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**Epidemiology and genomic characterisation of travel-associated and locally-acquired
influenza, Marseille, France**

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Abstract (210 words)

Background: The purpose of the study was to challenge the hypothesis of an introduction of influenza viruses by international travellers and subsequent local circulation in Marseille, France.

Methods: We analysed the epidemiological data of PCR-confirmed cases over an eight-year period and compared the genomic data of local and imported influenza viruses during a six-month period.

Results: Between June 2013 and December 2020, 12,434 patients in the Assistance Publique–Hospitaux de Marseille were diagnosed with an influenza virus infection at the laboratory of the Institut Hospitalo-Universitaire Méditerranée Infection of Marseille. Half of the patients were below the age of 20. Most of the imported cases were diagnosed outside of epidemic periods. Fourteen genomes of the influenza A virus, including six in international travellers returning from Europe or from the Arabian Peninsula and eight from patients who had not travelled were analysed. Sequences of influenza A/H1N1 virus genomes detected in subjects who had travelled to Saudi Arabia were in the same clade and differed from sequences detected later in a traveller returning from Italy, and in non-travellers who were infected in Marseille. This suggests that influenza viruses imported from Saudi Arabia did not subsequently circulate in Marseille.

Conclusion: Future studies with higher numbers of genomes are needed to confirm this result.

Keywords: *Influenza virus, travellers, genomic, epidemiological surveillance*

Introduction

International travellers are exposed to the acquisition of potential pathogens, including respiratory viruses, whether or not they present health problems during their trip. There is a risk, therefore, of pathogens being imported into France from endemic areas abroad, either from foreign travellers visiting France, from French travellers visiting foreign countries, or from migrants, with a potential risk of indigenous spread. In a study conducted on international travellers returning to Marseille, France between 2003 and 2015, respiratory/ear-nose-throat (ENT) illnesses accounted for 10% of syndromic diagnoses, ranking fourth after systemic febrile illness, and dermatological and gastrointestinal illnesses. Influenza-like illness accounted for 45% of respiratory/ENT presentations with 40 confirmed influenza cases, most of whom had travelled to the US (essentially during the H1N1 2009 pandemic) or to Saudi Arabia for the purpose of performing the Hajj pilgrimage [1]. In a retrospective study conducted among ill travellers returning from the Arabian Peninsula and presenting at our institute between 2013 and 2015, half of these patients had confirmed influenza [2].

In Marseille, influenza peaks during the winter months, as is the case across Western Europe, while travel-associated influenza cases are seen mostly during the summer holidays or during the Hajj seasons, which depend on the Islamic calendar. The objective of our work was to challenge the hypothesis that influenza viruses were being introduced by international travellers and subsequently circulated locally in Marseille. For that purpose, we analysed epidemiological data over an eight-year period and compared the genomic data of local and imported influenza viruses over a six-month period.

Materials and methods

Study design and clinical specimens

Between June 2013 and December 2020, we retrospectively extracted data about all patients who were diagnosed with a positive influenza virus real time-PCR (qPCR) result at the laboratory of the Institut Hospitalo-Universitaire Méditerranée Infection (IHU) in Marseille, which is the central laboratory for the Assistance Publique–Hospitaux de Marseille (AP–HM). The viruses were tested using FTD respiratory pathogens 33 (Fast-Track Diagnostics, Luxembourg) between 2013 and 2016. Testing of respiratory samples was performed using FTD Respiratory pathogens 21 (Fast-Track Diagnosis, Luxembourg) or Biofire FilmArray Respiratory panel 2 plus (BioMérieux, France) assays between 2017 and 2020. Patients whose samples were sent to the laboratory by external structures that are not part of AP–HM were not included in this analysis. Patients' age, gender, date of diagnosis, and virus type (A/B) were documented. In addition, electronic medical records from patients seen at the IHU (infectious diseases unit) were screened for a recent history of international travel (fewer than eight days between their return and the positive PCR) from 27 March 2016 to 31 December 2020.

Between June 2019 and February 2020, all influenza-positive specimens obtained from AP–HM patients returning to another European country or from the Arabian Peninsula were cell-cultured and sequenced. For each traveller's specimen, we randomly selected four or five positive specimens obtained during the winter epidemic from patients who had not travelled outside France.

Viral cultures

All samples were stored at -80°C until they were processed. For each clinical specimen, 100 µl of the sample was passed through a 0.22-µm pore sized centrifugal filter (Merck Millipore, Darmstadt, Germany) and then were inoculated into four wells of 96-well culture microplates containing MDCK cells (ATCC CCL-34) in MEM with 10% foetal calf serum and 1% L-glutamine. After centrifugation at 4000×g, microplates were incubated at 37°C. Each sample was cultured until cytopathic effect (CPE) was observed, usually requiring one to four passages (seven days per passage). Viral growth was detected by searching for CPE on a daily basis, using an inverted microscope then confirmed by specific qPCR. Results were considered positive when the cycle threshold (Ct) value of the qPCR was lower than that of the clinical sample inoculated.

Nucleic acid extraction from viral isolates

Viral RNA extraction was performed on 200 µl of cell culture virus isolate using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Genome sequencing

The viral genomes were sequenced on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA), according to the manufacturer's instructions. The DNA library was prepared using the commercial Nextera™ XT DNA library kit (Illumina, San Diego, CA, USA) consisting of enzymatic fragmentation, barcoding by PCR (12 cycles), and size-selection using magnetic beads. The sequencing run was then performed on a NextSeq500 using a mid-output cartridge resulting in 2×150 nucleotides paired-end reads. Fluorescent images were analysed using the Illumina RTA1.8/SCS2.8 base-calling pipeline to obtain demultiplexed FASTQ-formatted sequence data. The average read depth and average genome coverage were recorded across all segments.

The quality of the sequence data obtained was controlled using the CLC Genomics Workbench v7 (Qiagen, <https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/>) and the assembly was performed by SPADes V3.13.0 [3]. Sequences were trimmed with MiSeq and Trimmomatic [4] software, and untrimmed data were processed only using MiSeq software.

Phylogenetic analyses

All genomic sequences were aligned using MAFFT with default parameters [5]. The phylogenetic tree was reconstructed using the maximum likelihood method and a partial deletion (80%) setting [6].

Ethics

The data presented herein were collected retrospectively from the routine care setting using the hospital's electronic health recording system. This non-interventional retrospective study was approved by our institutional review board committee (Méditerranée Infection No. 2021-010). No additional samples were collected for this study. Viral cultures were obtained from biobanked samples collected in the routine care setting.

Results

Between June 2013 and December 2020, 12,434 AP-HM patients were diagnosed with influenza virus infection, including 8,625 which were due to influenza A, 3,774 which were due to influenza B virus, and 35 due to co-infections with influenza A and B viruses. 660 (5.3%) of these patients consulted or were hospitalised in the infectious diseases unit at the IHU. The demographic characteristics of the patients are presented in Table 1. Overall, half of the patients

were under the age of 20, with patients seen at the IHU being older. Figure 1 shows the weekly distribution of all cases in AP–HM patients. As expected, the epidemic peaked during the winter season, with slight yearly variations in terms of dates and total numbers of cases (Table 1). In 2020 no positive cases were identified after week 42.

Figure 2 shows the weekly distribution of cases in IHU patients including cases among international travellers between 27 March 2016 and 31 December 2020. Most of the imported cases were diagnosed outside the epidemic periods.

Between June 2019 and February 2020, we identified 1,699 confirmed influenza cases. 952 were due to influenza A virus, 739 to influenza B virus, and eight were co-infections with influenza A and B viruses. Of the 1,699 cases, 137 (8.1%) were seen at the IHU. 23/1,699 (1.4%) patients reported a recent history of travel, including 20 patients who were seen at the IHU. Viral cell culture was attempted from a total of 80 influenza-positive clinical specimens with 15 from international travellers returning from elsewhere in Europe or from the Arabian Peninsula, and 65 specimens were taken from patients who had not travelled. 26 viral cultures were successful, and 17 genomes were sequenced (Supplementary Figure 1). The demographics and clinical parameters of the 17 patients whose viral genomes were sequenced are presented in Table 2. Of these 17 patients, 14 cases were due to influenza A/H1N1 and three were due to influenza B virus. Ct values lower than 29.00 were observed with all virus isolates. Fourteen genomes of influenza A virus were used to build the phylogenetic tree. According to the phylogenetic tree (Figure 3), all genomes from five subjects who had returned from Saudi Arabia belonged to the same clade. The influenza A virus strain from the patient who had returned from Italy in September 2019 (Pat23) was closely related to two strains from patients who were infected in Marseille in November 2019 and January 2020 (Pat12 and Pat22).

Discussion

The age pattern of patients diagnosed with influenza at our institute is in line with French national data [7]. One study conducted in France between October 2014 and May 2019 showed that children under the age of 15 represented the most affected age group [8].

During the 2019–2020 influenza season, the first peak of locally-acquired cases diagnosed at our institute began in week 50 of 2019. This is consistent with national data with a pre-epidemic phase of influenza observed during weeks 50-51 of 2019 in the Île-de-France (North), Occitania (South-West), Pays-de-Loire (North-West) and Provence-Alpes-Côte d’Azur (PACA) (South-East) with the influenza epidemic starting during week 2 of 2020 in the Île-de-France and PACA and spreading across the whole country within three weeks [9]. Due to the COVID-19 pandemic, a strict lockdown was implemented in France on 17 March 2020, including the closure of schools and external European Union borders. This had probably a high impact on influenza circulation from week 13 of 2020 onwards [10]. We only identified three influenza cases after week 13 of 2020 (weeks 14, 17 and 42 of 2020), then no case was detected after week 42. This observation is in line with national data suggesting that the 2019–2020 influenza epidemic ended in week 12 of 2020 [9].

In our study, all sequences of influenza A/H1N1 viruses detected in subjects who had travelled to Saudi Arabia and returned to France in August 2019 were relatively homogeneous and pertained to the same clade. Viral sequences obtained from a traveller returning from Italy (September 2019) and from non-travellers infected in Marseille (November 2019 to January 2020) pertained to other clades. This result suggests that influenza viruses imported from Saudi Arabia did not

subsequently circulate in Marseille. One study carried out during the Hajj pilgrimages at Mecca in 2013–2015 showed that the influenza A virus diversity in Hajj pilgrims resulted from multiple introductions each year [11]. In 2015, data suggested an introduction of influenza A/H1N1 into Saudi Arabia by international pilgrims with a subsequent regional spread in the Arabian Peninsula. The specific mass gathering context of the Hajj might have triggered an amplification process responsible for geographical spread [11].

Concerning the proximity of the influenza A/H1N1 genome from Italy, with that from two non-travellers infected in Marseille, common mutations may have occurred leading to convergent evolution.

Our work has some limitations. Data surveillance from the laboratory was based on virological diagnosis, with little epidemiological information. Different types of qPCR were used for the detection of influenza viruses, which may have influenced the virus detection rate. In addition, the sample size of the genomes was small over the six-month study period. Future studies with higher numbers of genomic data are needed to confirm this result.

Notwithstanding these limitations, our data reflects the high susceptibility to influenza infection of younger individuals, including children under the age of 15. We recommend reinforcing vaccination coverage in this age group. This preliminary report also suggests that influenza viruses circulating in Marseille are not introduced by international travellers. Investigation of the source of local influenza viruses and their genomic characterisation is crucial for the control of the disease. Despite an increasing number of travellers worldwide, influenza vaccination guidelines for international travellers are still scarce, especially in European countries [12]. WHO recommends that “travellers [...] at high risk of influenza complications”, but not necessarily all international travelers should be vaccinated [13]. Also, travellers should be

informed that influenza seasonality may be different at their destination from that in their home countries [13]. According to the American Center for Disease Control and Prevention, influenza vaccines are recommended for travellers who have not gotten an influenza vaccine for the current season and who are travelling to parts of the world where influenza activity is ongoing [14]. In France, vaccination against seasonal influenza is recommended for all travellers undergoing organised group trip or using cruise ship, for cabin crew on cruise ships and airplanes, as well as tour guides [15]. The Saudi Ministry of Health recommends that international pilgrims be vaccinated against seasonal influenza before arrival into the Kingdom of Saudi Arabia, particularly those at increased risk of severe influenza diseases [16]. Finally, there is a need for a universal influenza vaccine for travellers that will address infections in both hemispheres [17].

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Table 1: Characteristics of Assistance Publique–Hôpitaux de Marseille patients with PCR confirmed influenza virus (June 2013–December 2020).

		Total AP–HM (N= 12,434) N (%)	Infectious diseases unit (Institut Hospitalo Universitaire Méditerranée Infection) (N= 660) N (%)
Number of positive cases during epidemic periods (N=10,803)			
Epidemic period duration ¹ (in weeks)			
2013–2014	5 (week 5/2014–9/2014)	441 (3.5)	10 (1.5)
2014–2015	9 (week 3/2015–11/2015)	1,504 (12.1)	27 (4.1)
2015–2016	11 (week 3/2016–14/2016)	1,516 (12.2)	15 (2.3)
2016–2017	10 (week 49/2016–6/2017)	1,088 (8.8)	46 (7.0)
2017–2018	16 (week 49/2017–12/2018)	2,297 (18.5)	101 (15.3)
2018–2019	8 (week 2/2019–9/2019)	1,970 (15.8)	115 (17.4)
2019–2020	9 (week 3/2020–11/2020)	1,987 (16.0)	212 (32.1)
Demographics			
Gender	Female	6,274 (50.5)	325 (49.2)
	Male	6,160 (49.5)	335 (50.8)
Age (years)	Mean	30.1	55.2

	Min-Max	0-106	2 - 100
Age group (years)	<5	4,714 (37.9)	12 (1.8)
	6–19	1,771 (14.2)	30 (4.5)
	20–39	1,630 (13.1)	156 (23.7)
	40–59	1,337 (10.8)	165 (25.0)
	60–79	1,625 (13.1)	156 (23.6)
	>80	1,357 (10.9)	141 (21.4)
Influenza virus type			
Influenza A mono infection		8,625 (69.4)	452 (68.5)
Influenza B mono infection		3,774 (30.4)	206 (31.2)
Influenza A and B co-infection		35 (0.2)	2 (0.3)

¹Source Santé Publique France (<https://www.santepubliquefrance.fr/maladies-et-traumatismes/maladies-et-infections-respiratoires/grippe>).

Table 2: Characteristics of 17 patients with sequenced influenza virus (23/08/2019–10/01/2020).

Patient code	Gender	Age (years)	Travel	Country of exposure	Date of return	Date of positive sample	Sample type	Influenza virus type	PCR Ct values of viral isolates
Pat01	Female	26	No	France	-	07/01/2020	Pharyngeal swab	Influenza B	21
Pat02	Male	0	No	France	-	10/01/2020	Pharyngeal swab	Influenza A	14
Pat03	Male	41	Yes	Saudi Arabia	19/8/2019	23/08/2019	Sputum	Influenza A	15
Pat04	Male	72	Yes	Saudi Arabia	20/8/2019	22/08/2019	Pharyngeal swab	Influenza A	15
Pat05	Female	64	Yes	Saudi Arabia	21/8/2019	23/08/2019	Sputum	Influenza A	29
Pat06	Male	69	Yes	Saudi Arabia	22/8/2019	23/08/2019	Pharyngeal swab	Influenza A	14
Pat07	Female	66	Yes	Saudi Arabia	24/8/2019	25/08/2019	Sputum	Influenza A	17
Pat09	Female	4	No	France	-	11/09/2019	Nasal swab	Influenza B	17
Pat10	Female	33	No	France	-	02/11/2019	Nasal swab	Influenza B	14
Pat11	Female	33	No	France	-	30/11/2019	Sputum	Influenza A	15
Pat12	Male	3	No	France	-	30/11/2019	Pharyngeal swab	Influenza A	13
Pat13	Male	0	No	France	-	04/12/2019	Nasal swab	Influenza A	11

Pat14	Male	24	No	France	-	04/12/2019	Pharyngeal swab	Influenza A	12
Pat16	Male	0	No	France	-	13/12/2019	Pharyngeal swab	Influenza A	15
Pat21	Female	32	No	France	-	27/12/2019	Pharyngeal swab	Influenza A	13
Pat22	Female	33	No	France	-	04/01/2020	Pharyngeal swab	Influenza A	22
Pat23	Male	84	Yes	Italy	23/09/2019	24/09/2019	Pharyngeal swab	Influenza A	11
Ct: Cycle threshold									

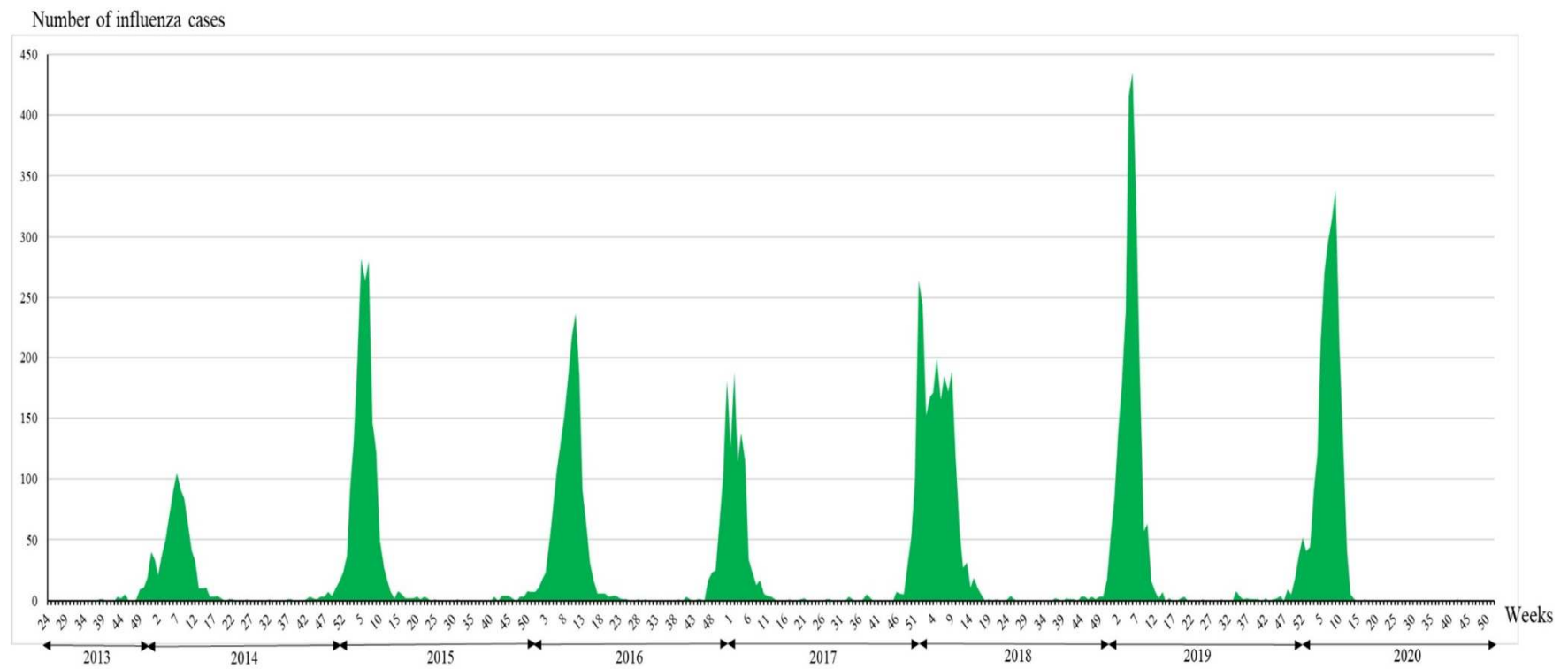


Figure 1: Weekly distribution of PCR-confirmed influenza cases in patients seen at the Assistance Publique–Hopitaux de Marseille (June 2013–December 2020, N=12,434).

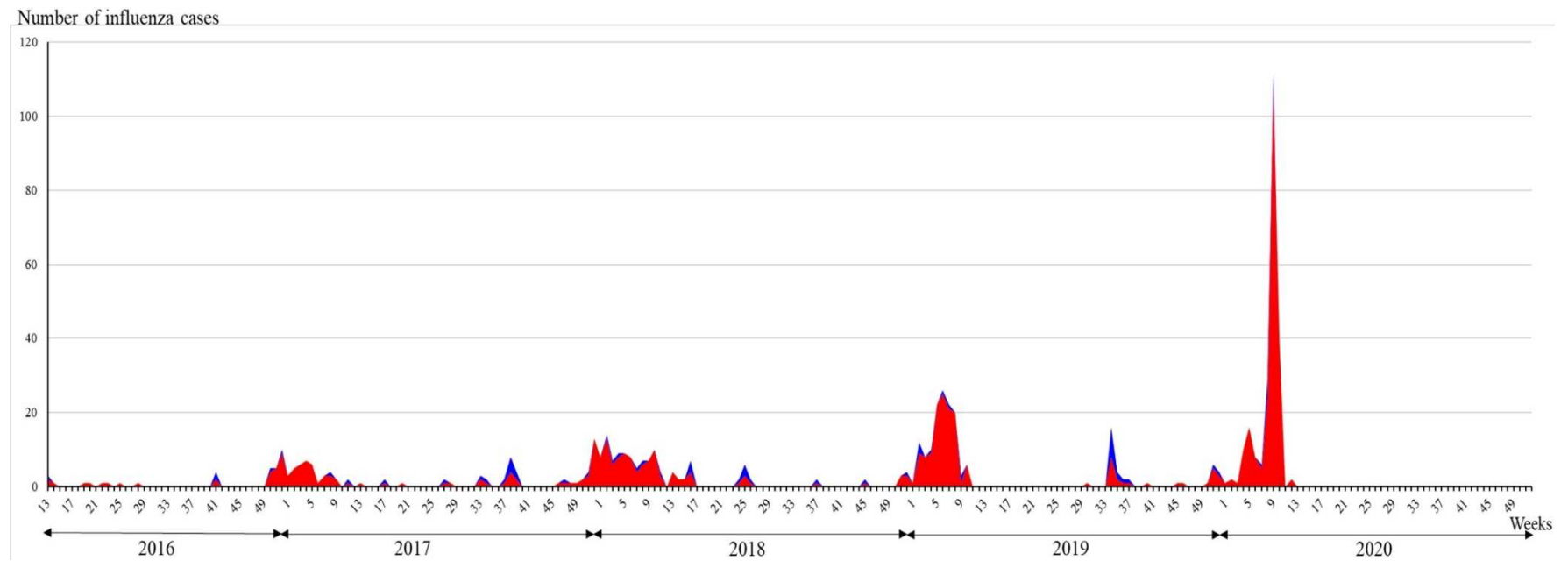


Figure 2: Weekly distribution of imported (blue curve) and locally acquired (red curve) PCR-confirmed influenza cases in patients seen at the Institut Hospitalo-Universitaire Méditerranée Infection (27/3/2016–December 2020, N=574).

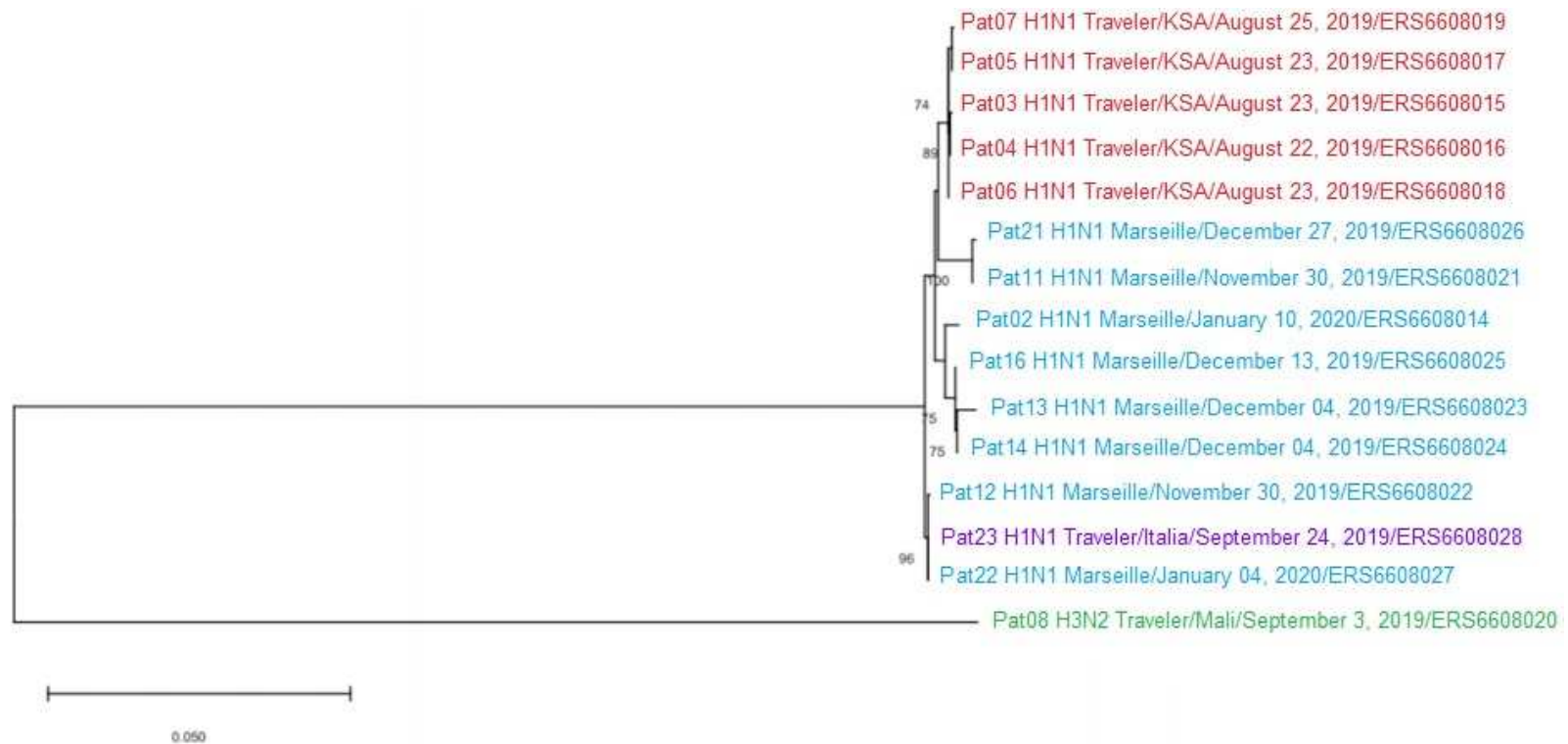


Figure 3: Molecular phylogenetic analysis of 14 sequences of influenza A/H1N1 viruses.

Note: - KSA: Kingdom of Saudi Arabia

- Patient code/Influenza virus subtype Traveller status/Place of exposure/Date of sample/Genbank accession number. One sequence of influenza A/H3N2 from a traveller returning from Mali (ERS6608020) was used as an outgroup.