>
</tr>
<tr>
<td>A(H1N1)pdm09</td>
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<tr>
<td>B/Victoria</td>
<td>459</td>
<td>304</td>
</tr>
<tr>
<td>B/Yamagata</td>
<td>660</td>
<td>954</td>
</tr>
<tr>
<td>Total</td>
<td>3,096</td>
<td>3,795</td>
</tr>
</tbody>
</table>

### Baloxavir susceptibility testing by cell-based phenotypic assays

As molecular mechanisms of reduced susceptibility to baloxavir are not yet fully elucidated, it is necessary to supplement NGS analysis with phenotypic testing. A phenotypic reduction in susceptibility is expressed as a fold change compared with a reference EC50. Following approval of baloxavir in Japan, the baseline susceptibilities for seasonal viruses circulating in Japan were established using a focus reduction assay (FRA) [10].

Previously, Stevaert et al. suggested using a one-cycle infection assay to test PA inhibitors [11]. Recently, we developed a one-cycle infection assay, known as high-content imaging neutralization test (HINT), for antigenic analysis [12]. A single infection cycle is achieved by omitting trypsin, thereby preventing virus spread to neighbouring cells (Supplemental Figure S1). Virus-infected cells are detected by immunofluorescence.
HINT was used in this study to establish type-/subtype-specific baseline EC50 values.

To this end, 116 viruses representing different subtypes (type A) and lineages (type B) circulating in the US during 2016/17 and 2017/18 seasons were tested (Table 4). EC50 values were similar to those reported for viruses tested using a plaque reduction assay [5]. As expected, type B viruses displayed ca four-fold greater EC50 compared with type A viruses. The consistency between HINT and plaque reduction assay was encouraging as EC50 values determined using a different assay, ViroSpot, appeared to be elevated [5].

Next, we tested 11 nasal washes from patients positive for influenza B (Ct values ranged 19.06–22.23). The EC50 values ranged from 2.5–6.7 nM, which was within the established baseline for type B virus isolates (data not shown).

Viruses flagged by NGS analysis were tested alongside their PA-matching counterpart (i.e. control viruses) (Table 3 and Supplemental Table S1). A/Louisiana/49/2017, containing I38M, displayed a 10-fold change in baloxavir susceptibility compared with the control virus A/Louisiana/50/2017, which is in agreement with a previous report for another A(H3N2) virus using a plaque reduction assay [5] (Table 1). We also tested this virus pair by a modified FRA [13]; an 11-fold change in baloxavir susceptibility was detected. Furthermore, Omoto et al. independently tested these viruses using a plaque reduction assay and reported a

### Table 3

<table>
<thead>
<tr>
<th>Type/subtype</th>
<th>Virus name</th>
<th>Codon*</th>
<th>PA AA</th>
<th>EC50 ± SD</th>
<th>Fold change to control</th>
<th>Fold change to median</th>
<th>EC50 ± SD</th>
<th>Fold change to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(H3N2)</td>
<td>A/Louisiana/50/2017</td>
<td>ATA</td>
<td>I38</td>
<td>1.33 ± 0.22</td>
<td>1</td>
<td></td>
<td>1.12</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A/Louisiana/49/2017</td>
<td>AIG</td>
<td>I38M</td>
<td>13.88 ± 2.25</td>
<td>10</td>
<td></td>
<td>11.75</td>
<td>11</td>
</tr>
<tr>
<td>A(H1N1)pdm09</td>
<td>A/Kentucky/13/2018</td>
<td>GAA</td>
<td>E23</td>
<td>2.39 ± 1.58</td>
<td>1</td>
<td></td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>A/Florida/20/2018</td>
<td>GGA</td>
<td>E23G</td>
<td>8.86 ± 1.62</td>
<td>4</td>
<td></td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>A/Texas/121/2018</td>
<td>GAA</td>
<td>E23</td>
<td>1.90 ± 0.43</td>
<td>1</td>
<td></td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>A/Arizona/35/2018</td>
<td>GGA</td>
<td>E23G</td>
<td>10.24 ± 2.78</td>
<td>5</td>
<td></td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>A/Illinois/08/2018</td>
<td>ATA</td>
<td>I38</td>
<td>1.61 ± 0.22</td>
<td>1</td>
<td></td>
<td>2.12</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A/Illinois/38/2018</td>
<td>CTA</td>
<td>I38L</td>
<td>12.95 ± 1.87</td>
<td>8</td>
<td></td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

AA: amino acid; FRA: focus reduction assay; HINT: high-content imaging neutralization test; NT: not tested; PA: polymerase acidic; SD: standard deviation.

* Underlined base indicates the nt change.

† Mean and SD of three independent tests.

‡ Fold change to EC50 of test virus compared with sequence-matched control virus. A/Texas/121/2018 and A/Arizona/35/2018 also contained E688G and E126D in PA, respectively.

§ Fold change to EC50 of test virus compared with baseline median values: 0.80 for A(H3N2) and 1.57 for A(H1N1)pdm09 (Table 4).

‖ Average of two results.

An arbitrary threshold (> three-fold EC50) was used to report PA amino acid substitutions that confer reduced susceptibility to baloxavir.

### Table 4

<table>
<thead>
<tr>
<th>Virus</th>
<th>Number of viruses</th>
<th>Baseline EC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(H3N2)</td>
<td>28</td>
<td>0.33–1.53</td>
</tr>
<tr>
<td>A(H1N1)pdm09</td>
<td>34</td>
<td>0.86–2.86</td>
</tr>
<tr>
<td>B Victoria</td>
<td>31</td>
<td>1.79–8.03</td>
</tr>
<tr>
<td>B Yamagata</td>
<td>23</td>
<td>1.72–8.29</td>
</tr>
</tbody>
</table>

SD: standard deviation.

* Seasonal United States influenza viruses collected during May 2017–August 2018 were propagated in cell cultures and used for baseline median EC50 determination.

† Viruses tested as single titration in a single assay to determine EC50.
combined seven-fold change [14]. Therefore, the similar results from HINT and multi-cycle infection assays re-enforce the utility of HINT for testing baloxavir susceptibility.

The new substitution, I38L, conferred an eight- and seven-fold change compared with the control virus in the HINT and FRA assays, respectively. Two A(H1N1)pdm09 viruses carrying E23G displayed a four–five-fold change by HINT (Table 3). When fold changes were calculated using type- and subtype-specific median EC₅₀ values (Table 4), the results were generally in agreement. However, a fold change for A/Louisiana/49/2017 I38M virus was higher when calculated against the median EC₅₀ of the matching control virus was catwo-fold above the median (Table 3 and 4). The presence of heterogeneous subpopulations had no apparent effect on baloxavir susceptibility (Supplemental Table S1); nevertheless, their relevance cannot be ruled out at this time. As expected, I38V conferred one–two-fold change compared to control viruses, which was previously reported [5].

Combining NGS and HINT data from two seasons, the frequency of viruses displaying reduced baloxavir susceptibility (> three-fold change) was low: 0%, 0.032% and 0.3%, for type B, A(H3N2) and A(H1N1)pdm09 viruses, respectively. Similar to a previous report, A(H1N1)pdm09 viruses exhibited a higher frequency of PA amino acid polymorphism [11].

Out of 26 A(H1N1)pdm09 viruses submitted from Illinois, two contained I38L, A/Illinois/37/2018 and A/Illinois/38/2018, which were identical on a nt level. Both were collected on 8 February 2018 from school age males residing in the same county. Because natural polymorphism at this residue is rare, we investigated for potential transmission.

The CDC requested additional A(H1N1)pdm09 viruses collected in this state during the 2017/18 season. Forty-one additional A(H1N1)pdm09 positive specimens were available for evaluation. To expedite testing, the pyrosequencing assay [15] was used to test for AAS at residue 38 (Supplemental Figure S2). Results were obtained for 30 specimens, all of which lacked I38L. The combined results from NGS and pyrosequencing estimated the rate of detection of I38L to be 3.6% (2/56) for Illinois and 4.3% (2/47) for this particular county.

Discussion and conclusion
In this study, we established the methodology for monitoring baloxavir susceptibility in the US ahead of the drug entering the national market. As NGS analysis has become the cornerstone of influenza surveillance, it created a platform for monitoring resistance for all FDA-approved anti-influenza therapeutics. The frequency of AAS associated with reduced baloxavir susceptibility should be determined by type and subtype. This will allow for accurate inter-seasonal comparisons, as different types and subtypes predominate season to season. In the event of suspected emergence of resistance, either NGS or pyrosequencing could support epidemiological investigations in a timely manner. Pyrosequencing has been employed by many US PHLs to conduct enhanced antiviral surveillance, so this assay can readily be implemented for detecting baloxavir resistant viruses. Phenotypic assays detect viruses with reduced drug susceptibility regardless of underlying mutations. Here, HINT was successfully applied to establish the baseline susceptibility of seasonal viruses. Moreover, we have shown that clinical specimens could be tested directly by HINT, which could expedite surveillance.

Overall, in the 2016/17 and 2017/18 seasons the frequency of AAS associated with reduced susceptibility to baloxavir was low. For the current 2018/19 season, we analysed PA sequences of 384 US viruses (accessed from GISAID on 11 January 2019) and found no AAS at positions of concern. Additionally, we tested 73 representative viruses by HINT and all EC₅₀ values were within their respective type/subtype baseline (data not shown).

The antiviral testing algorithm described above will be applied to both foreign and domestic viruses. In collaboration with the Association of Public Health Laboratories, CDC aims to advance antiviral surveillance by implementing HINT at designated National Influenza Reference Centers. To assist in baloxavir susceptibility testing at the WHO National Influenza Centers and other laboratories, a CDC panel of reference viruses with reduced susceptibility to baloxavir will be made available from the International Reagent Resource [16]. As baloxavir is expected to be more commonly prescribed in Japan and the US in the coming seasons, close monitoring of resistance is necessary to inform public health policies regarding antiviral use.

Acknowledgements
The authors are most grateful to Shionogi and Co., LTD. for kindly providing baloxavir acid for phenotypic testing. We are also thankful to US public health laboratories for timely submission of influenza viruses to the national surveillance. We are grateful to US Association of Public Health Laboratories and the National Influenza Reference Centers in New York, Wisconsin and California for productive collaboration with the CDC Influenza Division. We are thankful to Chris Vogt from Carbondale laboratory, Illinois Department of Health, for his technical assistance on this project. We would like to acknowledge the valuable contributions of our colleagues from the Virology, Surveillance and Diagnosis Branch and the Epidemiology and Prevention Branch of the CDC Influenza Division.

Conflict of interest
None declared.
Authors’ contributions

AC, VPM and JDLC conducted the antiviral experiments. VPM developed phenotypic test, HINT. LGV and DEW conceived the original idea and experimental design. HTN and RG conducted bioinformatics analysis. SS, APC, MS and HR conducted epidemiological investigations. LVG supervised the project. AC, VPM and LVG interpreted the results. MCP and AC compiled data and wrote the manuscript with support from LGV. RG, JMK, AMF, JB and DEW provided critical feedback. All authors discussed the results and contributed to the final manuscript.

References


The novel cap-dependent endonuclease inhibitor baloxavir marboxil was approved for the treatment of influenza virus infection in Japan in February 2018. Two influenza A(H3N2) viruses carrying an I38T substitution in the polymerase acidic subunit (PA) were detected in baloxavir-treated children in December 2018. This mutation is known to confer reduced susceptibility to baloxavir, and the two mutant viruses exhibited 76- and 120-fold reduced susceptibility to baloxavir.

The novel antiviral drug baloxavir marboxil was approved in Japan on 23 February 2018 for the treatment of influenza virus infection, in patients 12 years and older and children younger than 12 years weighing 10 kg or more; it became available on 14 March 2018 in Japan (Figure). The hydrolysed active form of baloxavir marboxil (baloxavir acid) inhibits the cap-dependent endonuclease of influenza A and B viruses [1]. In Phase II and III clinical trials, I38T, I38F and I38M substitutions in the polymerase acidic subunit (PA) were detected in A(H1N1)pdm09 and A(H3N2) influenza viruses [2,3]. Patients infected with these mutant viruses exhibited prolonged virus shedding, and the median time to symptom alleviation was longer in baloxavir recipients infected with viruses bearing these substitutions than in those infected with viruses that lacked these substitutions [2,3]. Therefore, we conducted nationwide monitoring of the baloxavir susceptibility of circulating influenza viruses by using a combination of phenotypic methods to analyse antiviral susceptibility and genotypic methods to detect amino acid substitutions [4].

**Detection of PA I38T mutant influenza A(H3N2) viruses**

In December 2018, influenza outbreaks occurred in two primary schools in Yokohama, Japan. We isolated four influenza A(H3N2) viruses, A/YOKOHAMA/133/2018, A/YOKOHAMA/134/2018, A/YOKOHAMA/135/2018 and A/YOKOHAMA/136/2018, from four children during these outbreaks (Table 1). Two patients aged 6 and 7 years, infected with A/YOKOHAMA/133/2018 respectively A/YOKOHAMA/135/2018, were treated with a single oral dose of baloxavir marboxil. The third patient, 7-years-old and infected with A/YOKOHAMA/136/2018, was treated with the neuraminidase (NA) inhibitor oseltamivir, whereas the last patient, aged 7 years and infected with A/YOKOHAMA/134/2018, had no exposure to antiviral drugs before specimen collection. Fever in the two children that received baloxavir resolved within 2 days of baloxavir administration, and in the child that received oseltamivir, it resolved within one day of oseltamivir administration. The child without treatment had a fever at the time of specimen collection.

Clinical specimens were collected 3 days after baloxavir administration, on day 2 of oseltamivir administration or on the day after onset. Sequencing analysis detected the PA I38T substitution in A/YOKOHAMA/133/2018 and A/YOKOHAMA/135/2018, but not in A/
YOHOKAMA/134/2018 or A/YOKOHAMA/136/2018. No amino acid substitutions associated with reduced susceptibility to NA inhibitors were detected. These results demonstrate that PA I38T mutant viruses were isolated from children 3 days after baloxavir administration.

Antiviral susceptibilities of the PA I38T mutant viruses

We compared the susceptibilities of the PA I38T mutant viruses and the wild-type virus to baloxavir and four NA inhibitors approved in Japan: oseltamivir, peramivir, zanamivir and laninamivir (Table 2). Antiviral susceptibilities were determined by using a focus reduction assay and a fluorescent NA inhibition assay with the NA-Fluor Influenza Neuraminidase Assay Kit (Applied Biosystems, California, United States) as previously described [4]. Baloxavir acid was purchased from MedChemexpress (New Jersey, United States). Oseltamivir carboxylate, peramivir and zanamivir were purchased from Sequoia Research Products (Pangbourne, United Kingdom), and laninamivir was provided by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Results are expressed as the 50% inhibitory concentration ($IC_{50}$).

The $IC_{50}$ values of the viruses to baloxavir and the NA inhibitors are shown in Table 2. Both the PA I38T mutant viruses and the wild-type viruses showed normal inhibition with all four NA inhibitors, whereas the PA I38T mutant viruses exhibited 76- and 120-fold higher $IC_{50}$ values to baloxavir compared with the mean value of wild-type viruses. These results indicate that the PA I38T mutant viruses had reduced susceptibility to baloxavir, but remained susceptible to NA inhibitors.

Discussion

A Phase II clinical trial of baloxavir marboxil was conducted in Japan during the 2015/16 influenza season and Phase III trials were conducted in Japan and the United States in the 2016/17 season [2,3]. The drug was approved in February 2018 in Japan and in
October 2018 in the United States. During the 2015/16 and 2016/17 seasons, influenza A(H1N1)pdm09 and A(H3N2) viruses, respectively, predominated in Japan. During the Phase II trial, the PA I38T and I38F substitutions emerged after baloxavir treatment in four (3.6%) of 112 A(H1N1)pdm09 viruses isolated from adults aged 20–64 years [4]. In the Phase III trials, the PA I38T and I38M substitutions emerged in 36 (9.7%) of 370 A(H3N2) viruses obtained from patients aged 12–64 years and in 18 (23.4%) of 77 A(H3N2) viruses obtained from children aged 6 months to < 12 years [2,3]. Cumulative data from clinical trials of oseltamivir, involving almost 2,000 oseltamivir-treated patients, indicate that the incidence of reduced susceptibility to oseltamivir is 0.32% in adults and 4.1% in children (if low-level mutants detected by genotyping alone in mixed virus populations are included, then the corresponding values are 0.4% and 5.4%, respectively) [5]. These results suggest that the incidence of influenza viruses exhibiting reduced susceptibility to baloxavir is higher than that to oseltamivir. In Japan, baloxavir marboxil became available at the end of the 2017/18 influenza season. We isolated two PA I38T mutant influenza A(H3N2) viruses from baloxavir-treated children in December 2018. PA I38 is highly conserved in influenza A and B viruses [3], and the I38T substitution was not detected among 17,227 PA sequences from A(H3N2) viruses in the National Institute of Allergy and Infectious Diseases (NIAID) Influenza Research Database (IRD) [6]. Furthermore, our sequencing analysis revealed that these two PA I38T mutant viruses possessed different PA sequences and thus originated from different viruses, suggesting no human-to-human transmission. Our findings indicate that these viruses emerged under the selective pressure of baloxavir marboxil. In contrast, no viruses exhibiting reduced susceptibility to NA inhibitors were detected among 90 influenza A viruses tested between September and December 2018 in Japan [7]. These observations suggest that the emergence of PA I38T mutant viruses may increase as the use of baloxavir marboxil increases in the 2018/19 influenza season. Therefore, the baloxavir susceptibility of influenza viruses should be closely monitored.

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
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<tbody>
<tr>
<td>Influenza A(H3N2) viruses detected in outbreaks, Yokohama, Japan, December 2018 (n = 4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GISAID isolate ID</th>
<th>Isolate name</th>
<th>Onset of symptoms</th>
<th>Antiviral treatment</th>
<th>Specimen collection</th>
<th>PA substitution</th>
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</thead>
<tbody>
<tr>
<td>EPI_ISL_332908</td>
<td>A/YOKOHAMA/133/2018</td>
<td>2 Dec 2018</td>
<td>3 Dec 2018 Baloxavir</td>
<td>6 Dec 2018</td>
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<td>A/YOKOHAMA/135/2018</td>
<td>4 Dec 2018</td>
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<td>7 Dec 2018</td>
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<td>6 Dec 2018</td>
<td>None</td>
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<td>38I (Y277F)</td>
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GISAID: Global Initiative on Sharing All Influenza Data; PA: polymerase acidic subunit.

*The amino acid residue at position 277 is not involved in PA–inhibitor interactions.

<table>
<thead>
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</table>

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>PA substitution</th>
<th>IC₅₀, nM</th>
<th>Baloxavir</th>
<th>Neuraminidase inhibitors (WHO criteria)</th>
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<tbody>
<tr>
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<td>I38T</td>
<td>227.08</td>
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<td>0.15 (NI)</td>
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<tr>
<td>A/YOKOHAMA/136/2018</td>
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<td>2.78</td>
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<td>0.28 (NI)</td>
<td>0.12 (NI)</td>
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</tbody>
</table>

PA: polymerase acidic subunit; IC₅₀: 50% inhibitory concentration; NI: Normal inhibition; WHO: World Health Organization.

*The median IC₅₀ values of 30 influenza A(H3N2) viruses isolated in the 2018/19 influenza season in Japan to oseltamivir, peramivir, zanamivir and laninamivir were 0.22 ± 0.08, 0.11 ± 0.02, 0.50 ± 0.26 and 0.98 ± 0.26, respectively.
In vitro studies using the plaque reduction assay revealed that influenza A/WSN/33(H3N1) viruses with the PA I38T or I38M substitutions show 27.2- and 10.6-fold higher EC$_{50}$ values (the 50% effective concentration) to baloxavir compared with the wild-type virus [3]. Furthermore, influenza A/Victoria/3/75(H3N2) viruses with the PA I38T or I38M substitutions showed 56.6- and 13.8-fold higher EC$_{50}$ values, respectively [3]. These results suggest that the PA I38T substitution has a marked impact on baloxavir susceptibility. In the present study, we obtained two influenza A(H3N2) clinical isolates possessing the PA I38T substitution. These viruses showed 76- and 120-fold higher IC$_{50}$ values to baloxavir compared with the mean value of wild-type viruses. Our data thus demonstrate that the PA I38T substitution is associated with reduced susceptibility to baloxavir in currently circulating influenza A(H3N2) viruses.

The Technical Expert Working Group of the World Health Organization’s (WHO) Global Influenza Surveillance and Response System (GISRS) for Surveillance on Antiviral Susceptibility (WHO-AVWG) has established a set of criteria to define the NA inhibitor susceptibility of influenza viruses based on the fold-change in IC$_{50}$ value compared with the median value for viruses from the same type/subtype/lineage [8]. For influenza A virus, use of the terms normal (<10-fold increase), reduced (10–100-fold increase) and highly reduced (>100-fold increase) inhibition is recommended when reporting and analysing surveillance data; for influenza B, the same definitions are used but for <5-fold, 5–50-fold and >50-fold increases. The WHO-AVWG is currently collecting more data on baloxavir marboxil to establish a similar set of criteria to define baloxavir susceptibility, which should be available in the near future.

In summary, our results indicate that continuous monitoring of the emergence of baloxavir-resistant viruses is important for public health planning and clinical recommendations for antiviral drug use.

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**Conflict of interest**

None declared.

**Authors’ contributions**

Designed the analyses: ET, SW, TO. Analysed and interpreted the data: ET, CK, HM, RO, SF, MS, HM, KN, NK, TK, KM, TA, MI, MY, SW, TO. Drafted the article: ET. Revised the article: SW, TO.

**References**


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Report of simultaneous measles outbreaks in two different health regions in Portugal, February to May 2017: lessons learnt and upcoming challenges

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In Portugal, measles vaccination coverage and population immunity are high, and no endemic measles cases had been reported since 2004. The World Health Organization classified measles as eliminated in the country in 2015 and 2016, based on data from the previous 3 years. However, in a context of increasing incidence in several European countries in 2016 and 2017, Portugal experienced two simultaneous measles outbreaks with a total of 27 laboratory-confirmed cases (0.3 cases/100,000 population) in two health regions between February and May 2017. Nineteen cases (70.1%) were adults, of whom 12 were healthcare workers. Overall, 17 cases (63.0%) were not vaccinated, of whom five were infants younger than 12 months of age. One unvaccinated teenager died. Genotype B3 was identified in 14 cases from both regions. Measles virus sequencing identified different possible origins of the virus in each region affected. Although measles transmission was stopped in less than 2 months from the first case being notified, these outbreaks represent an opportunity to reinforce awareness of measles diagnosis. We highlight the intensity of the control measures taken and their impact on the rapid control of the outbreaks and also the fact that high vaccination coverage was crucial to stop transmission.

Introduction

Measles is one of the most highly contagious infectious human diseases and can cause serious illness, lifelong complications and death. The widespread use of safe and cost-effective measles vaccines in national immunisation programmes globally has resulted in a steep decrease in measles cases and deaths worldwide [1]. Following the 2010 decision by the Member States in the World Health Organization (WHO) European Region to initiate the process of verifying elimination, the European Regional Verification Commission for Measles and Rubella Elimination was established in 2011 [1]. In this context, the Global Measles and Rubella Strategic Plan 2012–2020 and the European Vaccine Action Plan 2015–2020 both include measles elimination as a main objective [2,3].

Currently, in accordance with the Portuguese National Immunisation Programme (NIP), two doses of MMR vaccine are recommended for children (at 12 months and 5 years of age) [4]. Due to consistent and sustained high immunisation coverage against measles (> 95%), the number of measles cases has declined dramatically over the past two decades (Figure 1). The last major measles outbreaks took place in 1987–89 and 1993–94, and the last reported suspected endemic measles cases in Portugal were reported in 2003. Thus, WHO Europe classified measles as eliminated in the country in 2015 and 2016, based on data from the previous 3 years [5].

In a context of increasing number of outbreaks in European countries [6], with 30 European Union (EU)/European Economic Area (EEA) countries reporting 5,881 cases between 1 March 2016 and 28 February 2017 [7], two measles outbreaks were detected in Portugal at the beginning of 2017 [8]. The first outbreak was identified in the Algarve health region (southern Portugal) and another outbreak was identified in the Lisbon and Tagus Valley health region. The latter lasted until May 2017 and, overall, 27 confirmed cases were notified to health authorities.

The aim of this article is to describe, beyond the previous rapid communication [8] and in further detail, the two measles outbreaks that occurred in the Algarve
and Lisbon and Tagus Valley health regions between February and May 2017, the control measures taken, their impacts in the community, the workforce involved, the challenges faced in contact tracing and the lessons learnt.

**Methods**

**Measles epidemiological surveillance**

Physicians who suspect a measles case are expected to report to local public health authorities and to collect samples to send to the WHO-certified national reference laboratory for measles and rubella, the National Institute of Health (Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA) [9,10]. Notification to public health authorities is currently done electronically through the National System for Epidemiological Surveillance (Sistema Nacional de Vigilância Epidemiológica, SINAVE), which records clinical and laboratory notifications. Likewise, INSA also reports laboratory results electronically through SINAVE. Following each clinical notification, an automatic email alert is generated for local, regional and national public health authorities. Local public health authorities are responsible for undertaking epidemiological investigation and implementation of immediate control measures for each suspected case identified. All cases described in these outbreaks were notified through SINAVE. The first cases identified in both transmission chains were first notified by the laboratory, while the others were primarily notified by clinicians. For all confirmed cases, there was a clinical and a laboratory notification.

**Case definition and classification**

The measles case definition and classification used during these outbreaks meet the criteria of the European Union case definition [11] and have already been described in the previous rapid communication [8].

**Epidemiological investigation**

Local public health units were responsible for undertaking epidemiological investigation and implementation of control measures for each suspected measles case identified. Regional public health departments coordinated those investigations and communicated with the national level: INSA and DGS. For each suspected measles case, extensive contact tracing was carried out, which allowed, for confirmed cases, the identification of earlier cases who had not yet been diagnosed or notified. Epidemiological investigations also made it possible to identify and document clear links between confirmed cases in both transmission chains.
Laboratory investigation

Laboratory investigation was carried out by INSA. Laboratory tests included serum IgG and IgM measurements, or measles nucleic acid detection or measles virus isolation in oral fluids, throat swabs or urine. Genetic characterisation was carried out in all measles-RNA-positive cases. Genotype was determined by sequence analysis of the 450 nt that code the C-terminal of the nucleoprotein (N) according to WHO protocol [12].

Outbreak description

From 1 January until 30 June 2017, 243 suspected measles cases were reported in Portugal, of which 222 were laboratory investigated. During this period, 27 cases were confirmed, 5 were possible, and 211 were discarded. Among confirmed cases, two imported measles cases were identified in the North and Alentejo health regions, which corresponded to isolated cases with no epidemiological or genotypic links to the cases in the two outbreaks described in this paper.

Overall, the two outbreaks included 27 confirmed cases in two health regions: Algarve (7 cases, 1.58/100,000 population) and Lisbon and Tagus Valley (20 cases, 0.55/100,000 population) (Figure 2). Of the 27 confirmed cases, 17 were unvaccinated, 12 were healthcare workers, and one unvaccinated teenager died (Figure 3).

Algarve outbreak

The outbreak in the Algarve health region was notified to health authorities on 30 March.

Overall, this transmission chain comprised seven confirmed cases (Figure 3; Table). Cases 1, 2, 3 and 4 were infants younger than 1 year and therefore were not yet vaccinated with the measles-mumps-rubella (MMR) vaccine. Case 5 was an unvaccinated adult. Only Cases 6 and 7 had been vaccinated with two doses of the MMR vaccine, with the second dose given more than 10 years. Six of these cases acquired measles in a healthcare setting, including two healthcare workers.

As Case 1 had not travelled abroad, the most likely hypothesis, in a context of measles elimination with high population immunity and in a popular European touristic destination, is that an unknown case (Case A) who acquired measles abroad, came into contact with Case 1 around week 6 2017. However, Case A was neither diagnosed nor reported to health authorities.

Lisbon and Tagus Valley outbreak

The outbreak in the Lisbon and Tagus Valley health region was notified to health authorities on 6 April. This transmission chain comprised 20 confirmed cases (Figure 3; Table), including two infants, two adolescents and 16 adults. Of the 20 cases, 10 were healthcare workers, 12 were unvaccinated, 8 were hospitalised, and one died.

Cases 1 and 2 did not report recent travel abroad and did not have contact with each other. The fact that both had disease onset within 5 days suggests that they may have acquired measles from a common source. As in the Algarve health region, the most likely hypothesis is that an unknown measles case who acquired measles abroad (Case B) came into contact with these two cases in different settings. Case B was neither diagnosed nor reported to health authorities.

Characteristics of cases

The median age of the 27 confirmed cases was 25 years (range: 0–45 years). Most confirmed cases (n = 19) occurred in adults (≥ 18 years), two cases were adolescents, and six cases occurred in infants under 15 months of age (Table). Twelve cases were healthcare workers.

Of the 27 cases, 17 had not been previously vaccinated, while the remaining cases had documented evidence of one (n = 2), or two or more doses (n = 8) of a measles-containing vaccine, either single or combined (Table). Of the 10 cases who were previously vaccinated, nine were healthcare workers (Table).

Among the unvaccinated cases (n = 17), five were infants under 12 months of age and thus too young to be vaccinated, one was a 13-month-old infant, two were adolescents, and the remaining nine cases were adults (Table).
Two of the 12 healthcare workers had received one dose and seven two or more doses of measles-containing vaccine; three healthcare workers had not been previously vaccinated (Table).

### Laboratory results

Up to 30 June, samples from 222 suspected measles cases were sent to INSA for laboratory investigation, of which 27 cases related to these outbreaks were laboratory-confirmed measles cases. Eleven cases were confirmed by using PCR testing of oral fluids or urine specimens, while other 11 cases were confirmed by detection of measles-specific IgM antibodies in serum; in four cases, both IgM and PCR positive test results were reported, and one case was confirmed through elevation of IgM levels in a pair of titres.

For one of the 15 PCR-confirmed cases, the genotype could not be identified, because of low number of copies. In the remaining 14 cases, the sequence was identified as the B3 measles virus, which is the same genotype detected in other outbreaks in Europe in 2016 and 2017, including Belgium and Italy [13,14]. However, through the phylogenetic analysis of the measles virus, it was possible to identify two possible different origins.

In the Algarve health region, the identified sequences were phylogenetically similar to the virus type circulating in Germany in 2016 and 2017, which suggests that Case A (neither identified nor reported) could have travelled from Germany by the end of January 2017 and come into contact with Case 1 in the Algarve health region (Figure 3).

In the Lisbon and Tagus Valley health region, the 12 identified sequences were all the same, even though not all epidemiological links in this cluster were clearly documented. Theses sequences were phylogenetically identical to the virus type circulating in France and Italy in 2016 and 2017, which suggests that Case B could have travelled from France or Italy in end of February 2017 and come into contact with Cases 1 and 2 in the Lisbon and the Tagus Valley health region (Figure 3).
Control measures

The increasing number of measles cases reported in several European countries in 2016 and early 2017 led the Directorate-General of Health (Direção-Geral da Saúde, DGS) to issue an alert to healthcare services, followed by recommendations and guidelines about diagnosis, early detection and response to measles cases, within the scope of the National Measles Elimination Programme [10]. After the identification of the first measles case, a contingency plan was implemented, which included four main axes: (i) containment, prevention and control; (ii) training; (iii) information sources and (iv) communication.

A specific algorithm for early detection of measles was also created within the National Health Service contact centre (SNS 24) for triage of suspected cases by phone. In these outbreaks, all reported suspected measles cases were investigated and control measures were promptly implemented at the local level to contain transmission (Figure 4). According to national guidelines, when a suspected measles case is identified by a physician, the patient should be immediately isolated (at the hospital or at home) until 4 days after rash onset. Simultaneously, samples must be collected and sent to INSA for laboratory investigation and the case must be notified to public health authorities (Figure 4).

Several teams undertook extensive and rapid contact tracing for all measles cases. While public health units mainly conducted contact tracing in the community, occupational health teams and nosocomial infections teams undertook contact tracing at workplaces and hospitals, respectively.

Contacts and relatives of cases were informed about measles transmission, risk and prevention, and their vaccination status was assessed. When unvaccinated

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F: female; M: male; MMR: measles-mumps-rubella; NA: not applicable; NK: unknown.
contacts were identified, these were vaccinated whether the case was confirmed or not. Thus, this strategy was useful to enhance vaccination in the community. Close contacts of confirmed cases were offered either post-exposure vaccination or immunoglobulin.

Besides interviewing patients, hospital staff and family members, public health authorities had to liaise with airline companies and foreign public health authorities, as one confirmed case had travelled abroad during the incubation period (Case 16 from the Lisbon and Tagus Valley chain of transmission).

Epidemiological investigations and control measures were complemented with broader public health actions. Those were carried out by DGS and included the dissemination of key documents to support prevention and control measures, for example, posters about clinical features of measles, guidelines, epidemiological bulletins and background materials for healthcare services [15-17].

Additionally, DGS sent several emails to reinforce information about measles risk and prevention, targeting healthcare workers and schools, and raised public awareness about the importance of vaccination through numerous reports in national media. Following these outbreaks, and in the scope of the National Measles Elimination Programme, DGS also set up a measles vaccination catch-up campaign [18].

### Discussion

These outbreaks were the largest to have occurred in Portugal since 1993–94. The fact that the country had not had any endemic measles cases for more than a decade represented a challenge for health services in terms of diagnosis and sampling of all suspected cases. Despite extensive contact tracing and investigation of the possible source of infection for each measles case, this was the first time, since 2004, that outbreaks had occurred without identification of the imported primary cases. However, these two outbreaks were also an opportunity to increase measles diagnosis awareness among healthcare workers, as evidenced by the identification and investigation of more than 200 suspected measles cases between March and June 2017. Additionally, 1,200 and 1,600 contacts of suspected cases were investigated by local public health authorities in the Algarve and Lisbon and Tagus Valley health regions, respectively. Identifying close contacts represents a challenge and additional effort for health authorities, as health services need to be adjusted when faced with an outbreak, including human resource management (for the rapid identification of contacts, vaccination post-exposure in the first 72 hours, administration of immunoglobulin to susceptible persons), financial resources (strengthening vaccine stock, ensuring immunoglobulin availability). To this end, health service resilience is essential for quick control of the outbreak.

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**Figure 4**

Control measures during the measles outbreak, Algarve and Lisbon and Tagus Valley health regions, Portugal, February–May 2017
Immunity against measles is high among the Portuguese population, due either to the free circulation of the virus until 1972 or to sustained vaccination since 1973. In contrast to other European countries, Portugal has achieved sustained high immunisation coverage against measles. Vaccination coverage of two MMR doses in the population below 18 years of age has been at least 95% for more than two decades [19]. The third National Serological Survey (2015–16) showed a proportion of immune individuals of 94.2% in the general population [20], confirming the results of the second survey (2001–02) [21]. Results by age group show that those >55 years and <10 years were the age groups with the highest immunity (measles-specific IgG antibodies ≥ 200 mUI/mL) [20]. People aged between 20 and 29 years had the lowest immunity against measles [20]. In fact, most cases of the outbreaks presented here occurred in young adults who were either unvaccinated or had been vaccinated more than 10 years previously. This seems to be a possible consequence of lower antibody levels due to the absence of natural boosters (no circulation of the virus in the community) when the virus was circulating. In fact, the majority of vaccinated cases were healthcare workers who came into contact with measles cases. None of the vaccinated cases in both outbreaks was hospitalised or had complications.

The fact that healthcare settings were the main route of transmission in both outbreaks represented a challenge because any person in hospital environment, regardless of their role, can be affected, since measles is highly contagious and persists in the environment for up to 2 hours, requiring immediate implementation of control measures [22,23]. The NIP recommends two doses of MMR for healthcare workers in Portugal, but four cases were either unvaccinated or incompletely vaccinated. Therefore, verification of healthcare workers’ immunisation status and vaccination of unvaccinated or non-immune individuals is demonstrated to be a critical component of the National Measles Elimination Programme. Given the high risk of exposure and transmission that healthcare workers face, it is not surprising that even some who had received two doses of MMR vaccine became infected with measles virus. However, it is important to note that vaccinated healthcare workers experienced mild measles infection, did not need hospitalisation and did not transmit the disease.

These outbreaks and all the communication actions taken made healthcare workers and the general public aware that measles is still a threat and a serious disease, which can cause hospitalisations and deaths. As a result, not only during but also after the outbreak, demand for vaccination increased (data not shown).

Measures were taken to reinforce vaccination in communities where MMR coverage was lower than 95%. A national catch-up campaign was set-up covering the following groups: (i) children and adolescents younger than 18 years (recommended schedule of two doses), with focus on pockets of susceptible population; (ii) healthcare workers (complete two doses for those who have never had the disease); and (iii) adults (≥18 years old) born in or after 1970 (one dose for those who have never been vaccinated and never had the disease), with focus on those aged 18–30 years.

Although the primary cases in both outbreaks could not be identified, measles sequencing was crucial to document the introduction of two different B3 genotypes in Portugal, in the context of increasing numbers of outbreaks in European countries since 2016 [7]. Given the epidemiological situation in other European countries and the increasing popularity of Portugal as a travel destination, Portuguese public health authorities should remain alert and strengthen epidemiological surveillance to avoid future outbreaks.

**Lessons learnt**

Continuous maintenance of high vaccination coverage rates is critical to stopping transmission chains and controlling measles outbreaks. To this end, it is important not only to notify unvaccinated people but also to implement innovative strategies to raise awareness among the population.

It was found that persons adequately vaccinated and with a high level of exposure when providing healthcare to cases developed measles; however, they presented a mild clinical picture.

It was verified that the cases occurring in vaccinated persons did not generate secondary cases, which is important for the prevention of transmission chains in health services.

The resilience of health services to measles outbreaks is a challenge which needs to be given particular attention by policymakers because of the high cost of resources involved in control measures.

In terms of the international epidemiological context, EU/EEA countries should maintain a high alert level.

Portugal is strongly committed to meeting the criteria defined by the WHO to maintain the status of measles elimination.

**Conclusion**

High vaccination coverage, and early and effective implementation of control measures contributed to the rapid interruption of measles transmission in both regions affected. Although Portugal has been successful in meeting WHO’s objective of eliminating measles in the European Region, this disease is a challenge that requires a coordinated effort from all European countries due to the high risk of measles importation. Sustained high vaccination coverage, effective epidemiological surveillance and early implementation of control measures are critical to quickly contain
outbreaks such as the one described here, and to interrupt virus circulation. These outbreaks represent an opportunity to strengthen the existing National Measles Elimination Programme and to enhance vaccination both in the community and among healthcare workers.

Acknowledgments

The authors wish to thank all members of local public health units, regional public health departments and the Directorate-General of Health who participated in investigation and control measures in this outbreak. The authors also wish to thank to all members in the National Institute of Health Dr Ricardo Jorgé who were involved in the laboratory investigation and genotypic analysis of measles cases. Special thanks go also to all doctors, nurses and other healthcare workers in hospitals and primary care units who assisted with control measures, as well as all patients and their families who cooperated promptly in the epidemiological investigations.

Conflict of interest

None declared.

Authors' contributions

AS and PIN coordinated the investigations of the outbreak. GFA, BAA, AM, NP, TF, PV and AL contributed to data collection, case information and data analysis. PP, EV, RC and SL were involved in the laboratory investigations. GFA drafted the manuscript, with contributions by NP, TF, AL and PP. All authors were involved in revising the manuscript. All authors reviewed and approved the final version.

References

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Disentangling a complex nationwide *Salmonella* Dublin outbreak associated with raw-milk cheese consumption, France, 2015 to 2016

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On 18 January 2016, the French National Reference Centre for *Salmonella* reported to Santé publique France an excess of *Salmonella enterica* serotype Dublin (*S. Dublin*) infections. We investigated to identify the source of infection and implement control measures. Whole genome sequencing (WGS) and multilocus variable-number tandem repeat analysis (MLVA) were performed to identify microbiological clusters and links among cases, animal and food sources. Clusters were defined as isolates with less than 15 single nucleotide polymorphisms determined by WGS and/or with identical MLVA pattern. We compared different clusters of cases with other cases (case–case study) and controls recruited from a web-based cohort (case–control study) in terms of food consumption. We interviewed 63/83 (76%) cases; 2,914 controls completed a questionnaire. Both studies’ findings indicated that successive *S. Dublin* outbreaks from different sources had occurred between November 2015 and March 2016. In the case–control study, cases of distinct WGS clusters were more likely to have consumed Morbier (adjusted odds ratio (aOR): 14; 95% confidence interval (CI): 4.8–42) or Vacherin Mont d’Or (aOR: 27; 95% CI: 6.8–105), two bovine raw-milk cheeses. Based on these results, the Ministry of Agriculture launched a reinforced control plan for processing plants of raw-milk cheeses in the production region, to prevent future outbreaks.

**Background**

Nontyphoidal *Salmonella* is a main cause of bacterial food-borne infection in Europe [1,2]. The majority of human infections is caused by a limited number of *Salmonella* serotypes among the 2,600 described to date [3,4]. *Salmonella enterica* serotype Dublin (*S. Dublin*) is particularly invasive in humans and more often leads to severe disease and higher mortality rates compared with other serotypes [4-7]. *S. Dublin* is host-adapted to bovines and is frequently isolated from cattle, with raw milk or raw-milk cheeses as a typical vehicle for food-borne outbreaks [8,9]. In 2012, a major *S. Dublin* outbreak occurred in France, with 103 cases linked to Saint-Nectaire (bovine raw-milk cheese) consumption [10,11]. In 2015, 34 *S. Dublin* cases were reported linked to the consumption of Reblochon (bovine raw-milk cheese) (data not shown; Santé publique France).

In France, the National Reference Center for *Salmonella* (NRC) and the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) routinely collect and serotype human *Salmonella* isolates, respectively [12-14], using the Kauffmann–White–Le Minor scheme [3]. The *S. Dublin* isolates collected are frequently susceptible to all antibiotics and show an indistinguishable pulsed-field gel electrophoresis...
(PFGE) pattern. To better distinguish S. Dublin isolates, multilocus variable-number tandem repeat analysis (MLVA) has recently been used for surveillance and outbreak investigations [11,15]. Moreover, whole genome sequencing (WGS) of Salmonella has been shown to discriminate between closely related isolates of S. Dublin [16,17].

Outbreak detection
On 18 January 2016, the French NRC reported to Santé publique France (SpFrance, the French national public health agency) an excess of S. Dublin infections across the country, with 37 S. Dublin isolates identified between mid-November 2015 and mid-January 2016, compared with 10 S. Dublin isolates during the same period in the two previous years. An outbreak investigation team with experts from SpFrance, NRC, ANSES and the French Directorate General for Food (DGAL) launched extensive epidemiological, microbiological and food investigations to confirm the outbreak, identify the vehicle of transmission and propose appropriate control measures.

Methods
We carried out both epidemiological and microbiological investigations on subsets of S. Dublin cases and isolates, respectively.

Microbiological investigations
Salmonella Dublin isolates
During the years 2015 and 2016, a total of 324 S. Dublin isolates were collected, 223 from clinical NRC isolates (108 in 2015 and 115 in 2016) and 101 from non-human ANSES isolates (62 and 39, in each year respectively). Of those, a total of 235, including 147 (83 in 2015 and 64 in 2016) clinical and 88 (62 and 26, respectively) non-human isolates, were extensively studied by WGS and/or MLVA. We also analysed 54 ‘historical’ subtyped S. Dublin isolates collected between 1929 and 2014, 31 from humans and 22 from non-human specimens, as well as the human isolate which gave the name to the serotype in 1929 (number 65k) [18]. In total, we included 289 isolates in this study (Figure 1).
**Whole genome sequencing**

The NRC used WGS to subtype all the 289 isolates of which 116 corresponded to the outbreak period from November 2015 to March 2016. High-throughput genome sequencing was carried out at the ‘Plateforme de microbiologie mutualisée’ (P2M) of the Pasteur International Bioresources network (Institut Pasteur, Paris, France). After extraction with the MagNA Pure 96 System (Roche, Basel, Switzerland), DNA was further processed for sequencing with Illumina systems (libraries using the Nextera XT DNA Library Prep kit and the sequencing with the NextSeq 500 system) generating 100 to 146 bp paired-end reads. Reads were trimmed and assembled as previously described [19]. Genomic data as multilocus sequence typing (MLST) type and resistance genes were detected from assembled sequences using web-tools (http://www.genomiepidemiology.org/). For each isolate, the paired-end reads were aligned against the S. Dublin strain 3246 reference genome (GenBank accession number: CM001151.1) using Bowtie2 with default parameters [20]. A core-genome multi-alignment of assembled genomes was also done using Harvest v1.0.1 f ParSNP function [21]. For each approach, the resulting single nucleotide polymorphisms (SNPs) were concatenated to generate a filtered multiple alignment that was used as input for the construction of a phylogenetic tree using Molecular Evolutionary Genetics Analysis (MEGA)6 [22] with a maximum-likelihood (ML) approach. The final trees were visualised in the interactive Tree Of Life [23]. All reads generated in this study have been deposited in project PRJEB28817.

**Multilocus variable-number tandem repeat analysis**

ANSES used MLVA as described elsewhere [11], to analyse 241 isolates (including 148 human and 93 non-human), of which 110 corresponded to the outbreak period. The measured lengths for each fragment were obtained using an ABI3500 capillary electrophoresis system (Applied Biosystems, France). Data were imported into GeneMapper software (Applied Biosystems, France) where each fragment was identified according to colour and size. A normalisation of the results was done with the free access MLVA_Normalizer software [24].

**Epidemiological investigation**

**Case definition**

We defined cases as residents in mainland France with a S. Dublin infection reported to the NRC between 17 November 2015 and 11 March 2016, without travel history outside of France within 7 days prior symptom onset and without history of S. Dublin infection before 17 November 2015 (i.e. no S. Dublin strain ever isolated and received at the NRC before 17 November 2015).

**Cluster definition**

We defined a cluster as isolate sequences with < 15 SNP divergence obtained by core-genome comparison and/or with identical MLVA pattern. Among these clusters, we also defined subclusters as isolate sequences having < 5 intra SNPs.

**Study design**

We compared cases belonging to a specific cluster/sub-cluster with other cases belonging to all other clusters/subclusters in a case–case study and with controls in a case–control study. For the case–control study,

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**Table 1**

Characteristics of Salmonella Dublin outbreak cases, France, November 2015–March 2016 (n = 83, including 63 interviewed cases)

<table>
<thead>
<tr>
<th>Characteristics of cases</th>
<th>Category</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases (n = 83)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>44</td>
<td>83</td>
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<tr>
<td></td>
<td>1–17</td>
<td>6</td>
<td>83</td>
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<tr>
<td></td>
<td>18–44</td>
<td>10</td>
<td>83</td>
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<tr>
<td></td>
<td>45–64</td>
<td>19</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>65–84</td>
<td>31</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>85–94</td>
<td>17</td>
<td>83</td>
</tr>
<tr>
<td>Age</td>
<td>Auvergne-Rhône-Alpes</td>
<td>8</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Bourgogne-Franche-Comté</td>
<td>19</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Bretagne</td>
<td>6</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Grand-Est</td>
<td>6</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Haut-de-France</td>
<td>7</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Île-de-France</td>
<td>9</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Nouvelle-Aquitaine</td>
<td>9</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Pays-de-la-Loire</td>
<td>7</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Othera</td>
<td>12</td>
<td>83</td>
</tr>
<tr>
<td>Region of residence</td>
<td>Blood</td>
<td>39</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Stool</td>
<td>25</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>11</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Articular fluid</td>
<td>3</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Pus</td>
<td>2</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>3</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Deceased</td>
<td>Yesb</td>
<td>10</td>
</tr>
<tr>
<td>Type of human sample</td>
<td>Comorbidities</td>
<td>Yesc</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Feverd</td>
<td>38</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Nauseae</td>
<td>15</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Vomiting</td>
<td>19</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Abdominal pain</td>
<td>27</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Diarrhoea</td>
<td>30</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Blood in faeces</td>
<td>5</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Hospitalisation</td>
<td>Yes</td>
<td>41</td>
</tr>
</tbody>
</table>

NA: Not applicable due to low figure in the denominator (< 60).

a Number of cases for which the information was available.

b Centre Val-de-Loire, Normandie, Occitanie and Provence-Alpes-Côte d’Azur.

c Information provided by the National Reference Center for Salmonella (NRC), without confirmation that cause of death was attributable to S. Dublin infection.

d Asthma, cancer, cardiac problems, diabetes, renal failure.

e Cases could have more than one clinical symptom.

f Fever was defined as body temperature > 38°C or perception of having fever.
controls were recruited from a cohort of individuals with children and adults registered on GrippeNet.fr (https://www.grippenet.fr), an online population-based surveillance system for influenza-like illness [25,26]. During winter 2015/16, 6,515 participants reported online the presence or absence of basic symptoms on a weekly basis using a list of 19 predefined symptoms commonly or rarely related to influenza. We excluded controls reporting travel abroad during the Salmonella outbreak period and those with digestive symptoms.

Data collection
Epidemiologists from the regional offices of SpFrance interviewed the cases by telephone using a trawling questionnaire on clinical symptoms, medical history, detailed food consumption, contact with other persons experiencing diarrhoea, travel history and contact with animals. Loyalty card information was collected to trace-back supermarket purchases.

On week 8 (22–28 February), 2016, controls from the GrippeNet.fr cohort received a web link to complete an online questionnaire about health, travel history and food consumption during 11–17 January 2016 (a 7-day ordinary non-festive period).

Statistical analyses
We calculated proportions, using the number of non-missing values as denominators. For the case–case study, we calculated crude odds ratios (OR). For the case–control study, we calculated adjusted odds ratios (aOR) for age and sex using multivariable logistic regression. The initial regression models included age, sex and food items consumed by at least 50% of the cases. We performed this analysis for WGS clusters/subclusters and MLVA clusters with at least 10 cases.

We used STATA version 12.0 (Stata Corporation, Texas, United States) for this analysis.

Ethical considerations
The study was approved by the French Commission for Data Protection (Commission Nationale de l’Informatique et des Libertés). Interviewees or next of kin provided verbal consent. Only anonymised data were analysed and used for the purpose of the study.

Food production chain and animal trace-back investigations
DGAL conducted a trace-back investigation on potential contaminated products identified by the epidemiological investigations. Points of purchase, like supermarkets or cheese retailers, were reported by cases. Where possible, customer loyalty card numbers were used to identify the exact point of purchase and product batch numbers and specific production facilities. The trace-back investigation was conducted for a retrospective period of up to a month before symptom onset of given cases, if date of onset was available, or if not, up to two months before the date of isolation of S. Dublin in the patient specimen. Suspected food products were tested for Salmonella if food samples were available.

Results
Description of cases
Between 17 November 2015 and 11 March 2016, 83 cases were identified. Median age was 70 years (range: 1–94), 44 (53%) were female and respondents originated from 12/13 regions in mainland France, with 19 (23%) cases coming from the Bourgogne-Franche-Comté region (Table 1). S. Dublin was isolated from blood (n = 39; 47%) but also from stool (25; 30%), urine (11; 13%), and other samples (8; 10%). Ten (12%) deaths were reported with no information available on the cause of death. Questionnaires were not completed for 20 cases (9 deaths, 7 unreachable, 4 refusals), leading to 63 (76%) cases included in further analyses.

Among these cases, 39 of 59 with available information reported having pre-existing chronic medical conditions (asthma, cancer, cardiac problems, diabetes, renal failure). Most frequently reported symptoms included fever (either reported as a measurement of body temperature >38°C or reported as a perception; 38/55), abdominal pain (27/47) and diarrhoea (30/56). Among cases with data on hospitalisation, 68% (41/60) were hospitalised.

The number of cases peaked during week 53 (28 December–3 January) (Figure 2).
The WGS analysis of all the 289 S. Dublin isolates, including historical ones, indicated a unique MLST, S. Dublin str. 3246 reference genome (GenBank accession number: CM001151.1) showed high divergence with 19,213 SNPs, the core-genome multi-alignment of assembled genomes using ParSNP function was preferred. A generated matrix file revealed that maximum divergence for all isolates sequenced during the outbreak including the reference strain was 791 SNPs suggesting a relative homogeneous population of S. Dublin isolates that were circulating in France. We identified 28 different clusters with 15 SNPs, including five clusters with 10 isolates (clusters A, B, C, F and K). Three of those, A, B and C, accounted for the majority of human (70%, 125/179) and non-human isolates (46%, 51/110) (Figure 3).

A total of 18 isolates (17 human and 1 non-human) with date of isolation between October 2015 and March 2016 were identified as belonging to the WGS cluster A and were mainly associated to the MLVA cluster 20–8–10–7–5–4 (Table 2, Figure 3). In terms of food items, only one WGS cluster A isolate from October 2015 was found in raw milk.

The WGS cluster B comprised a total of 35 isolates (20 human and 15 non-human). Two main subclusters were identified; one B3 (mainly associated to the MLVA cluster 17–8–10–7–5–4) was found in relation to cattle, milk and raw-milk cheeses (Reblochon and Morbier) with date of isolation mainly in January 2016 and the other B4 (mainly associated to MLVA cluster 18–8–10–7–5–4) was found in cases with date of isolation mainly in December 2015 and January 2016.

The WGS cluster C was the most prevalent with 123 isolates (88 human and 35 non-human). It was subdivided into nine subclusters (25 SNPs), C3 and Cother (grouping eight smaller subclusters including C8). All the 35 isolates belonging to the C3 subcluster presented a very limited intra SNP difference (2) and a sufficient divergence (95 SNPs) to the other eight C subclusters (Cother), indicating high level of genetic relationship due to a putative common source of contamination. The WGS subcluster C3 was mainly associated with MLVA cluster 18–8–10–7–5–4 and was found in clinical isolates from patients between January and April 2016. This molecular signature was also found in one isolate from Morbier cheese in February 2016 during this investigation. It was found as well in several isolates in Morbier cheeses tested in 2015 through company internal microbiological monitoring system. The WGS subcluster C8 was mainly associated with MLVA cluster 19–8–10–7–5–3 and harbourd isolates from patients at the beginning of 2015. In that period, another S. Dublin outbreak had been investigated between February and April 2015 in seven French regions, and was found to be possibly associated with consumption of Reblochon cheese (data not shown; Santé publique France).

Among the non-human isolates, we revealed several other WGS cluster groups (in particular F and K) but no or few linked human isolates (data not shown). The review of the historical human and non-human

### Table 2

<table>
<thead>
<tr>
<th>MLVA</th>
<th>WGS</th>
<th>A</th>
<th>B</th>
<th>C3</th>
<th>Cother</th>
<th>F</th>
<th>K</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>15–8–10–7–5–3</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>17–8–10–7–5–4</td>
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<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
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<td>3</td>
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<td>2</td>
<td>47</td>
<td></td>
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<td>4</td>
<td>18</td>
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<td>0</td>
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<td>15</td>
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<td>40</td>
<td>83</td>
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<td>2</td>
<td>5</td>
<td>17</td>
<td>4</td>
<td>0</td>
<td>18</td>
<td>48</td>
</tr>
<tr>
<td>Total</td>
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<td>35</td>
<td>35</td>
<td>88</td>
<td>38</td>
<td>11</td>
<td>64</td>
<td>289</td>
<td></td>
</tr>
</tbody>
</table>

MLVA: multilocus variable-number tandem repeat analysis; WGS: whole genome sequencing.

* The study was conducted between 2015 and 2016, the subtyped isolates included historical strains isolated between 1929 and 2014.

* There were 23 other WGS subtypes.

* Among these 19, 13 were associated with WGS subcluster F4.

* All nine isolates with MLVA 17–8–10–7–5–4 pattern were associated with WGS subcluster B3.

* Eight isolates with MLVA 18–8–10–7–5–4 pattern were associated with WGS subcluster B4.

* Fourteen isolates with MLVA 19–8–10–7–5–3 pattern were associated with WGS subcluster C6.

* There were 42 other MLVA patterns.

### Description of controls

Among the 6,200 GrippeNet.fr participants (i.e. controls) who could be reached, 2,914 (47%) completed the questionnaire. Of those, 2,690 (92%) did this within 2 days; 1,916 (66%) were female; median age was 56 years (range: 2–90). The whole process, i.e. from the beginning of the recruitment of the controls to the end of data collection, took 12 days.

There were significant differences between cases and controls in terms of proportion of female (53% in cases vs 66% in controls; p value = 0.01) and age (58% of cases vs 29% of controls were >65 years-old; p value = 0.00).

### Microbiological findings

Among 241 MLVA performed for S. Dublin isolates, 49 different MLVA patterns were obtained and among these, seven gathered 10 or more isolates (Table 2).

The WGS cluster C was the most prevalent with 123 isolates (88 human and 35 non-human). It was subdivided into nine subclusters (25 SNPs), C3 and Cother (grouping eight smaller subclusters including C8). All the 35 isolates belonging to the C3 subcluster presented a very limited intra SNP difference (2) and a sufficient divergence (95 SNPs) to the other eight C subclusters (Cother), indicating high level of genetic relationship due to a putative common source of contamination. The WGS subcluster C3 was mainly associated with MLVA cluster 18–8–10–7–5–4 and was found in clinical isolates from patients between January and April 2016. This molecular signature was also found in one isolate from Morbier cheese in February 2016 during this investigation. It was found as well in several isolates in Morbier cheeses tested in 2015 through company internal microbiological monitoring system. The WGS subcluster C8 was mainly associated with MLVA cluster 19–8–10–7–5–3 and harbourd isolates from patients at the beginning of 2015. In that period, another S. Dublin outbreak had been investigated between February and April 2015 in seven French regions, and was found to be possibly associated with consumption of Reblochon cheese (data not shown; Santé publique France).
specimens suggested that WGS B and C cluster isolates have been circulating in France for decades, while WGS cluster A isolates seem to be more recent.

Among the 63 cases for whom a completed questionnaire was available, 58 had had an isolate subtyped by WGS, 55 had had an isolate subtyped by MLVA, and 55 both. Of the 58 cases with WGS subtype, 17 belonged to subcluster C3, 11 to cluster A, and 30 to 14 other subclusters. Of the 55 cases with MLVA pattern, 23 belonged to cluster 18–8-10–7-5–4, 10 to cluster 20–8-10–7-5–4 and 22 to six other clusters.

**Epidemiological investigations**

**Case–case study**

Compared with cases belonging to the other WGS clusters, WGS cluster A cases seemed to have consumed Vacherin Mont d’Or cheese more frequently, although this result was not statistically significant (odds ratio (OR): 5.1; 95% confidence interval (CI): 0.9–34). Similarly, WGS subcluster C3 cases appeared to be related to Morbier cheese consumption (OR: 3.3; 95% CI: 0.8–15) (Table 3). Compared with cases belonging to other MLVA patterns, MLVA cluster 18–8-10–7-5–4 cases (which frequently coincided with WGS cluster A) seemed to have more often consumed Morbier cheese, however this was not significant (OR: 2.1; 95% CI: 0.5–8.3) and MLVA cluster 20–8-10–7-5–4 cases (which
frequently coincided with WGS cluster C3) appeared as consuming more often Vacherin Mont d’Or cheese (OR: 5.1; 95% CI: 0.9–35). No associations were found with other raw-milk cheeses, nor with any other food items.

Case–control study

After adjustment for age and sex, compared with controls, cases belonging to the WGS cluster A were more likely to have consumed Vacherin Mont d’Or cheese (aOR: 27; 95% CI: 6.8–105) and cases belonging to the WGS subcluster C3 were more likely to have consumed Morbier cheese (aOR: 14; 95% CI: 4.8–42) (Table 3). Compared with controls, cases belonging to the MLVA cluster 18–8-10–7-5–4 were more likely to have consumed Morbier cheese (aOR: 11; 95% CI: 4.2–29) and cases belonging to the MLVA cluster 20–8-10–7-5–4 were more likely to have consumed Vacherin Mont d’Or cheese (aOR: 27; 95% CI: 6.8–104). No other significant associations were found with other raw-milk cheeses, nor with any other food items.

Food trace-back investigations

We collected 39 (62%) loyalty card numbers from 63 cases. Based on the available information, trace-back investigations were conducted among 10 supermarket brands. Twelve cheese producers were identified as potential origin of the cheeses consumed by the cases. The trace-back investigations linked one Morbier producer and three different Vacherin Mont d’Or producers, to 11, five, four and three cases, respectively. All those producers were located in the same region, i.e. Bourgogne-Franche-Comté (Eastern part of France).

Food and veterinary investigations

From the 101 non-humans isolates collected in 2015 and 2016, 82 (81%) were collected from milk products (54 from cheese, 27 from milk and one from other dairy), 12 (12%) from animal samples (cattle, meat, faeces) and seven (7%) from environmental samples (milk filter, trough). For the 54 cheese samples, S. Dublin was detected in Morbier (n = 37), in Saint-Nectaire (n = 6), in Reblochon (n = 5), in Vacherin Mont d’Or (n = 1) and in other or unknown cheeses (n = 5).

Discussion

We reported one of the largest S. Dublin outbreaks in France in the past few years. Two different bovine raw-milk cheeses, Morbier and Vacherin Mont d’Or, were the most likely vehicles of transmission for this food-borne outbreak. For the present outbreak investigation we used two different typing methods on a large panel of strains (both historical and obtained during the 2015–16 outbreak period, as well as from human and non-human origins). The first method was MLVA, which had already been used in previous investigations of other outbreaks in France [11]. The second, WGS, was used for the first time in the current S. Dublin investigation and demonstrated increased capacity to discriminate clusters. We also used two different epidemiological methods: a case–case study that allowed a rapid analysis and identification of suspected sources and a case–control study that was more statistically powerful to confirm the suspected associations.

In this investigation, MLVA was deemed sufficient to identify a link between human cases, food and animal sources. However, MLVA could not distinguish some of the clusters identified by WGS. Our results suggested that at least two outbreaks of S. Dublin occurred during the same period, and potentially originated from two different sources. WGS cluster A and subcluster C3 occurred in different periods indicating that they might belong to distinct outbreaks. The retrospective use of WGS also confirmed the occurrence of different S. Dublin outbreaks in 2012 [10,11]. High resolution molecular tools like WGS may facilitate linkage of human cases to sources, especially in serotypes with limited intrinsic genetic variation, and may also provide a more detailed picture of the extent and context of the outbreak.

Recruiting controls from an online health cohort survey for an ongoing outbreak investigation was novel in France and served as a pilot to evaluate the suitability of this method in future food-borne outbreak investigations. This method allowed conducting the case–control study in a timely manner with minimum resources, achieving a high response rate [28].

Our investigation pointed towards several cheese producers from the same region as sources of the outbreaks. In this region, an increase in salmonellosis incidence was observed in cattle at the end of summer 2015 (data not shown; Santé publique France). This could explain the increase of contaminated cheese batches in autumn and winter 2015. Veterinary and food investigations were challenging due to (i) high number of possibly implicated processing plants of raw-milk cheeses, and (ii) the high frequency of cheese consumption by cases and the variety of cheeses and places of purchase. It was difficult to identify the exact batches that cases consumed because some cheeses were sold at the deli counter, sliced on demand. Furthermore, the probable low levels of contamination of the implicated cheeses may have led to false negative test results, possibly allowing some contaminated batches to enter the market.

Previous studies indicated that S. Dublin is frequently isolated in live cattle, and that cheeses made with unpasteurised milk may be contaminated with S. Dublin [8,9]. S. Dublin infection is also responsible for substantial losses in the dairy industry [29]. A modelling exercise in Denmark [29] estimated the gross margin losses due to S.Dublin infection in a 200-cow stall-herd to be up to EUR 188 per stall annually averaged over the 10-year period following introduction of infection. In that study, relative simple and cheap control measures such as improving calving and colostrum management could lead to significant decreases in prevalence of S. Dublin in some herds. In other herds, it was reported that these measures might have to
be supplemented by changes in hygiene and feeding practices. It might be worthwhile conducting studies in France to evaluate the impact of recent or future measures on S. Dublin prevalence at herd level. In addition, infected cattle might carry chronic and possibly asymptomatic infections while still contributing to onwards transmission by excreting pathogens in faeces [30,31]. SNP-typing based on WGS is a promising tool to monitor the routes and the spread of S. Dublin between herds in traditional regions of cheese production, as already reported in previous studies [16,17]. The combination of epidemiological studies in human and non-human sectors and the use of WGS may improve the cost effectiveness of control measures for S. Dublin in France, by targeting contaminated herds.

Following the investigations currently reported here, the group of producers of Morbier and Vacherin Mont d’Or cheeses implemented an action plan, including (i) systematic testing for Salmonella of batches of Morbier and Vacherin Mont d’Or sold since 1 February 2016, (ii) more regular farm visits by veterinarians, (iii) detection and containment of infected cattle, (iv) expert mission from the Ministry of Agriculture and Food to support milk industry professionals in Bourgogne-Franche-Comté region to identify and recommend better practices for detection and management of contaminated raw-milk products and (v) creation of a working group with experts on Salmonella issues from different organisations. The Morbier processing plants union reinforced their sanitary protocols, including more frequent testing of milk.

<table>
<thead>
<tr>
<th>Type of cheese</th>
<th>Cluster cases</th>
<th>Other cases(^\text{a})</th>
<th>Controls</th>
<th>Case–case study</th>
<th>Case–control study(^\text{b})</th>
<th>Other cases(^\text{a})</th>
<th>Controls</th>
<th>Case–case study</th>
<th>Case–control study(^\text{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n %</td>
<td>n %</td>
<td>n %</td>
<td>OR</td>
<td>95% CI</td>
<td>n %</td>
<td>n %</td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Vacherin Mont d’Or</td>
<td>7 70 11</td>
<td>323 8</td>
<td>5.1</td>
<td>0.9–35</td>
<td>27</td>
<td>6.8–104</td>
<td>7 70 12</td>
<td>233 8</td>
<td>5.1</td>
</tr>
<tr>
<td>Comté</td>
<td>6 67 25</td>
<td>1,386 48</td>
<td>1.4</td>
<td>0.3–10.2</td>
<td>NS</td>
<td>NA</td>
<td>6 67 23</td>
<td>1,386 48</td>
<td>1.4</td>
</tr>
<tr>
<td>Gruyère</td>
<td>7 78 26</td>
<td>1,841 63</td>
<td>1.1</td>
<td>0.2–13</td>
<td>NS</td>
<td>NA</td>
<td>7 78 28</td>
<td>1,841 63</td>
<td>1.0</td>
</tr>
<tr>
<td>Camembert</td>
<td>6 67 23</td>
<td>756 26</td>
<td>1.0</td>
<td>0.2–7.6</td>
<td>NS</td>
<td>NA</td>
<td>6 67 25</td>
<td>756 26</td>
<td>1.0</td>
</tr>
<tr>
<td>Morbier</td>
<td>12 60 10</td>
<td>361 12</td>
<td>2.1</td>
<td>0.5–8.3</td>
<td>11</td>
<td>4.2–29</td>
<td>10 67 12</td>
<td>361 12</td>
<td>3.3</td>
</tr>
<tr>
<td>Goat cheese</td>
<td>9 53 13</td>
<td>1,384 47</td>
<td>1.0</td>
<td>0.2–4.0</td>
<td>NS</td>
<td>NA</td>
<td>Not consumed by 50% of the cases</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Camembert</td>
<td>12 63 17</td>
<td>756 26</td>
<td>0.8</td>
<td>0.2–3.4</td>
<td>NS</td>
<td>NA</td>
<td>11 73 20</td>
<td>756 26</td>
<td>1.6</td>
</tr>
<tr>
<td>Gruyère</td>
<td>13 68 20</td>
<td>184 63</td>
<td>0.4</td>
<td>0.1–2.3</td>
<td>NS</td>
<td>NA</td>
<td>10 71 25</td>
<td>184 63</td>
<td>0.6</td>
</tr>
<tr>
<td>Comté</td>
<td>Not consumed by 50% of the cases</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>7</td>
<td>50 22</td>
<td>1,386 48</td>
<td>0.5</td>
<td>0.1–2.3</td>
</tr>
</tbody>
</table>

**Table 3**
Frequency of reported cheese consumption\(^a\) according to MLVA and whole genome sequencing clusters for the case–case study and the case–control study, Salmonella Dublin outbreak, France, November 2015–March 2016 (n cases = 58; n controls = 2,914)

<table>
<thead>
<tr>
<th>Type of cheese</th>
<th>Cluster cases</th>
<th>Other cases(^\text{a})</th>
<th>Controls</th>
<th>Case–case study</th>
<th>Case–control study(^\text{b})</th>
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<tbody>
<tr>
<td></td>
<td>n %</td>
<td>n %</td>
<td>n %</td>
<td>OR</td>
<td>95% CI</td>
<td>n %</td>
<td>n %</td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Type of cheese</td>
<td>MLVA 20–8–10–7–5–4 (n = 10 cases)</td>
<td>WGS A (n = 11 cases)</td>
<td>WGS C (n = 17 cases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morbier</td>
<td>12 60 10</td>
<td>361 12</td>
<td>2.1</td>
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<td>11</td>
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<td>3.3</td>
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<td>Goat cheese</td>
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<td>NA</td>
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<tr>
<td>Comté</td>
<td>Not consumed by 50% of the cases</td>
<td>NA</td>
<td>NA</td>
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<td>7</td>
<td>50 22</td>
<td>1,386 48</td>
<td>0.5</td>
<td>0.1–2.3</td>
</tr>
</tbody>
</table>

\(^a\) Only cheeses consumed by at least 50% of the cases are included in the analysis; cases and controls could report more than one cheese consumed.

\(^\text{b}\) Cases who belong to the other known subtypes.

\(^\text{c}\) Compares cluster cases with other cases with other known subtypes.

\(^\text{d}\) Compares cluster cases with controls.

\(^e\) Adjusted for age and sex.

*aOR: adjusted odds ratio; CI: confidence interval; MLVA: Multilocus variable-number tandem repeat analysis; NA: not applicable; NS: did not remain significant in the final logistic regression model; OR: odds ratio; WGS: whole genome sequencing.*

**Limitations**
Our investigations suffered from several limitations. First, cases were interviewed by phone, while controls completed a shorter online questionnaire, which could have led to obtaining exposure data with different degrees of accuracy. To minimise this bias, we used the same questions for cases and controls. Second, age and sex distribution of controls differed from that of the cases. We thus included age and sex in the multivariable analysis to adjust for those characteristics. Third, it was difficult to identify the exact sources of contamination due to probably low levels of contamination by S. Dublin of the cheese batches. As the cattle contamination was diffuse, it was difficult to incriminate specific cheese producers in the Bourgogne-Franche-Comté region as sources of contamination. Furthermore, suspected batches of cheese remained untraced.
cheese identified through trace-back investigations were no longer available for testing. Then, even if epidemiological investigations were carried out within a very constrained period of time to allow the ad hoc microbiological analyses to be launched to support the generated hypotheses, we had to deal with the impossibility to get through the whole process due to the lack of products.

Conclusions and recommendations

Microbiological, epidemiological and environmental evidence pointed towards two raw-milk cheeses, Morbier and Vacherin Mont d’Or, as vehicles of the S.

Dublin infections. The use of MLVA and WGS subtyping methods allowed the identification of different clusters and of the potential vehicles of infection, highlighting the importance of adequate subtyping methods during Salmonella outbreaks and the relevance of company internal microbiological monitoring system. As a result, WGS has now been routinely implemented at the French NRC and findings of this multi-disciplinary investigation led to a reinforced control plan for processing plants of raw-milk cheeses to prevent future outbreaks.

Acknowledgements

We are grateful to all the microbiological laboratories processing human and non-human samples, who participate in the Salmonella network for isolates processing. We acknowledge Santé publique France epidemiologists from the regional offices who conducted the interviews, Edith Laurent who performed the data entry and Marie-José Letort who helped contacting laboratories. We thank all the persons involved in the microbiological investigations in Institut Pasteur (in particular Vincent Enouf, Estelle Serre and François Gravey) and in the French Agency for Food, Environmental and Occupational Health and Safety. We also thank the persons who conducted the food trace-back investigations in the Ministry of Agriculture and Food. We are grateful for the persons in the French National Institute of Health and Medical Research in the GrippeNet.fr project who worked with us in implementing the control recruitment method. Finally, we thank all the cases, their relatives and the nursing home staff and the controls who participated in the studies.

Conflict of interest

None declared.

Authors’ contributions

AU, KD and NJ coordinated the epidemiological part of the investigation. EL implemented the electronic version of the questionnaire, AU was in charge of the case–case study, DVC was in charge of the case–control study. DVC, LR and CG coordinated the part of the investigation regarding the GrippeNet.fr study. MLV, SCS, RL and SLH coordinated the microbiological part of the investigation regarding MLVA: MLV, SCS and RL collected food and animal samples and planned the MLVA analysis of human and non-humans isolates, AB, NF, LF and SLH coordinated the microbiological part of the investigation regarding WGS.

AM and MPD coordinated the food trace-back part of the investigation. AU and AB took lead in manuscript writing. NJ, KD and SLH the general coordination. All authors read, commented and approved the final manuscript.

References

11. Vignaud M-L, Cherchame E, Marault M, Chaing E, Le Hello S, Stoker AM and MPD coordinated the food trace-back part of the investigation regarding MLVA: MLV, SCS and RL collected food and animal samples and planned the MLVA analysis of human and non-human isolates, AB, NF, LF and SLH coordinated the microbiological part of the investigation regarding WGS.


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