**RAPID COMMUNICATION**

Increased detection of enterovirus A71 infections, Germany, 2019

Sindy Böttcher, Sabine Diedrich, Kathrin Keeren and the Laboratory Network for Enterovirus Diagnostic (LaNED)

**OUTBREAKS**

Listeriosis outbreak likely due to contaminated liver pâté consumed in a tavern, Austria, December 2018

Adriana Cabal, Franz Allerberger, Steliana Huhulescu, Christian Kornschober, Burkhard Springer, Claudia Schlagenhaufen, Marianne Wassermann-Neuhold, Harald Fotschl, Peter Pless, Robert Krause, Anna Lennkh, Andrea Murer, Werner Ruppitsch and Ariane Pietzka

**RESEARCH**

Shedding of OXA-181 carbapenemase-producing *Escherichia coli* from companion animals after hospitalisation in Switzerland: an outbreak in 2018

Aurélien Nigg, Michael Brilhante, Valentina Dazio, Mathieu Clément, Alexandra Collaud, Stefanie Gobeli Brawand, Barbara Willi, Andrea Endimiani, Simone Schuller and Vincent Perreten

**SURVEILLANCE**

New HIV-1 circulating recombinant form 94: from phylogenetic detection of a large transmission cluster to prevention in the age of geosocial-networking apps in France, 2013 to 2017

We report on the increased circulation of enterovirus A71 in Germany in 2019. Strains were mainly identified in hospitalised patients with suspected aseptic meningitis/encephalitis. Molecular analysis showed co-circulation of EV-A71 sub-genogroups C1 and C4, a signal for physicians and public health authorities to include/intensify EV diagnostic in patients showing signs of aseptic meningitis, encephalitis or acute flaccid paralysis/myelitis.

In Europe, enterovirus A71 (EV-A71) has mainly been detected sporadically in patients with neurological disorders. C2 has been the predominant sub-genogroup during the last decade [1]. A multi-recombinant C1 lineage (C1-like) emerged in 2015 in Germany [2-4]. Since then, it was detected in sporadic cases with severe neurological disease in several European countries [4-6], as well as in an outbreak in Catalonia, Spain in 2016 [7,8]. In south-eastern Asia, EV-A71, sub-genogroup C4 strains have been mainly associated with severe and even fatal courses of hand, foot and mouth disease (HFMD) in children [1].

Here we report on an increase of EV-A71 C1-like strains in Germany in 2019 and first detections of EV-A71 C4 strains in patients with neurological disease and HFMD.

Enterovirus surveillance in Germany

In Germany, enterovirus surveillance (EVSurv) is conducted within the framework of the Global Polio Eradication Initiative and provides continuous data on circulating enteroviruses since 2006. Stool or cerebrospinal fluid samples of hospitalised patients with signs of aseptic meningitis/encephalitis and/or acute flaccid paralysis are analysed within a quality-controlled laboratory network (LaNED). Laboratory results and pseudonymised patient data are reported to the national public health institute (Robert Koch Institute; RKI). Data are analysed and the results are publically available at evsurv.rki.de. Previous data on EV-A71 circulation suggested that EV-A71 strains circulate in upsurges every 3 years, with 2010 and 2013 being epidemic years in Germany [2].

Typing of enterovirus (EV) strains is performed according to the individual network laboratory algorithm, by sequencing the complete or partial VP1 or VP4/VP2 region and/or virus isolation and typing by neutralisation assay. Assignment of the EV-A71 strains to one of the seven known sub-genogroups was performed using the Enterovirus Genotyping Tool [9] and/or neighbour-joining algorithm-based phylogenetic tree analysis. All sequences were submitted to GenBank under accession numbers (MN397830-MN397906).

Since 2006, ca 2,500 samples have been tested annually within the EVSurv for a total of ca 34,000 analysed since that time. Of these, 25–30% were EV-positive. Overall, echovirus 30 was the most prevalent EV type (n = 2,194), followed by echovirus 6 (n = 775) and EV-A71 (n = 496). A total of 32 EV-A71 positive samples were documented in 2017 and 40 in 2018.

Enterovirus A71 detections through enterovirus surveillance

From January 2019 to the end July 2019, 38 EV-A71 positive patients were registered, including one fatal case. EV-A71-positive patients were observed in March (n = 2), April (n = 3), May (n = 6), June (n = 14) and July (n = 13). Besides EV-A71, CV-B5 was the second most common EV type (n = 24) within the EVSurv this year. Typing results are still pending for 35% (73/209) of EV-positive samples.

Of the 38 EV-A71 strains detected, 26 were analysed further at the National Reference Centre for Poliomyelitis and Enteroviruses (NRZ PE). Sequencing of the VP1 region was done as described previously [2]. Twenty-one strains were assigned to C1-like, four to sub-genogroup C4 and one to sub-genogroup C2 (Table 1).
The increased circulation of EV-A71 in 2019 has also been indicated by samples routinely sent to the NRZ PE, mostly from other laboratories sending in EV-positive samples for typing, but also from clinicians/hospitals not yet participating in EVSurv or those submitting samples from patients with diseases other than suspected aseptic meningitis/encephalitis or acute flaccid paralysis/myelitis. Of 126 EV strains typed from January through July 2019, 23 were assigned to EV-A71. Of these, 19 were assigned to EV-A71 lineage C1-like, three to EV-A71 sub-genogroup C4 and one to EV-A71 sub-genogroup C2 (Table 2). Notably, the number of EV-A71 detections in the first 7 months of 2019 (n = 23) has already exceeded the total of such detections for 2017 and 2018, as well as the totals from the epidemic years of 2010 (6/61), 2013 (12/206) and 2016 (17/144).

Germany does not conduct standardised HFMD surveillance, but occasionally, samples collected within nursery school outbreaks of HFMD are submitted to the NRZ PE by the local health authorities. From 2010 until 2018, between 15 and 82 samples from HFMD patients were submitted per year, with 6 to 57 patients being positive for EV and 2 to 8 patients being positive for EV-A71 annually. In 2018, there were 24 HFMD patients positive for EV, eight of whom had EV-A71. Of those eight, EV-A71 C4 was detected for the first time in four children with HFMD. For two strains (17672, 17673), the VP1 region was sequenced and compared with EV-A71 C4 strains identified in samples analysed within the EVSurv in 2018 and 2019. Close clustering indicates that there is no relationship between the VP1 region sequence and the presence of HFMD or symptoms related to aseptic meningitis/encephalitis (Figure 1B).

Discussion and conclusion

Our findings suggest an increased circulation of EV-A71 strains in Germany already in the first 7 months of the year. Considering the fact that the EV season is still ongoing, the data available suggest that 2019 will become an epidemic year similar to 2016, 2013 and 2010.

The emergence of new EV variants exhibiting altered antigenic sites might result in increased circulation and/or changed clinical manifestation. In Europe, EV-A71 was only sporadically associated with neurological disease during the past 30 years. In Germany, the predominant EV-A71 sub-genogroup C2 was replaced by C1-like strains in 2016. Emergence of EV-A71 sub-genogroup C4 strains in Germany was detected between 2011 and 2013 [2], with France [10], Russia [11] and Denmark [12] also reporting such detection around the same time.

One limitation of the EVSurv is that no detailed clinical information is available since the EVSurv request form deliberately only asks for basic cardinal symptoms: fever, nuchal rigidity, headache and vomiting. Since non-polio EV infections are not mandatorily notifiable at federal level in Germany, no data on severe cases or deaths are available. No routine testing for EV is performed in deaths so we therefore cannot exclude fatal cases with other EV than EV-A71. Nevertheless, we were informed of an additional fatal case where EV-A71 was detected in a German child hospitalised in Austria in July 2019 (personal communication, Franz Allerberger)

### Table 1

<table>
<thead>
<tr>
<th>EVSurv samples tested</th>
<th>2016</th>
<th>2017</th>
<th>2018</th>
<th>Jan–Jul 2019</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>2,444</td>
<td>2,188</td>
<td>1,959</td>
<td>1,160</td>
</tr>
<tr>
<td>EV-positive</td>
<td>527</td>
<td>448</td>
<td>416</td>
<td>209</td>
</tr>
<tr>
<td>EV-typed</td>
<td>445</td>
<td>380</td>
<td>349</td>
<td>136</td>
</tr>
<tr>
<td>EV-A71-positive</td>
<td>77</td>
<td>32</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td><strong>EV-A71 sub-genogroups</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lineage C1-like</td>
<td>57</td>
<td>18</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>C2</td>
<td>10</td>
<td>6</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>C4</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>ND</td>
<td>10</td>
<td>8</td>
<td>5</td>
<td>12</td>
</tr>
</tbody>
</table>

EV: enterovirus; EVSurv: enterovirus surveillance; Jan: January; Jul: July; ND: not determined.

Number of EV-A71 strains and sub-genogroup assignment based on the VP1 region is shown.

**Epidemiological data**

EV-A71-positive samples were reported from 11 of 16 federal states in Germany, with patients hospitalised in 20 different hospitals. All patients were between 0 and 10 years old, with 30 of 38 between 0 and 5 years old. Of these, 8 were younger than 1 year old. Twenty-one were male and 17 were female. All EV-A71 strains were detected in stool samples.

**Phylogenetic relation**

Sequence analysis of C1-like strains identified in 2019 showed high nucleotide (nt) identities with recently detected strains, indicating a rapid widespread circulation of this new recombinant variant [4]. Phylogenetic tree calculation showed several lineages either grouping separately or clustering to recent strains detected in France, Poland, Spain, Denmark, the United States (US) and Japan (Figure 1A).

Sequence analysis of sub-genogroup C4 showed separate clustering of the German EV-A71 C4 strains detected in 2011–2013 and 2018–July 2019. Currently circulating strains grouped together with EV-A71 C4 strains detected in south-eastern Asia (Figure 1B) with highest nt identities to MH716155, MH716157 and MG431943 detected in patients with severe HFMD. This cluster also includes strains isolated from patients with severe HFMD and even fatal courses (KT428649, KT428650).

**Enterovirus A71 detections in routine samples**

The increased circulation of EV-A71 in 2019 has also been indicated by samples routinely sent to the NRZ PE, mostly from other laboratories sending in EV-positive samples for typing, but also from clinicians/hospitals not yet participating in EVSurv or those submitting samples from patients with diseases other than suspected aseptic meningitis/encephalitis or acute flaccid paralysis/myelitis. Of 126 EV strains typed from January through July 2019, 23 were assigned to EV-A71. Of these, 19 were assigned to EV-A71 lineage C1-like, three to EV-A71 sub-genogroup C4 and one to EV-A71 sub-genogroup C2 (Table 2). Notably, the number of EV-A71 detections in the first 7 months of 2019 (n = 23) has already exceeded the total of such detections for 2017 and 2018, as well as the totals from the epidemic years of 2010 (6/61), 2013 (12/206) and 2016 (17/144).

Germany does not conduct standardised HFMD surveillance, but occasionally, samples collected within nursery school outbreaks of HFMD are submitted to the NRZ PE by the local health authorities. From 2010 until 2018, between 15 and 82 samples from HFMD patients were submitted per year, with 6 to 57 patients being positive for EV and 2 to 8 patients being positive for EV-A71 annually. In 2018, there were 24 HFMD patients positive for EV, eight of whom had EV-A71. Of those eight, EV-A71 C4 was detected for the first time in four children with HFMD. For two strains (17672, 17673), the VP1 region was sequenced and compared with EV-A71 C4 strains identified in samples analysed within the EVSurv in 2018 and 2019. Close clustering indicates that there is no relationship between the VP1 region sequence and the presence of HFMD or symptoms related to aseptic meningitis/encephalitis (Figure 1B).

**Discussion and conclusion**

Our findings suggest an increased circulation of EV-A71 strains in Germany already in the first 7 months of the year. Considering the fact that the EV season is still ongoing, the data available suggest that 2019 will become an epidemic year similar to 2016, 2013 and 2010.

The emergence of new EV variants exhibiting altered antigenic sites might result in increased circulation and/or changed clinical manifestation. In Europe, EV-A71 was only sporadically associated with neurological disease during the past 30 years. In Germany, the predominant EV-A71 sub-genogroup C2 was replaced by C1-like strains in 2016. Emergence of EV-A71 sub-genogroup C4 strains in Germany was detected between 2011 and 2013 [2], with France [10], Russia [11] and Denmark [12] also reporting such detection around the same time.

One limitation of the EVSurv is that no detailed clinical information is available since the EVSurv request form deliberately only asks for basic cardinal symptoms: fever, nuchal rigidity, headache and vomiting. Since non-polio EV infections are not mandatorily notifiable at federal level in Germany, no data on severe cases or deaths are available. No routine testing for EV is performed in deaths so we therefore cannot exclude fatal cases with other EV than EV-A71. Nevertheless, we were informed of an additional fatal case where EV-A71 was detected in a German child hospitalised in Austria in July 2019 (personal communication, Franz Allerberger)
The phylogenetic tree is based on complete viral protein 1 (VP1) nucleotide (nt) sequences (891 b corresponding to nt position 2439–3329 in the prototype BrCr ETU22521). Strains identified within the German enterovirus surveillance (EVSurv) and a representative set of enterovirus A71 strains retrievable from GenBank were used. Only sequences covering the complete VP1 region were included in the analysis. Subtrees including only EV-A71 sub-genogroup C1, including lineage C1-like (A) and EV-A71 sub-genogroup C4 (B) are shown. Strains identified within the EVSurv are marked with filled diamonds and strains identified in hand, foot and mouth disease (HFMD) patients with open diamonds. The tree was calculated using the neighbour-joining algorithm with 1,000 replicates in MEGA software version 7.0.26. Only bootstrap values > 70 are shown. Scale bars indicate nt substitution per site.
Number of EV-A71 strains and sub-genogroup assignment based on the VP1 region is shown.

EV: enterovirus; Jan: January; Jul: July; NRZ-PE: National Reference Centre for Poliomyelitis and Enteroviruses.

Furthermore, systematic studies are needed to assess reports of severe clinical presentation associated with detection of a EV-A71 C1-like strains [3,5-7], together with recent indications of long-term sequelae [13,14], should be a signal for physicians and public health authorities to include/intensify EV diagnostic in upcoming public health matters. (EV typing should be conducted to enable molecular epidemiology.

Continued virological data could provide first hints to upcoming public health matters. (EV typing should be conducted to enable molecular epidemiology. Furthermore, systematic studies are needed to assess the burden of EV infections in Europe.

Laboratory Network for Enterovirus Diagnostic (LaNED)

Nikolaus Ackermann, Maja Adam, Armin Baillot, Ingrid Ehrhard, Martin Enders, Wali Hafezi, Ralph Hahn, Jörg Hofmann, Klaus Korn, Joachim Kühn, Detlef Michel, Alexandra Pettke, Christiane Priifting, Holger F. Rabenau, Ursula Reif, Gabriele Rieder, Benedikt Weißbrich, Andreas Wille and Marta J Zuchowski.

Acknowledgements

We thank all clinicians participating in the German enterovirus surveillance; all cooperating labs sending enterovirus strains for typing; the local health authorities for collecting samples from hand, foot and mouth disease patients; and the technical staff in all labs for excellent performance.

Funding statement: Results were obtained during routine work under the auspices of the German Federal Ministry of Health.

Conflict of interest

None declared.

Authors’ contributions

Members of the Laboratory Network (LaNED) analysed samples at the laboratory level. KK coordinated the EVSurv and collected the data. KK, SD and SB analysed the data. SB wrote the manuscript draft, all co-authors commented and approved the final version.

References


Table 2

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
</tr>
<tr>
<td>Total</td>
<td>128</td>
<td>78</td>
<td>137</td>
<td>279</td>
<td>166</td>
<td>153</td>
<td>252</td>
<td>279</td>
<td>278</td>
<td>213</td>
</tr>
<tr>
<td>EV-positive</td>
<td>61</td>
<td>58</td>
<td>59</td>
<td>206</td>
<td>93</td>
<td>81</td>
<td>144</td>
<td>147</td>
<td>181</td>
<td>126</td>
</tr>
<tr>
<td>EV-A71 positive</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td>12</td>
<td>2</td>
<td>12</td>
<td>17</td>
<td>6</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>EV-A71 sub-genogroup</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lineage C1-like</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>9</td>
<td>2</td>
<td>7</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>C4</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

EV: enterovirus; NRZ-PE: National Reference Centre for Poliomyelitis and Enteroviruses.

Number of EV-A71 strains and sub-genogroup assignment based on the VP1 region is shown.


License, supplementary material and copyright
This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence and indicate if changes were made.

Any supplementary material referenced in the article can be found in the online version.

This article is copyright of the authors or their affiliated institutions, 2019.
Listeriosis outbreak likely due to contaminated liver pâté consumed in a tavern, Austria, December 2018

Adriana Cabal1,2, Franz Allerberger1, Steliana Huhulescu1, Christian Kornschober1, Burkhard Springer1, Claudia Schlagenhaufen1, Marianne Wassermann-Neuhold3, Harald Fötschl1, Peter Piess3, Robert Krause4, Anna Lennkh4, Andrea Murer1, Werner Ruppitsch1, Ariane Pietzka1
1. Institute for Medical Microbiology and Hygiene, Austrian Agency for Health and Food Safety, Vienna/Graz, Austria
2. European Public Health Microbiology Training Programme (EUPHEM), European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden
3. Department – Health and Nursing Management, Styrian Provincial Government, Graz, Austria
4. Section of Infectious Diseases and Tropical Medicine, Department of Internal Medicine, Medical University of Graz, Graz, Austria

Correspondence: Adriana Cabal (adriana@cabalrosel.com)

In late December 2018, an outbreak of listeriosis occurred after a group of 32 individuals celebrated in a tavern in Styria, Austria; traditional Austrian food (e.g. meat, meat products and cheese) was served. After the celebration, 11 individuals developed gastrointestinal symptoms, including one case with severe sepsis. Cases had consumed mixed platters with several meat products and pâtés originating from a local production facility (company X). Human, food and environmental samples taken from the tavern and company X were tested for L. monocytogenes. Whole genome sequence-based typing detected a novel L. monocytogenes strain of serotype IVb, sequence type 4 and CT7652 in 15 samples; 12 human, two food and one environmental sample from company X with an allelic difference of 0 to 1. Active case finding identified two further cases who had not visited the tavern but tested positive for the outbreak strain. In total, 13 cases (seven females and six males; age range: 4–84 years) were identified. Liver pâté produced by company X was identified as the likely source of the outbreak. Control measures were implemented and since the end of December 2018, no more cases were detected.

Background

Listeria monocytogenes is a Gram-positive bacterium that is typically transmitted to humans through the consumption of contaminated food products. Clinical symptoms of listeriosis vary depending on the immune status of the host, with those immunocompromised at higher risk of presenting severe symptoms [1]. In immunocompetent individuals, infection can be asymptomatic but it more often results in febrile gastroenteritis [2]. Febrile gastroenteritis usually resolves within 2–3 days after the onset of symptoms, while invasive forms of the disease can lead to meningoencephalitis, abortion, sepsis or even death [3,4]; other manifestations such as endophthalmitis have also been associated to infections with L. monocytogenes [1,5]. Foods that have been implicated with listeriosis outbreaks are ready-to-eat (RTE) products such as sliced meat, pâté and soft cheese varieties [6]. Since L. monocytogenes can persist in the environment for long periods due to its ability to form biofilms and its resistance to disinfectants, this pathogen can be difficult to eradicate from food-processing facilities [7].

In Austria, notification of invasive listeriosis cases is mandatory. The Austrian National Reference Laboratory for Listeria (NRL; Graz Austria) is responsible for performing whole genome sequence (WGS)-based typing of human and non-human (e.g. food, environmental) isolates. In recent years, WGS-based surveillance of L. monocytogenes has been successfully used in combination with analysis of epidemiological data in outbreak investigations [8-10].

Outbreak detection

On 21 December 2018, the local health authority of Styria (Directorate of Public Health, Graz, Austria) and the NRL confirmed the occurrence of an outbreak of febrile gastroenteritis, including one case of culture-confirmed L. monocytogenes bacteraemia, among 32 persons having attended a tavern in the province on 15 December 2018 as part of a celebration. Previously, a pregnant physician who had visited the tavern and was aware that L. monocytogenes had been isolated from a blood culture of a tavern guest had informed the local health authority of Styria about a possible listeriosis outbreak. According to her, more than half of the guests started showing symptoms of febrile gastroenteritis and vomiting within 2 days after the tavern visit.
On 29 January 2019, the Austrian Ministry of Health (Vienna, Austria) mandated the Austrian Agency of Health and Food Safety (AGES; Graz, Austria) to investigate the outbreak. The aim of the investigation was to identify the causative agent and the likely source of infection, in order to detect and prevent further cases.

Methods

Outbreak case definition
An outbreak case was defined as an individual who presented with febrile gastroenteritis at least 24 hours after visiting the tavern in Styria, Austria on 15 December 2018 and tested positive for \textit{L. monocytogenes} by either blood or stool culture.

Specimen collection and trace-back investigations
Of 32 individuals attending the celebration on 15 December 2018, 19 symptomatic individuals provided stool specimens. Of five individuals working in this tavern on this day, three asymptomatic individuals provided stool samples. The local food authority gathered information on involved food suppliers, restricting it to one local meat producer (company X) and three grocery shops (A, B and C).

Between 3 January and 25 January 2019, a total of 73 food and environmental samples were analysed for the presence of \textit{L. monocytogenes}: (i) 19 environmental (surface) samples and three food samples produced by company X were collected from the tavern, (ii) three samples were collected from food produced at other facilities (shops A to C) (iii) one sample was collected from food produced by the tavern itself, and (iv) 47 food and environmental samples were collected from company X at their production facility. Products from company X were only offered at six locations, including the affected tavern and sold directly from the factory.

Microbiological investigations and WGS-based typing
The detection of \textit{L. monocytogenes} in human samples was conducted as described elsewhere [11] and colonies were confirmed by Api-Listeria (BioMérieux, Marcy l'Etoile, France) or MALDI Biotyper (Bruker Daltonics, Hamburg, Germany). Food and environmental isolates received at the NRL were cultivated on RAPID-L.mono agar plates (Biorad, Munich, Germany) for species verification and subsequently subcultured overnight on Columbia Broth (BD Difco, Heidelberg, Germany) for extraction of high quality genomic DNA using the HMW MagAttract kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer for Gram-positive bacteria.

WGS was performed as described previously [12]. For sequencing, an Illumina MiSeq platform (Illumina Inc., San Diego, California, United States) was used. Library preparation was carried out using Nextera XT according to the instructions of the manufacturer (Illumina Inc.). For assembly into draft genomes, raw reads were de novo assembled using SPAdes version 3.11.1 [13]. Classical multilocus sequence typing (MLST) data were extracted from WGS sequence data [14]. A minimum spanning tree (MST) was generated in SeqSphere+ for visualisation of strain relatedness. Assessment of the core-genome multilocus sequence typing (cgMLST) was done as described by Ruppitsch et al. [8]. In parallel, a single nucleotide polymorphism (SNP) analysis was performed for comparison purposes with GenomeGraphR [15]. In addition, we created two task templates that were implemented with Seqsphere+ for detection of the pathogenicity islands LIPI-3 and LIPI-4 using the reference genomes from strain F2365 and strain CLIP 80459, respectively (NC_002973.6, NC_012488.1). Other virulence genes (VGs) were detected among the sequenced genomes by using VirulenceFinder [9].

Active case finding
We conducted active case finding to detect additional human cases (retrospectively and prospectively). A new case definition was created, which included any person testing positive to the outbreak strain by WGS since three months before the tavern gathering. We performed an intensive search and strain comparison using our local \textit{L. monocytogenes} database, which currently contains nearly 8,000 sequenced isolates.

Isolates show 0 to 1 alleles of difference. Accession numbers can be consulted in Supplementary Table S1.

**Figure 1**
Minimum spanning tree representing the genetic relatedness among the 17 sequenced isolates based on their core genome, Styria, Austria, 2018

\begin{figure}
\centering
\includegraphics[width=\textwidth]{mst.png}
\caption{Minimum spanning tree representing the genetic relatedness among the 17 sequenced isolates based on their core genome, Styria, Austria, 2018}
\end{figure}
Ethical statement

Ethical approval to conduct the study was not needed. In Austria, investigation of foodborne outbreaks is a legal obligation.

Results

Microbiological findings

Of 19 stool specimens collected from individuals with febrile gastroenteritis, 10 samples (specimen ID: H03–H10, H12, H14) were positive for *L. monocytogenes*. Of three stool specimens from asymptomatic staff members of the tavern, only one stool sample from a female in her early 60s (H11) tested positive for *L. monocytogenes*. In addition, one *L. monocytogenes* isolate came from a blood culture obtained from a male in his mid-20s who was hospitalised for febrile gastroenteritis 24 hours after the tavern visit (H02).

Of 73 non-human samples, two food samples and one environmental sample tested positive for *L. monocytogenes*. The two food samples (specimen ID: F01: smoked meat ('Geselchtes') and F02: liver pâté ('Leberstreichwurst') were collected at the tavern and produced by company X; both tested positive for *L. monocytogenes*. In addition, one *L. monocytogenes* isolate came from a blood culture obtained from a male in his mid-20s who was hospitalised for febrile gastroenteritis 24 hours after the tavern visit (H02).

In addition to the three non-human isolates (F01–F03), 12 human *L. monocytogenes* isolates (from specimens: H2–H12, H14) were available for sequencing. cgMLST performed as described by Ruppitsch et al. [8] revealed that all isolates belonged to the same genetic type: they were identified as genoserogroup IVb, clonal complex (CC)4, multilocus sequence type (ST)4 and cg complex type (CT)7652 [8] and displayed 0 to 1 allelic difference (Figure 1). In total, 1,701 loci composing the cgMLST scheme were detected. The SNP analysis revealed a maximum of four SNPs difference between the isolates and we did not find related strains in the database. All isolates yielded the same 72 virulence genes including LIPI-3 and LIPI-4 pathogenicity islands.

International alert and active case finding

Since this outbreak strain had not been detected previously in Austria, an urgent inquiry was launched through the Epidemic Intelligence Information System (EPIS) at the European Centre for Disease Prevention and Control (EPIS-UI-539) on 28 January 2019. Denmark, Finland, France, the Netherlands, Luxembourg, Portugal, Switzerland and the United Kingdom answered the inquiry via the EPIS platform; no cases were linked to this outbreak strain. The closest matches were found at the European Nucleotide Archive repositories showed at least 32 allelic differences when using the cgMLST scheme from Moura et al. [10].

Through active case finding, we identified two more cases of invasive listeriosis. These cases lived in the same two geographical districts ('Bezirk') as the tavern and company X. The first isolate was taken from a blood culture from a case in their early 80s (H1) who had not visited the tavern but developed symptoms...
in November 2018. The case, who later died from the infection, reported having repeatedly consumed liver pâté from company X purchased at a local market. The second isolate originated from an eye chamber aspirate from a case in their mid-50s with endophthalmitis (H13), with onset of clinical symptoms on 23 December 2018; the case had not visited the tavern. The two isolates differed by a maximum of one allele from the other clinical isolates and the food and environmental isolates.

In total, 13 individuals (seven females and six males; age range: 4–84 years) living within area radius of 24 km in Styria, were confirmed as outbreak cases. A timeline displaying all cases belonging to the outbreak is shown in Figure 2. A geographical representation of the residence for the confirmed *L. monocytogenes* cases, the tavern and the meat producer company X is shown in Figure 3.

**Outbreak control measures**

After the outbreak was confirmed on 21 December 2018, intensive cleaning of the tavern and at company X was performed by professional sanitation companies, under supervision of the local health authority. Only heat-treated products were allowed to be sold until confirmed sanitation (i.e. *L. monocytogenes* was no longer detected after repeated sampling). No more cases were detected by the end of December and the outbreak was declared over.

**Discussion**

The use of epidemiological data and WGS-based typing allowed us to confirm a local outbreak due to an *L. monocytogenes* IVb-CC4-ST4-CT7652 strain, not previously detected in Austria nor anywhere else. Trace-back investigations showed that meat products originating from company X were the most likely source of the outbreak. The 13 confirmed cases lived within a radius of 24 km, further suggesting that the outbreak was caused by a locally sourced ingredient. No more cases were detected after company X implemented control measures, supporting the hypothesis that meat products from company X were the likely source of the outbreak.

In contrast to classical restaurants, a tavern offers a limited selection of traditional food (especially local meat products and cheese) in a buffet-like manner but has a fully functioning kitchen including a fridge and a freezer. In Austria, gatherings of large groups at traditional taverns are common around festivities such as Christmas. It is possible that the time between food preparation and service might have been long increasing the possibility for *L. monocytogenes* to multiply.
Moreover, traditional Austrian meat products e.g. liver pâté and jellied pork do not require heating prior to consumption and have been previously identified as potential risk food for listeriosis due to their growth potential for *L. monocytogenes* [16,17]. In 2009, an outbreak of febrile gastroenteritis was reported that was also associated with a traditional Austrian tavern, this time locally produced jellied liver was identified as the vehicle of infection [11]. Liver pâté (‘Leberstreichwurst’) is a peculiar type of traditional Austrian meat product, made from cured, cooked, comminuted meat with fat as a binder. Its average pH value is 6.19 ± 0.15, with a corresponding water activity value of 0.963 ± 0.003, which provides *L. monocytogenes* with an optimal substrate for its growth [17]. Other types of pork pâté such as ‘pâté de campagne’ were found to be contaminated with *L. monocytogenes* [18]. Although enumeration of *L. monocytogenes* in the pâté seemed low (<10 CFU/gram), no Tween 80 was added to its initial suspension during the enrichment procedure, and that might explain the relatively low number of bacteria found per gram.

*L. monocytogenes* serotype IVb strains are commonly associated with outbreaks [22]. Moreover, serotype IVb CC4 is known to be widely distributed in food and it has been described as one of the IVb hypervirulent CCs [23,24]. All 14 human isolates (from the 13 confirmed cases plus the stool isolate from the asymptomatic case), the two food isolates and the environmental isolate carried LPI-3 and LPI-4 pathogenicity islands, which have been associated with high invasiveness and severe symptomatology [25]. In contrast, another article reported the hypervirulent CC4 was associated with dairy products, while hypovirulent clones, such as CC9 or CC121 were associated with meat products [26].

The only case without a proven connection to liver pâté of company X was the case with endophthalmitis; only 43 cases of endophthalmitis attributed to an *L. monocytogenes* infection have been reported in the literature so far [5,19-21]. While we were unable to elucidate any connection of this patient to company X, the case confirmed regular consumption of liver pâté so it is possible that the meat product may have been produced at company X.

*L. monocytogenes* is known to be widely distributed in food and it has been described as one of the IVb hypervirulent CCs [23,24]. All 14 human isolates (from the 13 confirmed cases plus the stool isolate from the asymptomatic case), the two food isolates and the environmental isolate carried LPI-3 and LPI-4 pathogenicity islands, which have been associated with high invasiveness and severe symptomatology [25]. In contrast, another article reported the hypervirulent CC4 was associated with dairy products, while hypovirulent clones, such as CC9 or CC121 were associated with meat products [26].

The main limitation of our study was associated with the closure of the tavern over the Christmas period. Due to the closure, the AGES outbreak investigation did not start until 45 days after the tavern visit. Nevertheless, control measures had been previously put in place preventing new cases to occur.

**Conclusion**

The isolate-based surveillance of *L. monocytogenes* using WGS-based typing and analyses of epidemiological data allowed us to confirm a local outbreak due to a *L. monocytogenes* IVb-CC4-ST4-CT7652 strain not previously detected elsewhere. Epidemiological and trace-back investigations showed the liver pâté produced at company X was the most likely source of infection. The applied control measures were effective in stopping the outbreak. Additional investigations are needed to estimate the risk of infection with *L. monocytogenes* when attending celebrations at taverns serving non-heated products that have been stored at room temperature for extended periods of time.

**Depositing of the sequences**

This whole-genome shotgun project has been deposited in DDBJ/EMBL/GenBank under the project number PRJNA528854 and corresponds to the study number SRP189297. Raw sequence data for each strain were deposited under SRA accession numbers SRR8776907, SRR8776908, SRR8776904, SRR8776910, SRR8776917, SRR8776915, SRR8776918, SRR8776920, SRR8776906, SRR8776905, SRR8776911, SRR8776912, SRR8776913, SRR8776914, SRR8776916, SRR8776919, SRR8776909.

**Acknowledgements**

All co-authors in this manuscript declare that no funding was received from any funding agency in the public, commercial or not-for-profit sectors. AC was supported by a grant from the European Public Health Microbiology Training Programme (EUPHEM), European Centre for Disease Prevention and Control (specific grant agreement number 1 ECD.7550 implementing ECDC/GRANT/2017/003).

**Conflict of interest**

None declared.

**Authors’ contributions**

AP, FA and WR were responsible for the concept and design of the study; AP, MW-N, AC and FA carried out the outbreak investigation; AP, AC, CS and WR conducted the microbiological analyses; AP, FA and WR were responsible for the concept and design of the study; AP, MW-N, AC and FA carried out the outbreak investigation. All authors have contributed to the writing and final version of the manuscript.

**References**


7. Leong D, Alvarez-Ordóñez A, Jordan K. Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and...


License, supplementary material and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence and indicate if changes were made.

Any supplementary material referenced in the article can be found in the online version.

This article is copyright of the authors or their affiliated institutions, 2019.
Shedding of OXA-181 carbapenemase-producing *Escherichia coli* from companion animals after hospitalisation in Switzerland: an outbreak in 2018

**Aurélien Nigg¹, Michael Brilhante ¹-², Valentina Dazio³, Mathieu Clément ²-³, Alexandra Collaud¹, Stefanie Gobeli Brawand¹, Barbara Willi⁴, Andrea Endimiani⁵, Simone Schuller³, Vincent Perreten¹**

1. Institute of Veterinary Bacteriology, Bern, University of Bern
2. Graduate School of Cellular and Biomedical Sciences, Bern, University of Bern
3. Department of Clinical Veterinary Medicine, Bern, University of Bern
4. Clinic for Small Animal Internal Medicine, University of Zurich, Zurich, Switzerland
5. Institute for Infectious Diseases, University of Bern, Bern, Switzerland

Correspondence: Vincent Perreten (vincent.perreten@vetsuisse.unibe.ch)


Article submitted on 22 Jan 2019 / accepted on 19 May 2019 / published on 26 Sep 2019

**Background:** Carbapenem-resistant Enterobacteriaceae pose a serious threat to public health worldwide, and the role of companion animals as a reservoir is still unclear. **Aims:** This 4-month prospective observational study evaluated carriage of carbapenem-resistant Enterobacteriaceae at admission and after hospitalisation in a large referral hospital for companion animals in Switzerland. **Methods:** Rectal swabs of dogs and cats expected to be hospitalised for at least 48 h were taken from May to August 2018 and analysed for the presence of carbapenem-resistant Enterobacteriaceae using selective agar plates. Resistant isolates were further characterised analysing whole genome sequences for resistance gene and plasmid identification, and ad hoc core genome multilocus sequence typing. **Results:** This study revealed nosocomial acquisition of *Escherichia coli* harbouring the carbapenemase gene *bla*<sub>OXA-181</sub>, the pAmpC cephalosporinase gene *bla*<sub>CMY-42</sub> as well as quinolone resistance associated with *qnrS1* and mutations in the topoisomerases II (GyrA) and IV (ParC). *bla*<sub>OXA-181</sub> and *qnrS1* genes were identified on a 51 kb IncX3 plasmid and *bla*<sub>CMY-42</sub> on a 47 kb IncI1 plasmid. All isolates belonged to sequence type ST410 and were genetically highly related. This *E. coli* clone was detected in 17 of 100 dogs and four of 34 cats after hospitalisation (21.6%), only one of the tested animals having tested positive at admission (0.75%). Two positive animals were still carriers 4 months after hospital discharge, but were negative after 6 months. **Conclusions:** Companion animals may acquire carbapenemase-producing *E. coli* during hospitalisation, posing the risk of further dissemination to the animal and human population and to the environment.

**Introduction**

Companion animals nowadays not only share the owner’s home environment but also benefit from intensive veterinary care in case of serious illness. Veterinary referral hospital environments are similar to human hospitals in that animal patients face similar risks of developing nosocomial infections. Bacterial infections in dogs and cats are frequently treated with critically important antimicrobials such as fluoroquinolones or cephalosporins. In very rare cases, even the last resort antibiotics of human medicine such as carbapenems may be used in companion animals in Switzerland to treat infections refractory to any other standard antimicrobial used in veterinary medicine (Ordinance on Veterinary Medicinal Products, SR 812.212.27, Art. 6).

Such treatments pose the risk of selecting resistance to these classes of antibiotics in Enterobacteriaceae through the acquisition of plasmid-mediated cephalosporinase genes (e.g. *bla*<sub>CTX-M</sub>, *bla*<sub>CMY</sub>) and fluoroquinolones resistance genes (e.g. *qnr*, *aac(6)-Ib-cr*), but also through chromosomal mutations in the promoter region of the AmpC β-lactamase and in the quinolone resistance-determining region (QRDR) of the topoisomerases II (GyrA) and IV (ParC) [1,2]. Resistances to carbapenems have also emerged in *Escherichia coli* and *Klebsiella pneumoniae* [3]. Three main groups of carbapenemases are referenced according to the Ambler classification: class A (e.g. KPC), class B (IMP, VIM, NDM) and class D (e.g. OXA) [4]. Among the OXA carbapenemases, OXA-48-like ones have been described as phantom carbapenemases since they...
may be difficult to detect using phenotypic susceptibility testing [5].

Until now, animals were considered to play a minor role in the dissemination of OXA-48-like carbapenemases [6], but reports of sporadic infections in dogs caused by carbapenemase-producing (CP) Enterobacteriaceae in Europe [7,8], in the United States (US) [9] and in Northern Africa [10] suggest that companion animals may represent an unsuspected reservoir of carbapenem-resistant bacteria. Recently, hospitalised companion animals in Italy were found to acquire carbapenem-resistant bacteria such as Acinetobacter spp. [11]. Nonetheless, very little is known about carriage and dynamics of CPE in dogs and cats before and after hospitalisation.

**Figure 1**
Temporal acquisition and carriage of carbapenemase-producing *Escherichia coli* ST410, Switzerland, May–August 2018 (n = 24)

Results as detected at admission and discharge to a companion animal clinic, as well as once or twice within 175 days after hospitalisation.

Shapes in green: test negative for CP *E. coli* carriage; shapes in red: test positive for CP *E. coli* carriage. Blue dotted line: time line of screened animals; grey dashed line: animals which were not resampled.
Here, we characterised by whole genome sequencing (WGS) CP E. coli isolated from cats and dogs at admission and after hospitalisation to a large veterinary referral hospital in Switzerland, identifying their antimicrobial resistance genes (ARG) and mobile genetic elements (MGE) as well as the phylogenetic relationship among the isolates.

Methods

Study design and sampling
This study was part of a larger prospective study which included three large veterinary referral clinics and two smaller veterinary practices in Switzerland. The present prospective observational study was performed at a university referral hospital with a 24/7 emergency service. The hospital provides advanced medical care for 6,000 dogs and cats annually. Dogs and cats were included irrespective of their underlying problems and previous treatments if they were expected to be hospitalised for at least 48 h and the owner gave informed consent. All animals presented here were recruited via the emergency service of the hospital. During the enrolment period of 4 months (1 May–31 August 2018), ca 2,000 animals were treated at the hospital and 134 of them fulfilled the study criteria and were screened.

Rectal swabs were collected in awake animals at admission to the hospital and at discharge, using polyurethane foam culture swabs with Ames transport medium (BBL CultureSwabs). If available, animals positive at discharge were retested once or twice within 36 to 166 days after their discharge of the clinic.

Isolation and identification of the strains
Swabs were placed into 5 mL of Luria-Bertani (LB) broth for overnight enrichment at 37°C. A loopful of the culture was streaked onto CHROMID OXA-48

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dogs (n=17)</th>
<th>Cats (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age median in years (IQR)</td>
<td>7.0 (4.0–9.0)</td>
<td>4.5 (2.3–10.5)</td>
</tr>
<tr>
<td>Weight median in kg (IQR)</td>
<td>16.6 (7.8–34)</td>
<td>4.2 (3.0–5.7)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (entire/neutered)</td>
<td>9 (5/4)</td>
<td>2 (0/2)</td>
</tr>
<tr>
<td>Male (entire/neutered)</td>
<td>8 (6/2)</td>
<td>2 (1/1)</td>
</tr>
<tr>
<td>Diagnoses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal disease</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Neurological disease</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Urinary tract disease</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Hospitalisation days; median (IQR)</td>
<td>4 (3–5)</td>
<td>5 (1.8–8.3)</td>
</tr>
<tr>
<td>ICU days; median (IQR)</td>
<td>3 (1–5)</td>
<td>2.3 (1.5–8.6)</td>
</tr>
<tr>
<td>Antimicrobial pre-treatment*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Antimicrobial treatment during hospitalisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Antimicrobials used during hospitalisationb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Sulfamethoxazole/trimethoprim</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

ICU: intensive care unit; IQR: interquartile range.

* Antimicrobials used for pre-treatments (i.e. prior to presentation/referral) were amoxicillin/clavulanic acid (n = 2), amoxicillin (n = 1), enrofloxacin (n = 1) and metronidazole (n = 1).

b Includes mono- and combination therapy.
and CHROMID CARBA (Biomérieux SA, Marcy-l’Étoile, France) selective plates, which were incubated at 37°C for 24 h under aerobic conditions.

Colonies were sub-cultivated onto trypton soy agar plates containing 5% sheep blood (TSA-S) (Becton and Dickinson Company, Franklin Lakes, US) and identified to the species level by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics GmbH, Bremen, Germany).

Biochemical and molecular tests
Carbapenemase production was detected using the Blue-Carba test [12]. Before WGS, carbapenemase-producing isolates were tested for the presence of blaOXA-48-like genes by PCR [5].

**Antimicrobial susceptibility testing**
Minimal inhibitory concentrations (MIC) of 16 antimicrobials (ampicillin, cefepime, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, ertapenem, gentamicin, imipenem, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, tigecycline and trimethoprim) were determined by broth microdilution using Sensititre EUVSEC and EUVSEC2 plates (Thermo Fisher Scientific, Waltham, US) and following the guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [13]. MIC results were interpreted using the EUCAST criteria, except for nalidixic acid, sulfamethoxazole and tetracycline, for which criteria from the Clinical and Laboratory Standards Institute (CLSI) were used [14].
E. coli isolated using the DNeasy Blood and Tissue Kit. Total DNA from OXA-48-like-positive strains was subjected to whole genome sequencing and in silico analysis. The genome assembly of strain AR24.2b was performed by Unicycler (v0.4.4), using both long (ONT) and short reads (Illumina), as well as by CANU (v1.7) that only uses the ONT long reads. The obtained scaffolds were polished by read-mapping with paired-end Illumina reads using Pilon (v1.22) and with Geneious software v10.1.3 (Biomatters Ltd, Auckland, New Zealand). Annotation was performed with PROKKA v1.12. The paired-end reads of all CP isolates were mapped to the final annotated plasmid sequence. Online tools available at the Center for Genomic Epidemiology (Technical University of Denmark DTU, Lyngby, Denmark) were used to detect known ARG (ResFinder 3.0) to determine incompatibility (Inc) groups of the plasmids, (PlasmidFinder 2.0) and for multilocus sequence typing (MLST 2.0).
Transposable elements present in the plasmids were identified using ISfinder (https://isfinder.biotoul.fr/). Blast ring image generator (BRIG) was used to generate circular map comparisons of plasmids (based on BLASTn) [15]. Comparison with closely related plasmids from the National Center for Biotechnology Information (NCBI) was achieved using the NCBI tool BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed November 2018).

Phylogenetic analyses
An ad hoc core genome multilocus sequence typing (cgMLST) scheme was created using SeqSphere+ (v. 4.01, Rindom GmbH, Münster, Germany). The E. coli of sequence type (ST) 410 from a dog in the UK (GenBank accession number CP031653) was defined as the reference genome from which 4,177 coding regions of genes were extracted by the cgMLST Target Finder v1.4. The contigs obtained from SPAdes of all 24 CP E. coli in this study, together with the reference genome and with six other genomes of E. coli ST410 isolated from humans or from the environment and published in GenBank (GenBank accession numbers CP029630, CP027205, CP026473, CP024801, CP023899 and CP018965), were loaded and searched for the 4,177 gene targets using built-in BLAST. Overall, 3,778 genes were shared among all isolates, which we defined as the core genome. The visualisation of the phylogenetic distance was achieved using the allelic profile of all strains to generate a neighbour-joining tree using SeqSphere+ with the parameters ‘pairwise ignoring missing values; % columns difference’ for the distance calculation.

Ethical statement
The study protocol was approved by the national ethics committee (BE 16/18) and signed informed consent was obtained from the owners before enrolment of the animals in the study.

Results
Occurrence of carbapenemase-producing Escherichia coli
Rectal swabs were taken from 100 dogs and 34 cats at admission to a large veterinary referral clinic in Switzerland between 1 May and 31 August 2018. Carriage of CP E. coli was detected in only one dog and none of the cats at admission (0.75%; 95% confidence interval (CI): 0–2.2).

In contrast, 17 of 76 dogs and four of 21 cats that were resampled at discharge were found to harbour CP E. coli (21.6%; 95% C: 13.4–29.8) indicating nosocomial acquisition of the bacteria. The 37 remaining animals were not available for resampling (n = 22) or deceased (n = 15).
**Figure 3**
Circular maps of the resistance plasmids pAN-OXA-181 and pAN-CMY-42, Switzerland, May–August 2018 (n = 2)

The coloured outer rings indicate regions of homology of the pAN-OXA-181 (GenBank accession number MK416154) and pAN-CMY-42 (GenBank acc. no. MK416155) plasmid of strain AR24.2b with other plasmids, based on BLASTn.

Left panel: pAN-OXA-181. The rings from the inner to the outer represent plasmids pAMA1167-OXA-181 (GenBank acc. no. CP024806; 99.99% identity, 1 single nucleotide polymorphism (SNP)), pOXA181_14828 (GenBank acc. no. KP400525; 100% identity), pKS22-OXA-181 (GenBank acc. no. KT005457; 99.98%, 3 SNP), pEC31346-OXA-181 (GenBank acc. no. KX894452; 99.99% identity, 1 SNP), pSTIB (GenBank acc. no. MG570092; 99.99% identity, 1 SNP) and pEC21-OXA-181 (GenBank acc. no. MG893567; 99.99% identity, 1 SNP).

Left panel: pAN-CMY-42. The rings from the inner to the outer represent plasmids pCMY42_M217 (GenBank acc. no. AP019190; 99% identity, 65% coverage), pCMY42_AR_0137 (GenBank acc. no. CP021882; 100% identity, 68% coverage), pCARB35_03 (GenBank acc. no. CP031666; 100% identity, 79% coverage), pCMY2_WCHEC13–8 (GenBank acc. no. KP789015; 100% identity, 71% coverage), pV233-b (GenBank acc. no. LC056425; 99% identity, 46% coverage) and pIBAC_IncI1_CMY-42 (GenBank acc. no. KY463221; 100% identity, 78% coverage).

The coloured outer rings indicate regions of homology of the pAN-OXA-181 (GenBank accession number MK416154) and pAN-CMY-42 (GenBank acc. no. MK416155) plasmid of strain AR24.2b with other plasmids, based on BLASTn.

The scale circle shows the coordinates in kbp of each plasmid. Genes are portrayed as coloured blocks in the inner ring of each circular map. Genes are coloured according to their classification. Red: antimicrobial resistance genes; dark purple: surface disinfectants resistance genes; orange: transposases; fuchsia: genes implicated in replication; green: transfer-associated genes; brown: partition genes; grey: other genes. The Δumud and ΔarsR genes are coloured in darker grey, flanking the composite transposon of pAN-OXA-181. The blc gene of pAN-CMY-42 is indicated in the map to better represent the composition of the native 4 kb element.
The dog positive for CP *E. coli* at admission was not under antimicrobial treatment at arrival and had no previous history of hospitalisation at this clinic, but whether it was previously hospitalised in another clinic is not known. Nor was it the first animal tested positive during the study period. This dog was still positive at discharge (Figure 1).

The demographics, hospitalisation details and antimicrobial treatments of the CP *E. coli*-carrying animals are summarised in Table 1. Animals were presented to the emergency service of the hospital with a wide range of complaints. All cats and 13 of the 17 dogs spent time in the intensive care unit of the hospital. The majority of dogs (14/17) and cats (3/4) were treated with antimicrobials during their hospitalisation; however, none of them received carbapenems.

Follow-up samples were obtained within 36 to 101 days after discharge from 12 of 21 CP *E. coli*-positive animals. The other nine animals could not be retested because they had died (n=3), or because the owners withdrew their participation in the study (n=6). Two cats still carried CP *E. coli* 36 days and 99 days after discharge (Figure 1). Seven of the 12 animals that underwent a first follow-up could be retested for a second time within a time interval of 109 to 166 days after discharge of the clinic, including the two cats that were still positive after the first follow-up. None of them were found to be shedding CP *E. coli* at the second follow-up (Figure 1).

**Phylogenetic analyses**

All 24 CP *E. coli* detected in this study belonged to ST410. Analysis of the 24 strains using cgMLST confirmed that they were all highly related, all clustering in the same branch of the phylogenetic tree, in contrast to the sequences of seven other and independent *E. coli* ST410 from animal, environmental and human origin. The most closely related strain was a canine *E. coli* isolated in the UK in 2018, which carried the carbapenemase gene *bla* _KPC_ , the plasmid-mediated AmpC (*pAmpC*) cephalsporinase gene *bla* _CMY-42_ , and the β-lactamase gene *bla* _TEM-199_ [16] (GenBank accession number CP031653) (Figure 2).

**Antimicrobial resistance profile of the carbapenemase-producing *E. coli***

All CP *E. coli* exhibited the same antimicrobial resistance profile; they were non-susceptible to ampicillin, cefepime, cefotaxime, ceftazidime, ciprofloxacin, ertapenem and nalidixic acid, except for one isolate (AR144.2) which was susceptible to ceftazidime and resistant to tetracycline (Table 2).

All isolates carried the carbapenemase gene *bla* _OXA-181* which has been associated with a low level of resistance to carbapenems [4,17]. The isolates had MICs below the resistance breakpoints set by EUCAST for imipenem and meropenem but were resistant to ertapenem, with MICs above the resistance breakpoint (Table 2). All isolates also contained the _qnrS1_ gene encoding a DNA gyrase protection protein which confers low-level resistance to fluoroquinolones, as well as a serine to leucine substitution at position 83 and an aspartic acid to asparagine substitution at position 87 in GyrA, and a serine to isoleucine substitution at position 80 in ParC. These amino acid substitutions are known to confer high-level resistance to fluoroquinolones in *E. coli* [1].

The pAmpC cephalsporinase gene *bla* _CMY-42_ was also found in all isolates, except in the two isolates (AR89.2b and AR144.2) which were not resistant to cefepime and ceftazidime. The tetracycline resistance found only in AR144.2 was associated with the tetracycline efflux gene _tet(A)_ . This isolate was also the only one that contained the _bla_ _TEM-18_ β-lactamase gene (Table 3).

**Characterisation of the plasmids containing resistance genes**

The _bla_ _OXA-181_ and _qnrS1_ genes were co-localised on a 51 kb IncX3 plasmid (pAN-OXA-181) in strain AR24.2b (Figure 3). Plasmid mapping showed that all other 23 isolates also harboured this same plasmid. Plasmid pAN-OXA-181 was 99.9% similar to other IncX3 plasmids from human *E. coli* strains in China (pOXA181_14828 and pEC21-OXA-181 [18,19], Denmark (pPAM167-OXA-181) [20], and Lebanon (pSTIB) [21], as well as from a porcine *E. coli* strain in Germany (pEc31346-OXA-181) [22]. Plasmid pAN-OXA-181 differed by 3 SNPs from pKS22-OXA-181 which was detected in a *Klebsiella variicola* isolated from fresh vegetables imported into Switzerland [23] (Figure 3).

Both _bla_ _OXA-181_ and _qnrS1_ were found within a composite transposon in pAN-OXA-181, which was integrated between _umuD_ and _asrR_ , similarly to pOXA181_14828 [18]. The composite transposon was flanked by two copies of the insertion sequence IS26 which were oriented in the same direction. Two 50 bp inverted repeats IRl and IRR, each preceded by a 2 bp AC direct repeat, were found at both ends of the IS26-flanked composite transposon, which is indicative of a transposition event [19]. Plasmid pAN-OXA-181 also contained the replication gene _repB_ and a 23 kb transfer region containing the _virB1–2–4–5–6–8–9–10–11_ , _traG_ and _trmB_ genes (Figure 3).

Strain AR24.2b also contained a 47 kb IncI1 plasmid (pAN-CMY-42) which harboured the pAmpC gene _bla_ _CMY-42_ (Figure 3). The _bla_ _CMY-42_ gene is commonly found in plasmids within a 4 kb element composed by _ISEcp1_ , an outer membrane lipoprotein gene _b1c_ and a quaternary ammonium compound (QAC) resistance gene _sugE_ ( _ISEcp1 – bla_ _CMY-42 – b1c_ – _sugE_ ) [24]. In pAN-CMY-42 however, _ΔISEcp1_ of this element was split by the insertion of a copy of _ISe1_ , and subsequent homologous recombination with another copy of _ISe1_ led to its modified structure, as previously described [24]. This recombination is supported by the inversion of the insertion repeats (GATAA and TTATA) (Figure 3).
Plasmid pAN-CMY-42 also contains the replication gene repA and transfer genes traA, traE, traI, traJ, traY and trbA.

Plasmid pAN-CMY-42 differed from other CMY-42-containing IncI1 plasmids by its size and structure. The most closely related IncI1 CMY-42 plasmids deposited so far in GenBank were larger than pAN-CMY-42 by 1–50 kb, including plasmids isolated from canine E. coli in the UK (pCARB35_03) [16], human patients in Myanmar (pCMY42_M217) [25], China (pCMY42_WCHEC13–8) [24] and Italy (pIBAC_IncI1_CMY-42) [26], as well as from E. coli isolated from the environment (pV233-b) [27], and from an unknown source (pCMY42_AR_0137 (Figure 3).

Plasmid mapping of the other 23 E. coli showed that 21 isolates also contained this same plasmid (pAN-CMY-42), whereas the two isolates AR89.2 and AR144.2 lacked the 4 kb element (ISEcp1 – blaCMY-42 – blc – sugE) on their IncI1 plasmid.

Plasmids pAN-OXA-181, pAN-CMY-42 and the chromosome of E. coli strain AR24.2b have been deposited in GenBank under accession numbers MK416154, MK416155 and CP035944, respectively.

**Discussion**

Surveillance of enteral carriage of antimicrobial-resistant bacteria in dogs and cats before and after hospitalisation in a companion animal hospital in Switzerland revealed the potential role of small animal clinics as an underestimated hotspot for acquisition of CP E. coli. In the particular situation presented here, almost one quarter of the hospitalised animals acquired a specific carbapenem-, cephalosporin- and fluoroquinolone-resistant clone of ST410 within a time span of 1-9 days of hospitalisation. The only dog found to be carrier of this clone at admission was chronologically not the first to be found positive during the study period, indicating that it was not the primary source of this strain. It may, however, have contributed to further enhance dissemination of the clone into the hospital. Long-term carriage beyond 108 days was only documented in one cat among the 10 dogs and two cats that were followed up. Nevertheless, all 21 animals that returned home with CP E. coli posed a potential risk of disseminating hospital-acquired CP E. coli into the environment and possibly to other animals and humans (Figure 4).

Although the risk of CP Enterobacteriaceae transmission may be related to their relative abundance in faeces, our study was not set up to quantify the effective number of CP Enterobacteriaceae in faeces because it used enrichment and selective plates.

This figure shows the results of the screening of 134 animals at admission to the veterinary hospital and of 97 animals at discharge. It illustrates the potential risk for the environment, humans and other companion animals with regard to the carriage of CP E. coli by animals that have returned to their homes after having acquired CP E. coli during hospitalisation.
It is important to extend screening and detection methods in veterinary settings and diagnostic laboratories to detect such bacteria of medical and public health importance [4]. Detection of carbapenemases of the OXA-48 family may be challenging because of their low hydrolysing activity which specifies low MIC to carbapenems [4,5]. Application of low MIC screening values would allow identifying the OXA-181-producing E. coli isolates in our study since they exhibited MICs higher than the resistance breakpoint of >0.5 μg/mL for ertapenem and higher than the meropenem screening cut-off value of >0.125 μg/mL recommended by EUCAST [13].

Phylogenetic analyses of the CP E. coli using 3,778 genes of the core genome (cgMLST) confirmed high genetic relationship shared among all the E. coli isolates of ST410 from this study. This sequence type has recently been reported as a new emerging international high-risk clone, non-susceptible to critically important antibiotics such as fluoroquinolones, third-generation cephalosporins and carbapenems and with the potential of cross-sectorial transmission between wildlife, humans, pets and the environment [28,29]. Escherichia coli ST410 harbouring blaOXA-181 was first described in human infections in China in 2015 [18], then in Denmark in 2017 [20] and in Italy in 2018 [30], but so far it has not been reported in humans in Switzerland. These previously described human E. coli ST410 producing OXA-181 clustered into other branches of the cgMLST phylogenetic tree than the strain isolated in our study, indicating an independent origin. Another CP E. coli ST410 was identified in a dog in the UK in 2018 [16], GenBank accession number CP031653), emphasising the potential of ST410 for dissemination in the veterinary setting. This canine E. coli ST410 from the UK exhibited a slightly different cgMLST clustering and contained NDM-5 as carbapenemase rather than OXA-181. Although the two canine E. coli ST410 from the UK and Switzerland contained CMY-42 Inc1 plasmids which were highly similar, the one from Switzerland was lacking a 12 kb region compared with the CMY-42 Inc1 plasmid of the canine E. coli from the UK.

The present study revealed for the first time an OXA-181-producing E. coli ST410 associated with hospitalisation of companion animals. Carriage of carbapenemase-producing bacteria has so far not been reported among animals in Switzerland, but one study already reported OXA-181 in K. variicola isolated from imported fresh vegetables [23]. The presence of the same plasmid containing blaOXA-181 (pAN-OXA-181) in Enterobacteriaceae from companion animals, humans and vegetables suggest that interspecies transmission of this plasmid between E. coli and other Enterobacteriaceae is very likely. Nevertheless, the origin of the E. coli ST410 containing the carbapenemase plasmid pAN-OXA-181 in the veterinary setting is intriguing. Carbapenems are not used in this clinic and are therefore not likely to be the driving force for the selection and maintenance of this carbapenem-resistant E. coli ST410. The use of fluoroquinolones and β-lactams (Table 1) may have contributed to the selection of this ST410 clone or its plasmids, since the isolates also exhibited resistance to these classes of antimicrobials. That more animals were carriers of the CP ST410 clone at discharge than at admission as well as the high genetic relatedness of all isolates (including the identical plasmids) strongly indicate a common source of contamination within the hospital. Nevertheless, no cases of infection caused by an OXA-181-producing E. coli were revealed in dogs and cats during the 7 months following the outbreak using the same selective agar plates as those used in this study at the diagnostic unit of our institute. Of note, this referral hospital faced several cases of infections caused by a nosocomial clone of third-generation-cephalosporin-resistant K. pneumoniae ST11 producing the pAmpC DHA in 2010–2013 [31].

As an infection control measure, thorough disinfection of the hospital environment was performed after this outbreak in 2018 using disinfectant without QAC since ST410 also contained a QAC resistance gene (sugE) on the cephalosporinase plasmid pAN-CMY-42. Furthermore, stringent hospital hygiene and environmental cleaning protocols were introduced and a staff screening campaign was initiated. Staff training was also intensified to improve hand and environmental hygiene. The results and the impact of these measures are still being analysed and will be part of a follow-up study. Although no conclusion can be drawn at this stage of the study on the impact of possible colonisation of the staff or the pet owners, the results indicate that companion animals probably disseminated CP Enterobacteriaceae via faeces in the environment after hospitalisation and may represent a potential risk for transmission of CP Enterobacteriaceae to other animals and humans in the community and a serious One Health concern.

This outbreak stresses the need for national routine monitoring of carbapenemase-producing bacteria in companion animals using a representative sampling strategy analogous to the national surveillance of antibiotic resistance in food-producing animals at slaughterhouses in Switzerland and the European Union [32]. Based on the results of our study, we strongly recommend monitoring hospitalised companion animals for CP Enterobacteriaceae carriage at discharge using a sampling strategy representative of the number of hospitalised animals in the enrolled clinics. Such a surveillance programme should identify high-risk companion animal clinics and improve infection prevention and control as well as hygiene standards.

**Conclusion**

This study provided evidences that dogs and cats can acquire CP Enterobacteriaceae within a short time of hospitalisation. Even if the colonisation of the animals did not last longer than 40 days in the majority of the animals, shedding of CP Enterobacteriaceae after
hospitalisation represents a public health and environmental concern. Early detection and nationwide surveillance of carbapenemase-producing isolates, as well as effective infection prevention and control measures, need to be implemented in veterinary settings to limit the spread of high-risk clones in animals, humans and the environment.

Acknowledgements
This study was financed by the Swiss Federal Food Safety and Veterinary Office (FSVO) Grant no. 18.10 “Prevention and identification of critical points for transmission of multidrug-resistant bacteria in small animal clinics - towards evidence-based guidelines for infection prevention and control” to SS, BW, SGB, AE and VP, and by the Swiss National Science Foundation (SNSF grant no. 177378 within the National Research Programme NRPP “Antimicrobial Resistance” to AE and VP). We are grateful to all participating owners who kindly made the sampling of their animals possible.

Conflict of interest
None declared.

Authors’ contributions
SS, BW, SGB, AE and VP contributed to the design of the study. BD sampled the animals. AN and AC isolated and identified the strains. SS and BD collected and analyzed the clinical data. AN and MB performed experiments and genomic analysis. MB and MC performed MinIon sequencing. AN, MB and VP interpreted bacteriological and molecular data, and AN and VP wrote the manuscript. All authors reviewed the manuscript.

References


License, supplementary material and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence and indicate if changes were made.

Any supplementary material referenced in the article can be found in the online version.

This article is copyright of the authors or their affiliated institutions, 2019.
New HIV-1 circulating recombinant form 94: from phylogenetic detection of a large transmission cluster to prevention in the age of geosocial-networking apps in France, 2013 to 2017

Marc Wirden¹, Fabienne De Oliveira², Magali Bouvier-Alias³, Sidonie Lambert-Nicolot⁴, Marie-Laure Chaix⁵, Stéphanie Raymond⁶, Ali Si-Mohammed⁷, Chakib Alloui⁸, Elisabeth André-Garnier⁹, Pantxika Bellecave¹⁰, Brice Malve¹¹, Audrey Mirand¹², Coralie Pallier¹³, Jean-Dominique Poveda¹⁴, Theresa Rabenja¹⁵, Veronique Schneider¹⁶, Anne Signori-Schmuck¹⁷, Karl Stefic¹⁸, Vincent Calvez¹⁹, Diane Descamps²⁰, Jean-Christophe Plantier²¹, Anne-Genevieve Marcelin²², Benoit Vissieux²³, on behalf of the French National Agency for Research on AIDS and Viral Hepatitis (ANRS) AC43 Study Group²⁴

1. Sorbonne Université, INSERM, Institut Pierre Louis d’Épidémiologie et de Santé Publique IPLESP, AP-HP, Hôpital Pitié Salpêtrière, Laboratoire de virologie, Paris, France
2. Normandie Université, UNIROUEN, EA2656 GRAM, CHU de Rouen, Laboratoire de virologie associé au CNR VIH, Rouen, France
3. HU Henri Mondor, Laboratoire de virologie, Créteil, France
4. AP-HP, Hôpital Saint-Antoine, Laboratoire de virologie, Paris, France
5. AP-HP, Hôpital Saint-Louis, Laboratoire de virologie, INSERM U944, Paris, France
6. Laboratoire de virologie, CHU Purpan de Toulouse, Toulouse, France
7. Laboratoire de virologie CHU de Dijon, Dijon, France
8. Laboratoire de virologie, Hôpital Avicenne, Bobigny, France
9. Laboratoire de virologie, CHU Hôtel Dieu, Nantes, France
10. Laboratoire de virologie CHU de Bordeaux, Bordeaux, France
11. Laboratoire de virologie CHU de Nancy, Nancy, France
12. Laboratoire de virologie CHU de Clermont-Ferrand, Clermont-Ferrand, France
13. Laboratoire de virologie, Hôpital P. Brousse, Villejuif, France
14. Laboratoire CERBA, Saint-Ouen-l’Aumône, France
15. Laboratoire du Grand Hôpital de l’Est Francilien, Jossigny, France
16. Laboratoire de virologie, Hôpital Tenon, Paris, France
17. Laboratoire de virologie CHU de Grenoble, Grenoble, France
18. Laboratoire de virologie CHU de Tours, Tours, France
19. Laboratoire de virologie, AP-HP, Hôpital Bichat Claude Bernard, Univ Paris-Diderot, INSERM, IAME, CRN VIH, Paris, France
20. Members are listed in the acknowledgements

Correspondence: Marc Wirden (marc.wirden@psl.aphp.fr)


Background: Ending the HIV pandemic must involve new tools to rapidly identify and control local outbreaks and prevent the emergence of recombinant strains with epidemiological advantages. Aim: This observational study aimed to investigate in France a cluster of HIV-1 cases related to a new circulating recombinant form (CRF). The confirmation this CRF’s novelty as well as measures to control its spread are presented. Methods: Phylogenetic analyses of HIV sequences routinely generated for drug resistance genotyping before 2018 in French laboratories were employed to detect the transmission chain. The CRF involved was characterised by almost full-length viral sequencing for six cases. Cases’ clinical data were reviewed. Where possible, epidemiological information was collected with a questionnaire. Results: The transmission cluster comprised 49 cases, mostly diagnosed in 2016–2017 (n=37). All were infected with a new CRF, CRF94_cpx. The molecular proximity of this CRF to X4 strains and the high median viraemia, exceeding 5.0 log₁₀ copies/mL, at diagnosis, even in chronic infection, raise concerns of enhanced virulence. Overall, 41 cases were diagnosed in the Ile-de-France region and 45 were men who have sex with men. Among 24 cases with available information, 20 reported finding partners through a geosocial networking app. Prevention activities in the area and population affected were undertaken. Conclusion: We advocate the systematic use of routinely generated HIV molecular data by a dedicated reactive network, to improve and accelerate targeted prevention interventions. Geosocial networking apps can play a role in the spread of outbreaks, but could also deliver local targeted preventive alerts.
**Figure 1**

Phylogenetic tree obtained from the sequences of the HIV-1 protease plus reverse transcriptase regions, which were collected during the investigation of a transmission cluster, France, 2013–2017 (n = 49)

<table>
<thead>
<tr>
<th>Patients</th>
<th>Year of diagnosis</th>
<th>Risk group</th>
<th>Stage of infection</th>
<th>HIV viral load log 10 copies/mL</th>
<th>CD4 cells count/mm³</th>
<th>V3 gene pattern</th>
<th>HIV tropism</th>
<th>False positive rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>D31</td>
<td>2016</td>
<td>MSM</td>
<td>&lt; 1 year</td>
<td>4.23</td>
<td>658</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D30</td>
<td>2017</td>
<td>MSM</td>
<td>&lt; 1 year</td>
<td>6.41</td>
<td>742</td>
<td>2M/25Q</td>
<td>8.1 (X4)</td>
<td></td>
</tr>
<tr>
<td>D29</td>
<td>2016</td>
<td>MSM</td>
<td>Primary</td>
<td>6.05</td>
<td>349</td>
<td>2M/25E</td>
<td>8.5 (X4)</td>
<td></td>
</tr>
<tr>
<td>D28</td>
<td>2017</td>
<td>MSM</td>
<td>Chronic</td>
<td>3.38</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D27</td>
<td>2016</td>
<td>MSM</td>
<td>Primary</td>
<td>&gt;7.00</td>
<td>298</td>
<td>2M/25E</td>
<td>8.5 (X4)</td>
<td></td>
</tr>
<tr>
<td>D26</td>
<td>2016</td>
<td>MSM</td>
<td>Primary</td>
<td>6.31</td>
<td>320</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D25</td>
<td>2016</td>
<td>MSM</td>
<td>Primary</td>
<td>5.18</td>
<td>740</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D24</td>
<td>2016</td>
<td>MSM</td>
<td>Chronic</td>
<td>5.42</td>
<td>538</td>
<td>2M/25Q</td>
<td>7.8 (X4)</td>
<td></td>
</tr>
<tr>
<td>D23</td>
<td>2016</td>
<td>Primary</td>
<td>&gt;7.00</td>
<td>793</td>
<td>2M/25E</td>
<td>8.5 (X4)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D22</td>
<td>2016</td>
<td>MSM</td>
<td>Primary</td>
<td>4.88</td>
<td>550</td>
<td>2M/25E</td>
<td>8.5 (X4)</td>
<td></td>
</tr>
<tr>
<td>D21</td>
<td>2016</td>
<td>MSM</td>
<td>&lt; 1 year</td>
<td>5.65</td>
<td>215</td>
<td>2M/25E</td>
<td>8.1 (X4)</td>
<td></td>
</tr>
<tr>
<td>D20</td>
<td>2017</td>
<td>MSM</td>
<td>Advanced</td>
<td>6.44</td>
<td>23</td>
<td>2M/25E</td>
<td>1.7 (X4)</td>
<td></td>
</tr>
<tr>
<td>D19</td>
<td>2016</td>
<td>MSM</td>
<td>Chronic</td>
<td>5.68</td>
<td>374</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D18</td>
<td>2016</td>
<td>MSM</td>
<td>Chronic</td>
<td>5.71</td>
<td>540</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D17</td>
<td>2017</td>
<td>MSM</td>
<td>Primary</td>
<td>6.19</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D16</td>
<td>2016</td>
<td>MSM</td>
<td>Primary</td>
<td>5.78</td>
<td>252</td>
<td>2M/25E</td>
<td>8.5 (X4)</td>
<td></td>
</tr>
<tr>
<td>D15</td>
<td>2017</td>
<td>MSM</td>
<td>Primary</td>
<td>6.90</td>
<td>561</td>
<td>2M/25E</td>
<td>8.5 (X4)</td>
<td></td>
</tr>
<tr>
<td>D14</td>
<td>2016</td>
<td>MSM</td>
<td>Primary</td>
<td>5.42</td>
<td>535</td>
<td>2M/25E</td>
<td>8.5 (X4)</td>
<td></td>
</tr>
<tr>
<td>D13</td>
<td>2017</td>
<td>MSM</td>
<td>Chronic</td>
<td>4.77</td>
<td>358</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D12</td>
<td>2017</td>
<td>MSM</td>
<td>Primary</td>
<td>5.81</td>
<td>402</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D11</td>
<td>2017</td>
<td>MSM</td>
<td>Primary</td>
<td>4.04</td>
<td>647</td>
<td>2M/25Q</td>
<td>7.8 (X4)</td>
<td></td>
</tr>
<tr>
<td>D10</td>
<td>2017</td>
<td>MSM</td>
<td>&lt; 1 year</td>
<td>5.97</td>
<td>190</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D9</td>
<td>2017</td>
<td>MSM</td>
<td>&lt; 1 year</td>
<td>5.60</td>
<td>607</td>
<td>2M/25E</td>
<td>8.5 (X4)</td>
<td></td>
</tr>
<tr>
<td>D8</td>
<td>2016</td>
<td>MSM</td>
<td>Primary</td>
<td>5.92</td>
<td>201</td>
<td>2M/25E</td>
<td>8.5 (X4)</td>
<td></td>
</tr>
<tr>
<td>D7</td>
<td>2017</td>
<td>MSM</td>
<td>Primary</td>
<td>5.72</td>
<td>551</td>
<td>2M/25K</td>
<td>2.8 (X4)</td>
<td></td>
</tr>
<tr>
<td>D6</td>
<td>2017</td>
<td>MSM</td>
<td>Primary</td>
<td>4.89</td>
<td>333</td>
<td>2M/25K</td>
<td>2.8 (X4)</td>
<td></td>
</tr>
<tr>
<td>D5</td>
<td>2017</td>
<td>MSM</td>
<td>&lt; 1 year</td>
<td>5.86</td>
<td>110</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D4</td>
<td>2016</td>
<td>MSM</td>
<td>&lt; 1 year</td>
<td>3.90</td>
<td>236</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>2017</td>
<td>MSM</td>
<td>Chronic</td>
<td>5.39</td>
<td>538</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>2016</td>
<td>MSM</td>
<td>Chronic</td>
<td>5.51</td>
<td>384</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>2016</td>
<td>MSM</td>
<td>Chronic</td>
<td>4.99</td>
<td>533</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>2016</td>
<td>MSM</td>
<td>&lt; 1 year</td>
<td>6.53</td>
<td>52</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>2015</td>
<td>MSM</td>
<td>Primary</td>
<td>5.20</td>
<td>1,081</td>
<td>2M/25K</td>
<td>2.8 (X4)</td>
<td></td>
</tr>
<tr>
<td>C2 w</td>
<td>2015</td>
<td>Hetero.</td>
<td>Chronic</td>
<td>5.07</td>
<td>533</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>2016</td>
<td>MSM</td>
<td>Advanced</td>
<td>6.00</td>
<td>46</td>
<td>2M/25K</td>
<td>1.7 (X4)</td>
<td></td>
</tr>
<tr>
<td>B10</td>
<td>2017</td>
<td>MSM</td>
<td>Chronic</td>
<td>5.32</td>
<td>113</td>
<td>2/25K</td>
<td>10.5 (R5)</td>
<td></td>
</tr>
<tr>
<td>B9</td>
<td>2015</td>
<td>MSM</td>
<td>Chronic</td>
<td>5.20</td>
<td>406</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>B8</td>
<td>2015</td>
<td>Primary</td>
<td>6.85</td>
<td>282</td>
<td>2I/25E (R5)</td>
<td>60 (R5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7</td>
<td>2014</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>2015</td>
<td>MSM</td>
<td>&lt; 1 year</td>
<td>5.16</td>
<td>371</td>
<td>2I/25K (R5)</td>
<td>10.5 (R5)</td>
<td></td>
</tr>
<tr>
<td>B5</td>
<td>2015</td>
<td>Primary</td>
<td>4.78</td>
<td>543</td>
<td>2I/25K (R5)</td>
<td>10.5 (R5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td>2016</td>
<td>MSM</td>
<td>&lt; 1 year</td>
<td>5.23</td>
<td>190</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>2016</td>
<td>MSM</td>
<td>Chronic</td>
<td>5.06</td>
<td>259</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>2016</td>
<td>NA</td>
<td>Hetero.</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>2016</td>
<td>MSM</td>
<td>Chronic</td>
<td>4.52</td>
<td>370</td>
<td>2M/25E</td>
<td>9.0 (X4)</td>
<td></td>
</tr>
<tr>
<td>A9</td>
<td>2015</td>
<td>MSM</td>
<td>Primary</td>
<td>5.64</td>
<td>407</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>2013</td>
<td>MSM</td>
<td>Chronic</td>
<td>4.73</td>
<td>231</td>
<td>2I/25E</td>
<td>60 (R5)</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>2013</td>
<td>Hetero.</td>
<td>Chronic</td>
<td>2.94</td>
<td>578</td>
<td>2I/25E</td>
<td>56 (R5)</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>2015</td>
<td>MSM</td>
<td>Chronic</td>
<td>4.83</td>
<td>550</td>
<td>2I/25E</td>
<td>83 (R5)</td>
<td></td>
</tr>
</tbody>
</table>

Hetero.: heterosexual; MSM: men who have sex with men; NA: not available; w: women.

* Direct or indirect link to a specific company.

a The 11/25 rule would predict an X4 strain.

Bootstrap values ≥ 80% defined four sub-clusters, A, B, C and D. Colours (reused in Figure 3) indicate the same year of diagnosis. Some parameters at the time of diagnosis are reported. The amino acids in positions 2 and 25 of the V3 protein are shown, when known.

In the last column, the false positive rate and the tropism in parentheses are depicted according to the output of the Geno2pheno algorithm (with a threshold of 10%) which reported also the result of the 11/25 rule when this one predicted an X4 tropism (footnote b).

The scale bar indicates the length of horizontal branches corresponding to a genetic distance of 0.003 substitution per nucleotides.
Introduction
In 2014, a '90-90-90' target was proposed by UNAIDS to help end the global acquired immunodeficiency syndrome (AIDS) epidemic by 2020 [1,2]. According to this target, 90% of infected people must be diagnosed, 90% of diagnosed people must be on antiretroviral treatment, and 90% of treated patients must display viral suppression. In countries of western Europe, highly efficient antiretroviral drugs, easy access to care, and the 'treat all' approach have made it possible to attain or get close to the last two of these objectives. Nevertheless, the incidence of HIV-1 infection has not decreased sufficiently, particularly among men who have sex with men (MSM) [3,4]. Screening for infection and upstream prevention are the weak links in the chain. In France, self-testing kits for HIV infection are now available over-the-counter and pre-exposure prophylaxis (PrEP) is supported by the healthcare system [5-7].

Recent advances in molecular epidemiology could facilitate the identification of transmission clusters and outbreaks, as well as the initiation of rapid and focused preventive activities to limit HIV spread, but their routine use is not easy [8-12]. Furthermore, the sustained HIV epidemic may allow new circulating recombinant forms (CRF) to emerge, particularly if different HIV subtypes are present, as in Europe [10,13-16]. Such genetic recombination may lead to the selection of more virulent HIV strains in the infected population. This study, reporting the discovery of a recent highly active transmission cluster, with factors boosting the spread of a new HIV recombinant, illustrates all these points.

The objectives of this work were to confirm the novelty of the recombinant and to estimate the extent of this cluster in France, as well as its origins and transmission pathways. Targeted prevention activities guided by results of phylogenetic and epidemiological data analyses to control the spread of this new HIV-1 strain were attempted.

Methods
Detection of a transmission cluster
Between 2015 and 2017, the Pitié Salpêtrière Hospital laboratory received 10 plasma samples for routine HIV-1 resistance genotyping — recommended at time of diagnosis — harbouring virus strains with identical subtyping discordances on analysis with the Los Alamos laboratory subtyping tools (https://www.hiv.lanl.gov/content/sequence/BASIC_BLAST/basic_blast.html). While the protease (PR) and integrase (INT) genes in the sequences derived from those samples, exhibited perfect matches with the CRF02_AG references, the reverse transcriptase (RT) gene matched the subtype B references. Moreover, the Basic Local Alignment Search Tool (BLAST) revealed high levels of similarity between the 10 strains: 100% for PR nt sequences, and 100 to 99.6% for the RT and INT sequences. These observations were suggestive of an active transmission cluster involving a new CRF of HIV-1 combining both the subtype B, the major HIV lineage in countries of western Europe but absent from Africa, and the CRF02_AG associating subtypes A and G, one of the oldest HIV recombinant lineage, which is mostly present in West Africa and is the main non-B lineage in France.

Study population
In September 2017, to verify the hypothesis of a cluster related to a new CRF, a prototype set of sequences was sent to all laboratories participating to the Agence Nationale de Recherche sur le SIDA et les Hépatites Virales (ANRS) AC43 resistance study group, nationwide, for testing and identification of similar strains. All available HIV-1 nt sequences displaying the same subtyping discordances as the reference set were collected, prospectively and retrospectively, until December 2017. Patients were included in the study if these similarities were confirmed by phylogenetic analyses, as described below.

Clinical data, sex, age, together with HIV-1 viral loads and CD4 cell counts, were collected at diagnosis and after treatment initiation. Where possible, epidemiological information was collected with an anonymous questionnaire and the patient’s consent. The questions included the date of the last HIV negative test, the probable transmission pathway, the place of residence and where infection probably occurred, the circumstances of dating (casual or regular partner or stranger, with dating apps, or cruising area or other), and the knowledge of PrEP. Because a specific company with clearly identified activities had been frequently and spontaneously reported by the first patients, we also asked if the patients, or their partners, or the place where infection may have occurred, had a link with any company with such activities in the suburbs of Paris.

Ethical statement
The study was based on routine patient follow-up biological data and did not involve any additional sampling. Ethical approval was not needed for this study.

HIV resistance genotyping and tropism determination
In the ANRS AC43 study group laboratories, the PR, RT, INT and V3 env genes had been routinely sequenced with the ViroSeq HIV-1 Genotyping System (Applied Biosystems, Foster City, California (CA)), and/or the ANRS method (http://www.hivfrenchresistance.org), depending on the laboratory and the amplification failures encountered. HIV tropism, C-C chemokine receptors type 5, CCR5 (R5) or C-X-C chemokine receptors type 4, CXCR4 (X4), had been determined with V3-loop sequencing and the Geno2pheno algorithm, with a false-positive rate (FPR) threshold of 10% (https://coreceptor.geno2pheno.org/ ). A recombinant virus phenotypic entry test result was also available for three patients [17].
Characterisation of the circulating recombinant form 94 composed of subtype B, CRF02_AG and F2 fragments with recombination breakpoints, France, 2013–2017

**Figure 2**

A. Bootscan analysis results with subtype references CRF02_AG (AB485636), B (K03455) and F2 (KU749422)

B. Identified breakpoints positions according to HXB2 subtype B reference

C. The origin of each fragment was confirmed by maximum likelihood phylogenetic analysis

LTR: long terminal repeat; vif: virion infectivity factor; vpr: virion-associated protein; vpu: viral protein U.

The percentages on the trees presented in panel 2C indicate the branch support of the node including the CRF94 sequences with the corresponding HIV subtype (ultrafast bootstrap).
Phylogenetic and epidemiological investigation of the new circulating recombinant form cluster

For confirmation that all the collected viral strains belonged to the same cluster, the PR+RT and INT sequences were aligned with all the HIV-1 group M subtype and CRF reference sequences from the Los Alamos National database (http://www.hiv-web.lanl.gov; n = 181). The seven previously described unique recombinant forms including both subtype B and CRF02_AG sequences were also included in the comparison [18]. Phylogenetic trees were constructed by maximum likelihood methods with IQ Tree 1.6.1 under the General Time Reversible with Gamma distribution of rates across sites (GTR-G) nt substitution model, with ultrafast bootstrapping and 1,000 replicates. This phylogenetic analysis was combined with temporal and geographical information to improve the characterisation of transmission within this cluster.

Analysis of the new circulating recombinant form

Sanger sequencing of seven overlapping fragments, as previously described, enabled to recover near-to-complete genome sequences for six plasma samples [19]. The samples were respectively obtained from six patients who were not directly linked epidemiologically and belonged to the four main sub-clusters of the PR+RT phylogenetic tree.

The six sequences were aligned with all full-length genome group M reference sequences of the Los Alamos database, for determination of the recombination breakpoints. This alignment was analysed with the Recombination Detection Programme (RDP) version 4.95. For each fragment identified, the corresponding lineages were confirmed by maximum likelihood phylogenetic reconstruction with IQ Tree 1.6.1, as described above. The six near-complete genome sequences were deposited in the GenBank database (accession numbers MH141491 to MH141494 and MH683549, MH683550).

To check the novelty of the identified recombination pattern, it was compared with all previously described CRFs and, more particularly to the CRF56_cpx, the only one containing both subtype B and CRF02_AG ancestors. All previously described unique recombinant forms (URF) containing both CRF02_AG and subtype B ancestors were also extensively retrieved from the scientific literature to verify any potential previous identification [13,18].

Results

Patient characteristics

A total of 49 patients were included in the study (Figure 1). Their infection was diagnosed in 2013 (n = 2), 2014 (n = 1), 2015 (n = 8), 2016 (n = 22), and 2017 (n = 15), with year of diagnosis unknown for one patient. All but one of the patients were male, and all were Caucasians of French origin. Median age was 35 years (interquartile range (IQR): 28–43 years), and 41 of the 49 patients were diagnosed in the Île-de-France region. The transmission route was MSM for 45 patients, heterosexual for three, and unknown for one. The reasons for HIV testing were clinical symptoms of primary infection (n = 19), systematic HIV testing (n = 12), other sexually transmitted infections (n = 8), regular partner testing positive for HIV (n = 3), or not reported (n = 7). HIV-1 infection was recent in 29 patients, including 19 with primary infection and 10 with a negative test result within the preceding year. In the 24 cases for which information was available, risky sexual behaviour was reported during the probable infection period, with strangers (20/24), and/or acquaintances (12/24), and/or a regular partner (5/24). Casual partners were mostly encountered through a geosocial networking app (20/24), and/or in gay cruising areas (7/24). None of the patients was on PrEP, and 18/24 were unaware of this preventive method at the time of diagnosis.

Biological parameters and treatment outcomes

For non-primary and primary infections, respectively, the median HIV-1 load at diagnosis was 5.28 (IQR: 4.80–5.89) and 5.78 (IQR: 5.19–6.28) log10 copies/mL, and the median CD4 cell count was 371 (IQR: 196–539) and 407 (IQR: 303–558) /mm3. No drug resistance mutations other than those in the genetic sequence leading to the classical CRF02_AG polymorphic L10I, K20I and M36I mutations of the PR protein were identified among the 49 samples. The ViroSeq genotyping system, which is routinely used in many laboratories, failed to amplify the PR and RT genes in seven of 11 cases, even with viraemia above 5.0 log10 copies/mL. In these cases, the ANRS method was used as second test to obtain the data.

HIV tropism was determined for 26 patients. V3 amino-acid (AA) sequences displayed five different patterns, according to the residues in positions 2 and 25. These two positions drove major FPR changes, leading to a change in tropism from R5 strains in patients diagnosed in 2013, to X4 strains in those diagnosed in 2016 and 2017 (Figure 1). The two patients (C1 and D20) diagnosed at an advanced stage with fewer than 50 CD4+ T-cells/mm3, harboured a mixture of the two X4 patterns (2M/25K and 2M/25E). The recombinant phenotype tests yielded conflicting results relative to those of the Geno2pheno algorithm, with dual R5/X4 usage for D20 who harboured the two X4 patterns and only R5 coreceptor usage for patients C3 (2M/25K) and D27 (2M/25E).

HIV viral loads after 6 and 12 months of treatment were available for 36 and 31 patients, respectively. Before treatment, median viraemia was 5.42 log10 copies/mL (IQR: 5.04–5.94), and CD4 cell count was 371/mm3 (IQR: 235–550). The first combined antiretroviral therapy (cART) received systematically consisted of two nucleoside reverse transcriptase inhibitors (NRTIs), www.eurosurveillance.org
plus an integrase inhibitor (for 21 of the 36 patients), a protease inhibitor (13 of 36 patients), or a non-NRTI (2 of 36 patients). After 6 and 12 months of treatment, 27 and 25 of these patients, respectively, had a viraemia below 50 copies/mL, with median CD4 counts of 634/mm³ (IQR: 478–950) and 777/mm³ (IQR: 498–900), respectively. Thus, six of 31 patients did not reach the therapeutic goal after 12 months on cART, but their viraemia was < 100 copies/mL except for patient D20 (550 copies/mL and without resistance mutations).

Before treatment, the median viraemia and CD4 cell count of these six individuals were 5.82 log₁₀ copies/mL and 478/mm³, respectively. Only two of them were in primary infection at baseline.

Characteristics of the new circulating recombinant form

The full-length genome analysis, confirmed the discovery of a new CRF, CRF94_cpx, composed of subtype B, CRF02_AG and F2 segments (Figure 2). All B/CRF02_AG identified recombination breakpoints were identified with more than six of the nine methods included in RDP 4.95. The F2 fragment was identified only with RDP and bootscan methods. However, the Los Alamos BLAST tools and the phylogenetic trees reconstructed for the corresponding genome fragment confirmed the strong sequence similarity between the CRF94_cpx strains and the F2 lineage. This CRF is the first containing a fragment of this rare sub-subtype F2 (belonging to subtype F), originally described in Cameroon according to the Los Alamos laboratory database (https://www.hiv.lanl.gov/components/sequence/HIV/search/search.html), to be reported.

Transmission cluster evolution in the context of geographical data

The PR+RT phylogenetic analysis showed that all strains formed a single recent transmission cluster (RTC) defined as a monophyletic clade with a branch support value > 95% (98%) and a maximum genetic distance < 4.5% (3.4%). Based on an analysis of time to most recent common ancestor (tMRCA), this RTC was estimated to have emerged in July 2012. In the phylogenetic tree, this RTC is divided into four distinct sub-clusters named A, B, C and D, with respective nodes supported by bootstrap values ≥ 80% (Figure 1). Interestingly, the patients diagnosed in 2013–2015 were all included in the first three sub-clusters (A–C), whereas the last sub-cluster (D) harboured only the patients diagnosed in 2016–2017. Moreover, the sub-clusters also had different geographical distributions (Figure 3), as patients from sub-clusters A and B resided in an area centred on the Val-de-Marne, whereas the patients in sub-cluster D were mostly from the north of the Seine-et-Marne or more distant French regions. We found that at least 14 of the 31 patients in sub-cluster D had links to a single company in north of Seine-et-Marne, having worked for this company or engaged in risky sexual behaviour with employees of the company during the likely period of their infection. Eight patients were diagnosed outside Ile-de-France. One (C1) of these had moved from the area concerned, and three others (D8, D14 and D15) were seasonal workers in the company in the month preceding primary infection (Figure 3). No information was available for the four other individuals.
These findings made it possible to identify a precise area and population playing a major role in this outbreak and its sudden acceleration since 2016.

Outbreak control measures
French preventive care organisations, and local healthcare organisations in the north of Seine-et-Marne, as well as associations involved in HIV prevention were informed of our observations. The local organisations were already aware of the existence of a large gay community working for this company and living in this area, displaying high-risk sexual behaviours. Preventive activities had been implemented within this population in the past, but had been stopped for several years. The discovery of this very active transmission cluster was the trigger for new targeted preventive activities in and around the company, and the places of residence of its staff, as well as in the gay cruising areas known by the local associations. These activities began in 2018 and consisted of HIV screening days, the promotion of condom use, as well as the distribution of information about post- and pre-exposure prophylaxis. As commonly done, the HIV screening days were broadcasted through one of the gay dating apps, but without any specific warning regarding this outbreak.

Discussion
We show here that, even in a setting in which HIV prevention and treatments are widely available, important HIV transmission chains can still emerge. These transmission chains may lead to the selection of new recombinant strains with a potentially greater capacity to spread, and new tools are required to detect them early and to optimise prevention of their spread.

The cluster identified was exceptional in its size, comprising 49 patients, two-thirds of whom were diagnosed, and probably infected, within a two-year period (2016–17). A recent retrospective study including only patients infected for less than 6 months but diagnosed over a period of 15 years, resulted in the finding of 44 French clusters, with a median of only four individuals (range: 3–41) per cluster [10]. The real number of people involved in the CRF94_cpx outbreak is undoubtedly higher, as some infected individuals may not yet have been tested for HIV, or may have been diagnosed outside the French ANRS network. This may apply particularly to foreign seasonal workers, who are frequently hired by the company concerned and who subsequently return to their home countries. The large size of this cluster may reflect a large number of MSM with risky sexual behaviour within a limited geographical area, particular characteristics of the new emerging recombinant strain, or both.

As frequently reported for HIV transmission clusters, most of the patients identified were MSM, and the proportion of primary and recent HIV infections was high [10]. The high levels of HIV viraemia observed in the patients with primary infections in this cohort were similar to those reported in comparable cohorts, but the patients diagnosed during chronic infection had a higher median viral load: 5.3 log10 (IQR: 4.8–5.9), vs only 4.6 (IQR: 4.0–5.1) log10 copies/mL (p<0.0001, data not shown) reported in the same ANRS network [20]. Normally, the first antiretroviral regimen leads to HIV RNA suppression before 6 months [21]. The high level of viraemia before treatment initiation may account for the inability to achieve this goal in six of 31 patients even after 12 months of treatment. The higher levels of replication in these patients may be a consequence of CRF94_cpx genetic recombination [22], increasing the risk of transmission of strains with an epidemiological advantage, capable of rapid spread.

The switch from R5 to X4 tropism over time between the A/B and C/D sub-clusters is also concerning. In cases of infection via sexual intercourse, R5 viruses generally predominate, accounting for more than 90% of cases, particularly in contexts of transmission clusters and primary infections [23–26]. X4 viruses are more prevalent in untreated patients at late stages of disease, with immunodeficiency [27,28]. In this cluster, most of the patients, including those in sub-cluster D in particular, were diagnosed with primary or recent infections. The FPR threshold of 10% must be sufficiently specific for the identification of X4 strains, but even with a more conservative 5% threshold, 5/26 patients harboured an X4 virus. The major change in the GenozhenoPheno tropism result is caused by the modification of residues at only two positions in V3-loop sequences. This small genetic difference clearly shows that CRF94_cpx is genetically close to both R5 and X4 viruses. This may explain the discordant results between Geno2Pheno tool predicting the strain to be X4 and phenotypic recombinant assays indicating R5 coreceptor usage for the 2M/25E pattern, which was observed in almost all branch D patients. This strain may have emerged during the outbreak in a patient at an advanced stage of immunodeficiency harbouring diversified X4 and R5 variants, like patient D20, for example. This hypothesis is consistent with recent suggestions that, in a context of transmission chains, the transmission bottleneck results in the selection of strains displaying R5 tropism [24]. X4 viruses are associated with a higher risk of progression to clinical AIDS [29]. The genetic proximity of CRF94_cpx to such strains is, thus, a matter of concern. Given the high levels of viraemia, facilitating transmission, and the difficulties decreasing viral replication to undetectable levels on treatment, these observations highlight the need to diagnose and treat individuals infected with this recombinant rapidly, to prevent further infection.

The high replication rate of this strain may not be the only characteristic favouring its spread. CRF94_cpx spread slowly through the southern suburbs of Paris in 2013–2014 (Figure 3, sub-clusters A, B and C). When the CRF94_cpx outbreak reached the eastern suburbs of Paris (sub-cluster D), the rate of infection increased due to faster spread through the population of MSM living or working in this area. Thus, the geographical concentration of MSM with risky behaviour was probably
one of the factors promoting the spread of this particular strain. The well-localised nature of the outbreak can be explained by the proximity of the patients’ workplace to their accommodation in nearby residences.

The use of geosocial networking apps may also have favoured the spread of this strain. In this study, the vast majority of cases used such apps to identify sexual partners nearby, leading to the infection of other inhabitants of the area without links to the company concerned. Conversely, French seasonal workers not from this suburb were diagnosed after returning home. This would also be the case for foreign seasonal workers, who might be diagnosed later. These findings highlight the importance of acting rapidly at the source of the outbreak to prevent its spread, and of surveys of the emergence of this strain in other European countries. The identification of this CRF94_cpx in the Los Alamos National Laboratory HIV database and the publication of this study could contribute to this.

This work also suggests that better use of new tools could help to end HIV outbreaks. Most of the patients of the CRF94_cpx cluster were unaware of PrEP at diagnosis. Condom use remains the cornerstone of HIV prevention, but awareness of the existence of PrEP should be increased in at-risk populations, and PrEP prescription should probably be increased to a larger population, as recently suggested [30]. A national information campaign was launched in the 2018 summer by the French AIDS association. The widespread use of apps to have a date with casual sex partners must also be taken into account when preventing and controlling outbreaks [31]. App users are more difficult to contact through traditional physical means, so collaboration with application editors may be required. These tools are sometimes used to encourage screening tests, but their geotargeting aspects could also be used to send focused alerts in cases of active outbreaks. More targeted actions are likely to be more effective. As shown here, combinations of phylogenetic and epidemiological analyses can help to identify the areas and populations involved in outbreaks.

One of the limitations of this study is that targeted activities began late in this outbreak, in 2018, partly due to the fortuitous discovery of this transmission chain and the improvised nature of the data collection and analyses. Moreover, the effectiveness of implemented prevention activities will be also difficult to assess without the possibility to compare the outbreak evolution without such activities. Another limitation is the unknown number of patients infected with CRF94_cpx strains and involved in this transmission cluster, because they were not recognised or not diagnosed. It was impossible to search these patients in all laboratories throughout France and Europe.

In conclusion, routinely generated HIV sequence data and epidemiological information for newly diagnosed patients can be used to focus preventive activities to limit HIV spreading. For optimal efficiency, data collection and analysis must be more systematic, with modern tools and connected epidemiological and interventional frameworks. A coordinated network of professionals, including virologists, clinicians and actors in local prevention (professional or associative) should be involved and supported by regional health authorities. In France, discussions about the reorganisation of existing national epidemiological surveillance HIV networks are underway, with a view to incorporating these new possibilities.

Acknowledgements

Members of the ANRS AC43 Resistance Study Group, by location: Amiens (C. Roussel; Angers, H. Le Guillou-Guillemette, A. Ducannelle); Argenteuil (L. Coudravault); Avicenne (C. Alloul, P. Honore); Besançon (Q. Lepeiller, D. Bettinger); Bordeaux (P. Bellecave, P. Pinson-Recordon, C. Tumiotto, S. Reigadas); Brest (S. Vallet, C. Payan, J.C. Duthe; Caen, M. Leroux, J. Dina, A. Vabret); Clermont-Ferrand (A. Mirand, C. Henquell); Créteil-Henri Mondor (M. Bouvier-alias); Dijon (A. Si-mohamed); Fort de France (G. Dos Santos); Genève (S. Yerly); Grenoble (A. Signori-Schmuck, P. Morand); HU Paris Sud (C. Pallier, M. Raho-Moussa, M. Mole, J-M. Dulucq); Lille–Tourcoing (L. Bocket, K. Aldijinou); Limoges (S. Ranger-Roger); Lyon (M. A. Trabaud, V. Icard, I.C. Tardy); Marseille (C. Tamalet); Metz/Thionville (C. Delamare); Montpellier (B. Montes); Nancy (E. Schwoerer, H. Fenaux); Nantes (A. Rodallec, E. André-Garnier, V. Ferré); Nice (A. De Monte, J. Dufayard); Orléans (A. Guigon, J. Guinard); Paris-Bichat Claude Bernard (D. Descamps, C. Charpentier, B Viseaux, G. Peytavin); Paris-Necker (M. Fillion); Paris-Pitié-Salpêtrière (C. Soulé, I. Malet, M. Wirden, A. G. Marcelin, C. Calvez, L. Assoumou, D. Costagliola); Paris-Saint Antoine (L. Morand-Joubert, S. Lambert-Nicot, D. Fofana); Paris-Saint Louis (C. Delaugerre, M. Chaix, N. Mahjoub); Paris-Tenon (V. Schneider, C. Amiel); Poitiers (G. Giraudou, A. Beby-Defaux, D. Plainchamp); Rennes (A. Maillard); Rouen (E. Alessandri-Gradt, M. Leoz, J. C. Plantier); Strasbourg (P. Gantner S. Fafi-Kremer, P. Fischer); Toulouse (S. Raymond, J. Izopet, J. Chiabrando); Tours (F. Barin, G. Fajole, O. Burgault); Versailles (S. Marque Juillet).

Members of the ANRS Clinical Centres and other, by location: Angers (J.M. Chenebault); Besançon (B. Hoen); Bordeaux (M. Dupon, P. Morlat, D. Neau); Brest (M. Garré, V. Bellein); Caen (R. Verdon, A. De la Blanchardière, S. Dargère, A. Martin, V. Noyou); Clermont-Ferrand (C. Jacomet); Créteil (J.D. Lelièvre, J.L. Lopez-Zaragoza); Dijon (B. Lorcerie); Fort de France (A. Cabié); Genève (S. Yerly); Grenoble (P. Leclercq, M. Blanc); Jossigny (E. Froguel, B. Hillion, P. Simon, S. Tassi); Le Kremlin-Bicêtre (C. Goujard); Lille–Tourcoing (O. Robineau); Limoges (P. Weinbreck); Lyon (L. Cotte; D. Makhloufi); Marseille (I. Poizot-Martin, I. Ravaud); Montpellier (J. Reynes); Nancy (H. Fenaux); Nantes (F. Raffi); Nice (E. Cua, J. Durant, P. Pugliese); Orléans (L. Hocquelloux, T. Prazuck); Paris-Bichat Claude Bernard (Y. Yazdanpanah, R. Landman, S. Legac); Paris-HEGP (L. Weiss, M. Karmochkine); Paris-Jean-Verdier (J. Bottero); Paris-Necker-Enfants Malades (C. Duvivier); HU Paris-Sud (C. Bolliot, M. Malet, D. Fofana); Paris-Sud (C. Pallier, M. Raho-Moussa, M. Mole, M-J. Dulucq); Paris-Saint Aimée (C. Delaugerre, M. Karmochkine); Paris-Necker (M. Fillion); Paris-Pitié-Salpêtrière (C. Katlama, R. Palich, S. Seang); Paris-Saint Antoine (M. Girard, J. L. Meynard); Paris-Saint Louis (J. M. Molina, N. Mahjoub): Paris-Tenon (V. Berrebi, G. Pialloux); Pointe à Pitre (I. Lamaury), Fort de France (A. Cabié); Poitiers (G. Le Moal, D. Plainchamp); Rennes (C. Michélet, J-C. Duthe); Rouen (F. Caron, Y. Debab, G. Unal); Strasbourg (M. Partisani, D. Rey, P. Fischer); Tours (B. Marchou, P. Delobel); Tours (V. Schneider, C. Amiel); Poitiers (G. Giraudeau, A. Beby-Defaux, D. Plainchamp); Rennes (A. Maillard); Rouen (E. Alessandri-Gradt, M. Leoz, J. C. Plantier); Strasbourg (P. Gantner S. Fafi-Kremer, P. Fischer); Toulouse (S. Raymond, J. Izopet, J. Chiabrando); Tours (F. Barin, G. Fajole, O. Burgault); Versailles (S. Marque Juillet).
Conflict of interest
None declared.

Authors’ contributions
MW, FDO, MBA, JCP, AGM and BV were involved in the conceptualisation of the research question and coordinated the data collection. MW, FDO and BV contributed with molecular analysis, analysed the data and wrote the manuscript. MABA, SLN, ML, SR, AS, MA, EAG, PB, BM, AM, CP, JDP, TR, VS, ASS, KS, VC and DD contributed with epidemiological data collection from respective region. All authors reviewed and approved the final version of the manuscript.

References
2. Eisinger RW, Fauci AS. Ending the HIV/AIDS Pandemic. 1.
3. 90-90-90 - An ambitious treatment target to help end the AIDS epidemic. 1.
4. McCormack S, Dunn DT, Desai M, Dolling DI, Gafos M, Gilson MW, FDO, MBA, JCP, AGM and BV were involved in the conceptualisation of the research question and coordinated the data collection. MW, FDO and BV contributed with molecular analysis, analysed the data and wrote the manuscript. MABA, SLN, ML, SR, AS, MA, EAG, PB, BM, AM, CP, JDP, TR, VS, ASS, KS, VC and DD contributed with epidemiological data collection from respective region. All authors reviewed and approved the final version of the manuscript.
15. QAD.0000000000001906  PMID: 21522007


License, supplementary material and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence and indicate if changes were made.

Any supplementary material referenced in the article can be found in the online version.

This article is copyright of the authors or their affiliated institutions, 2019.