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Clinical management and viral genomic diversity analysis of a child's influenza A(H1N1)pdm09 infection in the context of a severe combined immunodeficiency

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ABSTRACT

Introduction: A child with severe combined immunodeficiency (SCID) had an influenza A(H1N1)pdm09 infection with viral excretion longer than 6 months, during 2013–2014 influenza season, despite cord blood transplantation and antiviral treatments.

Methods: Conventional real-time RT-PCR methods were used to estimate viral load and to detect the presence of the common N1 neuraminidase (NA) H275Y substitution responsible for oseltamivir resistance. Next-generation sequencing (NGS) of influenza viruses was performed retrospectively to characterize viral quasispecies in specimens.

Results: The patient was first treated with oral oseltamivir, leading to detection of low-levels of NA-H275Y substitution. Concomitant cord blood cell transplantation, intravenous administration of zanamivir and immunoglobulins led to an increase in white blood cells and influenza viral load decrease. A viral rebound occurred as soon as the antiviral treatment was discontinued. Eventually, influenza viral load was negated with immune reconstitution. NGS found influenza quasispecies harboring NA-E119A substitution (10.3%). Moreover, NGS showed that viral genomic diversity evolved under antiviral treatment and immune status.

Conclusions: Conventional virological techniques were sufficient for influenza infection follow-up but NGS performances allowed characterization of viral variants evolution in this specific case of prolonged influenza virus infection. New and efficient treatments against influenza in immunocompromised patients are needed.

1. Introduction

The duration of influenza viral shedding is usually about 6 days in immunocompetent patients but increase in function of the immunity impairment (Baz et al., 2006; Boivin et al., 2002; Memoli et al., 2014).

In immunocompromised patients, influenza virus infections are more severe, with an elevated risk of antiviral resistance after selection in treated patients (Memoli et al., 2014).

Neuraminidase (NA) inhibitors (NAI) are the only anti-influenza drugs recommended by WHO. In France, only oral oseltamivir and

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intravenous (IV) zanamivir (upon special authorization) are available. In immunocompromised patients, IV zanamivir may be considered an option for the first line of treatment (Marty et al., 2017), but there is currently no consensus recommendation. However, oseltamivir and zanamivir combined is not recommended as it could lead to a competitive antagonism (Duval et al., 2010). Furthermore, a phase II clinical trial conducted in patients at risk of complications that investigated the combination oseltamivir, amantadine and ribavirin versus oseltamivir monotherapy did not find any clinical benefit of the combination treatment; the median duration of symptoms was 4.5 days in the combination arm and 4.0 days in the monotherapy arm (Beigel et al., 2017).

The 2007/2008 winter season was marked by the emergence of seasonal A (H1N1) influenza viruses bearing a NA-H275Y substitution responsible for oseltamivir resistance in untreated patients. However, since the 2009 pandemic, oseltamivir treatment in immunocompromised patients is the leading cause of oseltamivir resistance (Hurt et al., 2012; Okomo-Adhiambo et al., 2015; Takashita et al., 2013).

Currently, influenza virus genomic diversity study is focused on hemagglutinin antigenic evolution and matching with current vaccines. Next-generation sequencing (NGS) allows precise influenza genomic diversity assessment of individual specimens and a longitudinal evaluation over the course of infection and the immune status of a patient. However, NGS has been rarely used to characterize prolonged influenza A(H1N1)pdm09 or A(H3N2) infection in immunocompromised patients (Eshaghi et al., 2014; Ghedin et al., 2011, 2012; Trebbien et al., 2017), and only two papers report viral quasispecies investigation in severe combined immunodeficiency (SCID) patients, infected with respiratory syncytial virus (RSV) (Grad et al., 2014) or influenza A(H3N2) virus (Rogers et al., 2015).

In this paper, we describe severe influenza A(H1N1)pdm09 virus infection in a SCID child with more than 6 months of viral excretion. Viral genomic diversity under immune and antiviral treatment selection was studied using conventional techniques and NGS.

2. Materials and methods

2.1. Ethics

This study was approved by the ethics committee of Hospices Civils de Lyon on July 18, 2017. To protect anonymity of the patient the precise dates of specimen collection was not given. Respiratory samples were collected for regular disease management during hospital stay and no additional samples were taken.

2.2. Virological diagnosis of influenza virus infection

A total of 12 nasopharyngeal aspirates (NPA) and 21 nasopharyngeal swabs (NS) were collected from March to August 2014. Subsequently, virus culture medium was added to obtain at least 1.5 mL final volume. All clinical samples were screened for the presence of influenza virus using the respiratory MWS Influenza A/B R-GENE® real time reverse transcriptase quantitative polymerase chain reaction (rt RT-qPCR) assay (Argène bioMérieux, Marcy-l'Étoile, France) that can detect both influenza A and B viruses. RNA was extracted from 200 µL of sample using the automated NucliSENS® easyMAG® system (bioMérieux, Marcy-l'Étoile, France). Elution was performed in 50 µL. Influenza A subtyping was performed using in-house RT-qPCR (CNR des virus des infections respiratoires, Institut Pasteur, Paris).

2.3. Cells and viral culture

Madin-Darby canine kidney (MDCK) cells (obtained from ATCC; CCL34) were maintained in serum-free medium (EMEM; Lonza, Verviers, Belgium) supplemented with 1% L-Glutamine (200 nM;

Lonza), 2% of penicillin-streptomycin (10,000U penicillin/mL; 10,000U streptomycin/mL; Lonza). Cells were maintained at 37 °C and 5% CO₂. Respiratory samples were cultured on MDCK cells to isolate virus; two passages were performed prior to NA inhibition assays. Viral culture was performed at 34 °C and 5% CO₂.

2.4. NA activity and inhibition assays

Oseltamivir carboxylate was provided by Hoffmann-La Roche (Roche Diagnostics GmbH, Mannheim, Germany), and zanamivir by GlaxoSmithKline (GSK, Brentford, UK). The fluorometric inhibition assays were performed using a MFX fluorometer (DyNex technologies, Chantilly, VA, USA) as described previously, except MES buffer was at pH 6.4 (Ferraris et al., 2005). The NA inhibition assay was performed using a standardized amount of NA activity (10 nmol/h/mL). The NA activity was calculated as the quantity of 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA) substrate (Sigma-Aldrich, St Louis, MO, USA) degraded to 4-methylumbelliferone in 1 h per mL of viral suspensions (nmol/h/mL). The inhibitory concentration (IC₅₀) is the drug concentration (nM) able to inhibit 50% of the NA activity and was calculated using SigmaPlot software v 8.0 (Systat software, San Jose, CA, USA).

2.5. RT-qPCR for quantification of the NA-H275Y substitution in clinical samples

RT-qPCR for NA-H275Y substitution quantification in N1 was performed on 28 specimens as previously described (Escuret et al., 2012). Briefly, probes were specific for 275Y (FAM-probe) or H275 (VIC-probe). The assay was performed on the ABI7500 thermocycler (Applied Biosystem, Foster city, CA, USA) that was programmed as follows: 50 °C for 15 min (RT), 95 °C for 2 min (polymerase activation), then 45 cycles at 95 °C for 15 s (denaturation), and 60 °C for 40 s (annealing; elongation; reading of fluorescence). A Ct value higher than 45 was considered as negative for the rest of the study.

2.6. Influenza A(H1N1)pdm09 next-generation sequencing

Six NPA samples (collected on post-infection day 14, 99, 111, 115, 121, and 136) and two NS specimens (collected on post-infection day 40 and 72) were included in this study. Extraction was performed as for the diagnosis step. Nucleic acids were stored at –80 °C. Sequences corresponding to each influenza A genomic segment were obtained by a commercial multiplex RT-PCR using the PathAmp™ Flu A reagents kit (Life Technologies, Carlsbad, CA, USA), controlled on a 1% agarose gel. After purification using Agencourt AMPure XP beads (Beckman Coulter, Beverly, MA, USA). Quality and quantity of DNA was evaluated using a NanoDrop1000 spectrophotometer and Qubit 2.0 HS DNA kit (Thermo Fisher Scientific, Waltham, MA, USA).

Sequencing of these amplicons was performed using two different NGS methodologies. On the one hand, samples were sequenced using PGM technologies, as previously described (Pichon et al., 2017). Library was prepared manually using 100ng of DNA; using Ion Xpress™ plus Library Preparation kit (Life Technologies). After purification using Agencourt AMPure XP beads, quality and quantity of each library was verified using the 2100 Bioanalyzer (DNA High Sensitivity Chip, Agilent Technologies, Santa Clara, CA, USA). They were then diluted and pooled in equimolar amounts (26pM) before emulsification and sequencing. Emulsified libraries were finally loaded onto a Ion 318v2 chip (Life Technologies) then sequenced on an Ion-Torrent sequencer following the manufacturer's instructions (Ion PGM™ 200 Sequencing v2 protocol, Life Technologies).

On the other hand, amplicons were sequenced using an Illumina sequencing platform (Illumina, San Diego, CA, USA) to reinforce observed results on PGM platform. Briefly, after mechanical shearing on M220 Focused-ultrasonicator™, (Covaris Inc., Woburn, MA, USA), end-

repair, adenylation, and adapter ligation were performed using NextFlex™ Rapid DNA Sequencing Bundle (Bio Scientific, Austin, TX, USA). Size selection was performed to obtain 150–250 to 700 bp DNAC (corresponding to a 150-bp-insert). Amplification was performed according to the manufacturer's recommendations, quality was then checked using Agilent 2100 Bioanalyzer (Agilent Technologies) and followed by quantification of each library on Lab Chip® GX Touch DNA High-Sensitivity (PerkinElmer, Waltham, MA, USA). All libraries were pooled and sequenced on Illumina NextSeq 500® (Illumina) using a MidOutput cartridge (Illumina). The reads obtained on the sequencing platform were submitted to NCBI's Sequence Read Archive and can be found under project number SUB3709326.

2.7. Bioinformatic analysis

The sequence reads, obtained from Ion PGM and Illumina NextSeq, were analyzed separately but followed the same bioinformatic procedure except for technology-dependent settings. The reads were trimmed using cutadapt (v0.4.4) (Martin, 2011), for adapters and low base quality removal (Phred score < 20). Reads smaller than 50 bases and, for Illumina NextSeq only, the paired reads with which they were associated, were excluded. Since the studied strain belonged to clade 6, its representative, A/South Africa/3626/2013 (GISAID Isolate 175880), was used as reference for further steps (Supplementary Figure) (Bogner et al., 2006). Mapping was conducted with the BWA-MEM algorithm (v0.7.15) (Li, 2014) using default settings except for gap opening and extension penalties set to 10 and 2, respectively, to increase indel stringency. Sorted mapped reads were manually inspected through Fastqc (v0.11.5) to verify read quality. Single nucleotide polymorphism (SNP) calling was divided into two steps. First, variant calling files (vcf) were generated with naive variant caller (Biomina Galaxy platform) (Blankenberg et al., 2014)), only excluding bases presenting a base- or a mapping- Phred score < 20. Second, a homemade vcf analysis python script was used that applied filters to validate SNPs retrieved previously and generate end-user workable files. Briefly, to this purpose, a quantitative strand bias (SB) score was calculated to estimate an uncertainty for variants frequencies reported (Guo et al., 2012a, 2012b). For each position, a depth-adapted threshold was calculated following a *t*-test distribution and corresponded to the lowest variant frequency detected with 99% power of detection for a given depth. These SNPs required to have a SB score lesser than 1.0, corresponding to an uncertainty at least smaller than reported frequency, and an estimated minimal frequency greater than both an arbitrary (5%) and a depth-adapted threshold. SB score could only be calculated for paired-end files; therefore, no correction could be applied to Ion PGM SNP frequencies. Results reported in this study were based on validated SNPs found in both Ion PGM and Illumina Nextseq sequencing and their estimated minimal frequency. Segment sequences with a low coverage (mean depth < 2,000X) were excluded from analysis.

3. Results

3.1. Patient clinical history

A five-month-old child was admitted for oxygen-requiring interstitial pneumonia associated with bronchiolitis symptoms. These respiratory symptoms had started 14 days before hand (considered as Day 0, DO) and were due to an influenza virus infection diagnosed on NPA at D14 (Fig. 1A). Within his first months of life, he had presented two episodes of acute pyelonephritis and an episode of wheezing complicated by oral candidiasis due to steroid treatment. These iterative infections led to immunological analyses to be performed that found agammaglobulinemia and a defect of T and NK cells suggesting an X-SCID.

In order to correct the SCID, and despite the ongoing influenza infection, the patient underwent allogeneic cord blood transplantation on

D83, previously conditioned by fludarabine, melphalan and anti-lymphocyte serum association. Immunosuppressive therapy was based on cyclosporin and steroids. Aplasia ended on D97 with an increase in neutrophils and monocytes but lymphopenia persisted until lymphocytes increased on D173 and from D206 to D219 (Fig. 1B). The patient's condition deteriorated in the weeks following cord blood transplantation; severe respiratory distress (due to influenza infection concomitant with innate immunity reconstitution but with persistent lymphopenia) leading to intensive care unit (ICU) admission from D99 to D138. A graft-versus-host-disease (GvHD) was suspected due to hepatic impairment characterized by moderate hepatic cytolysis (2-fold increase of liver transaminases on D123) and important cholestasis (35-fold increase of gamma glutamyl transferases on D129). Thus, immunosuppression with cyclosporin and steroids was maintained but these drugs were responsible for hypertension. Due to persistent cholestasis, the patient was admitted again to the ICU on D220 to undergo plasmapheresis, and cyclosporin was replaced by tacrolimus on D222. The patient was also infected by a bocavirus and norovirus II whose viral loads also varied according to the immunity and were still detected on D236 and D240. Finally, the patient died on D243.

3.2. Influenza viral infection and antiviral treatment

Influenza virus detection remained positive in respiratory samples for 6 months (last positive specimen on D177) despite NAI treatment (Fig. 1A). Oral oseltamivir (15 mg twice daily) was administered from D35 to D40. A temporary authorization for the compassionate use of IV zanamivir was obtained on D100 since an oseltamivir-resistant subpopulation was detected. This treatment, associated with IV immunoglobulins, was extended during 15 days. This treatment, concomitant with partial immune reconstitution, allowed an influenza viral load decrease around 2 log₁₀ copies/mL on NPA (Fig. 1A, Table 1). However, insufficient data on a potential toxicity during extended use led to discontinue IV zanamivir and initiate another oseltamivir therapy from D118 to D123, as the oseltamivir-resistant subpopulation had lowered, but this treatment was inefficient. The viral load was correlated to immune reconstitution (Fig. 1). The white blood cells decrease between D117 and D146, associated to the antiviral stop led to a viral load rebound (very close Ct for NPA specimens at D136 and D100). Finally, the viral clearance was obtained when immune reconstitution was sufficient.

3.3. Evaluation of susceptibility of influenza virus to NAI using conventional techniques

During the clinical management of the patient, 11 NPA and 17 NS, collected between D14 to D177 after the onset of symptoms, were found to be positive for influenza A viruses (Table 1) and secondarily subtyped as an A(H1N1)pdm09 virus. The viral loads for NPA samples were higher as this sampling method allows recovering more cells than NS (Fig. 1A). A proportion of 11%–29% of influenza viruses bearing a NA-H275Y substitution were detected, after oseltamivir therapy, on NS performed on D40, D44, D51, D66 and D72 but not D58 when viral load was very low (Ct = 34). This subpopulation appeared just after oseltamivir treatment (from D35 to D40), persisted at least up to D121 (Table 1) during which there was low immune pressure (Fig. 1B). After cord blood allograft transplant, on D83, the respiratory distress worsened as viral load increased at D86 at which time no 275Y variants could be detected. On D107, the highest proportion of 275Y variants (24%) were detected and the viral load was the lowest since transplant (Ct = 29; Table 1).

The 275Y substitution was detected on several specimens but only at a low proportion (≤29%) (Table 1, Table 3) and only wild-type viruses were selected after cell culture. Fourteen influenza virus isolates were obtained after culture and all presented normal inhibition by oseltamivir (mean ± SD oseltamivir IC₅₀: 0.38 ± 0.18 nM) and

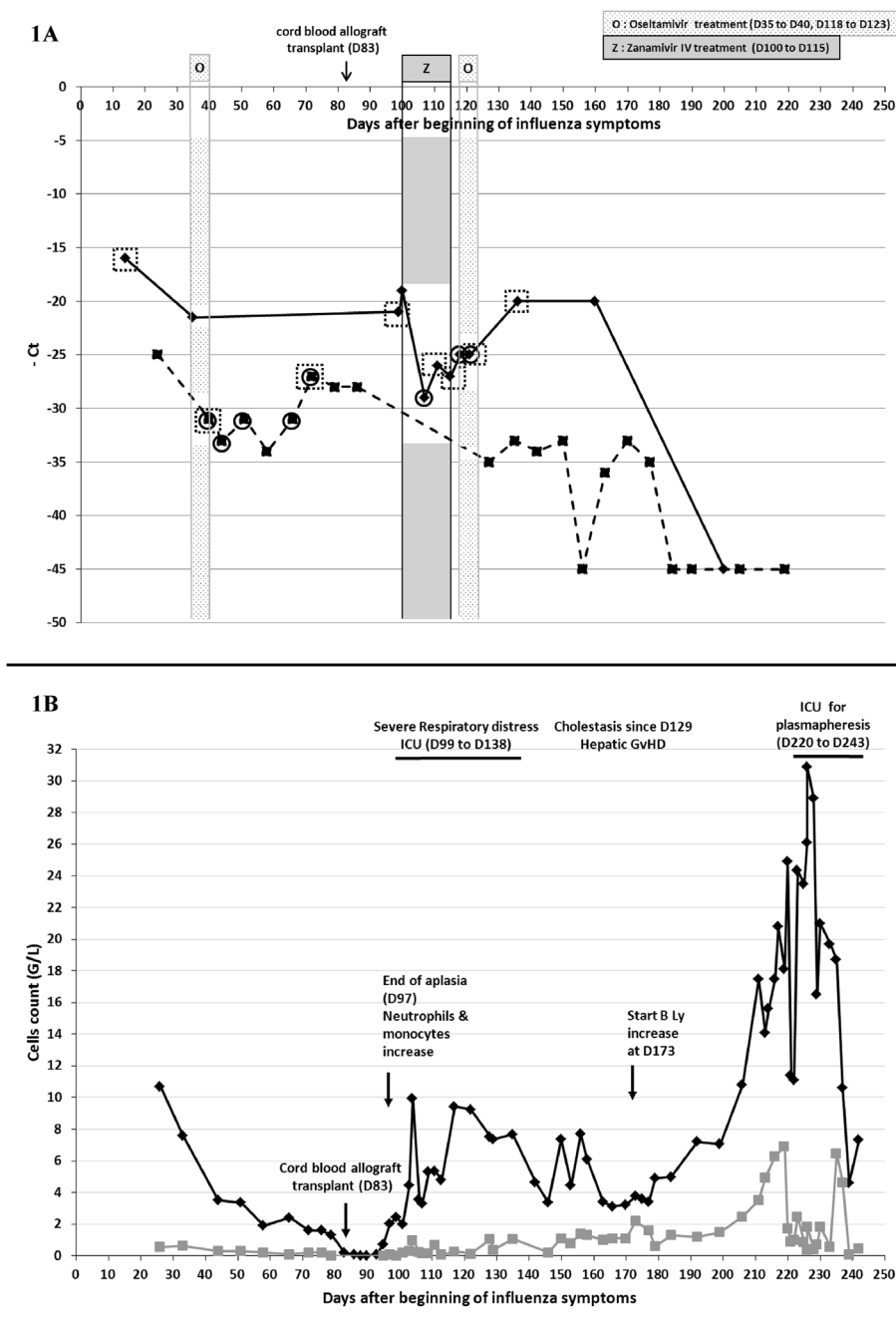


Fig. 1. Influenza viral load in clinical specimens (1A) and white blood cell counts (1B) during the course of influenza A(H1N1)pdm09 infection follow-up. (1A) Real-time RT-qPCR cycle thresholds (Ct) were inversely proportional to the influenza virus load in the respiratory sample. Inverted y-axis allows an easier comprehension of viral load kinetics. The x-axis represents days of specimen sampling after beginning of influenza clinical symptoms considered as D0. Diamonds represent nasopharyngeal aspirates (NPA) and squares nasopharyngeal swabs (NS). Viral load kinetics were represented by discontinuous or continuous lines depending of the sample nature (NPA and NS respectively). The H275Y NA substitution presence was tested by RT-qPCR in all specimens collected until D156. Specimens with detection of a proportion of 275Y NA substitution are circled. Specimens used for influenza deep genome sequencing are indicated with a dashed square. Treatments by oseltamivir or zanamivir are noted by shades of gray (light gray for oseltamivir and dark gray for zanamivir). Note that Ct could not be strictly superposed between NPA and NS, due to the difference between these sampling methods. (1B) The x-axis represents days after beginning of influenza clinical symptoms considered as D0. Absolute counts are expressed in G/L (corresponding to 10^6 Cells/ml). White blood cells are presented with a black line and include polynuclear cells, lymphocytes and monocytes. Lymphocytes are presented with a gray line. Aplasia ended at D97 with a rapid increase in the neutrophil count followed by an increase in monocyte count. Lymphopenia persisted on T and NK cells. Note that lymphocytes increase was due to B lymphocytes increase at D173 and from D206 to D219. Abbreviations: ICU, intensive care unit; Ly, lymphocytes; GvHD, graft versus host disease.

zanamivir (mean \pm SD zanamivir IC_{50} : 0.51 ± 0.13 nM).

3.4. Influenza A(H1N1)pdm09 virus deep sequencing

Whole genome sequencing of influenza A was performed by NGS on the first specimen (D14) after the onset of symptoms and on a selection of the next following specimens with a possible variant selection by antiviral treatment (D40, D72, D99, D111, D115, D121, and D136).

The sequencing depth and coverage was better in specimens with a higher viral load (D14, D99, and D136 specimens; Fig. 2A) and for shorter segments (Fig. 2B). In particular, PB1 and PA segments had a low mean depth ($< 2,000\times$) at D111 and D115 (Fig. 2B). Viral genomic diversity was the greatest in the D115 specimen at the end of zanamivir treatment, especially on the NP segment (Fig. 2C). Hemagglutinin (HA) and NA segments presented the highest number of substitutions on the first D14 specimen compared to the A/South Africa/3626/2013 virus

reference strain (GISAID reference 175880) (Table 2). PB2, NP then PA and NS1 segments presented four and three substitutions respectively; PB1, NS2 and M segments were very conserved (Table 2). Within-host influenza virus genomic diversity revealed that NP, HA, PB2 and NS1 segments were the most variable (Table 3). The analysis of PB1 and PA genome segments found a limited number of mutations and two distinct deletions on PB1 segment at D136 (Table 3). Matrix segment harbored few minor polymorphisms but no amino-acid substitutions of their consensus sequence. The NA sequencing was consistent with the specific H275Y RT-qPCR with 29% of 275Y variants in specimen at D72 and only H275 variants in specimens at D111 and D115 (Table 1, Table 3). A low proportion (10.3%) of NA-E119A substitution was detected at D111 during IV zanamivir treatment (Table 3).

Table 1
Influenza A(H1N1)pdm09 analysis and phenotypic characterization of sequential specimens collected from the patient and course of antiviral therapy.

Days ^a	Specimen origin	H or Y275 NA substitution quantification ^b (log ₁₀ copies/mL of specimen)		Viral Culture result	NA activity (nmol 4Mu/h/mL) ^c	Mean IC ₅₀ ± SD (nM) ^c	
		H275	Y275(%)			oseltamivir	zanamivir
14 ^f	NPA	7.38	0	IAV	164	0.21 ± 0.11	0.32 ± 0.04
24	NS	4.84	0	IAV	117	0.26 ± 0.11	0.42 ± 0.06
35 ^d	NPA	7.71	0	IAV	124	0.22 ± 0.01	0.44 ± 0.04
40 ^{d,f}	NS	3.89	3.00 (12%)	N	ND	ND	ND
44	NS	3.04	2.66 (29%)	N	ND	ND	ND
51	NS	3.36	2.73 (19%)	IAV	88	0.63 ± 0.18	0.49 ± 0.01
58	NS	2.91	0	N	ND	ND	ND
66	NS	3.36	2.46 (11%)	N	ND	ND	ND
72 ^f	NS	4.71	4.16 (22%)	IAV	68	0.41 ± 0.15	0.81 ± 0.28
79	NS	4.16	0	IAV	76	0.81 ± 0.16	0.50 ± 0.03
86	NS	5.09	0	IAV	101	0.31 ± 0.04	0.63 ± 0.18
93	NS	3.10	0	N	ND	ND	ND
99 ^f	NPA	6.63	0	IAV	96	0.51 ± 0.10	0.60 ± 0.06
100	NPA	7.77	0	IAV	85	0.35 ± 0.11	0.57 ± 0.07
107 ^e	NPA	5.13	4.63 (24%)	N	ND	ND	ND
111 ^{e,f}	NPA	6.30	0	IAV	19	0.25 ± 0.03	0.45 ± 0.06
115 ^{e,f}	NPA	5.73	0	N	ND	ND	ND
118 ^d	NPA	5.03	3.79 (5%)	N	ND	ND	ND
121 ^{d,f}	NPA	4.95	3.93 (9%)	IAV	38	0.27 ± 0.04	0.50 ± 0.25
127	NS	0	0	N	ND	ND	ND
135	NS	3.92	0	N	ND	ND	ND
136 ^f	NPA	7.59	0	IAV	55	0.36 ± 0.18	0.43 ± 0.16
142	NS	3.12	0	IAV	ND	ND	ND
150	NS	3.47	0	N	ND	ND	ND
156	NS	0	0	N	ND	ND	ND
160	NPA	ND	ND	IAV	ND	ND	ND
163	NS	ND	ND	N	ND	ND	ND
170	NS	ND	ND	N	ND	ND	ND
177	NS	ND	ND	N	ND	ND	ND

We used reference strains for validation of the NA inhibition assays.

For A/California/07/2009 reference strain (reference for wild-type H275 NA), the mean oseltamivir IC₅₀ was 0,44 ± 0,21 nM and zanamivir IC₅₀ was 0,65 ± 0,15 nM.

For A/LyonChu/48.307/2009 isolate (reference for variant 275Y NA) (Escuret et al., 2012), the mean oseltamivir IC₅₀ was 147,87 ± 45,80 nM and zanamivir IC₅₀ was 0,52 ± 0,14 nM.

Abbreviations: Ct, cycle threshold; IC₅₀, NAI concentration able to inhibit 50% of the NA activity; IAV, Influenza A Virus; N, Negative; NA, neuraminidase; ND, not determined, NPA Naso Pharyngeal Aspirates, NS: nasal swabs; 4Mu, 4-methylumbelliferone.

Samples D184, D190, D200, D205, and D219 were not represented as influenza detection remained negative at these time points.

^a Days after beginning of influenza symptoms.

^b The quantification of the proportion of a wild type H275 NA or variant 275Y NA substitution were performed using an in house RT-qPCR. As only low proportion (≤29%) of 275Y NA substitution were detected, only wild type viruses were selected after cell culture.

^c The NA activity and IC₅₀ were determined using the MUNANA substrate as described in materials and methods section. The mean IC₅₀ + SD of influenza A isolates are duplicates.

^d Sampled under antiviral treatment (oseltamivir).

^e Sampled under antiviral treatment (zanamivir).

^f Samples that were sequenced using NGS.

4. Discussion

The present study combines specimen whole influenza genome sequencing, clinical data, and antiviral treatment of a SCID patient infected by influenza A(H1N1)pdm09 virus.

The late SCID diagnosis led to perform the cord blood transplantation with an ongoing infection, which is known to have a poor outcome (Rivers and Gaspar, 2015). Influenza infections in immunocompromised patients require effective antiviral agents but only oseltamivir and zanamivir are available.

IV zanamivir as late add-on therapy has been described to have a limited effectiveness; however most patients were immunocompromised (Fraaij et al., 2011). In the case presented here, we observed a partial viral response with a 2 log₁₀ copies/mL decrease in seven days. This observation is consistent with a median decrease in viral load of 1.81 log₁₀ copies/mL after 2 days of IV zanamivir treatment of children (phase II assay) (Bradley et al., 2017). In previously published cases, IV zanamivir was also associated with viral load decrease although with WB cells increase and persistent lymphopenia

(Dulek et al., 2010). It was reported to be effective in stem cell transplanted teenagers; however, although IV zanamivir decreased influenza viral load, genome clearing was only observed more than 4 months later, following immune reconstitution (Ghosh et al., 2012). IV zanamivir was discontinued after the usual 15 days of treatment (Dohna-Schwake et al., 2010; Dulek et al., 2010; Gaur et al., 2010). In another clinical case, IV zanamivir was administered for a total of 34 days without notification of particular adverse events (Ghosh et al., 2012). In this situation, owing to important cholestasis on D129, the hepatotoxicity of IV zanamivir was questioned. Hepatotoxicity of IV zanamivir was hypothesized in a case of pediatric liver transplant (Dohna-Schwake et al., 2010) without any serious adverse events detected in other patients (Dulek et al., 2010; Fraaij et al., 2011). IV zanamivir in adults led to liver enzyme elevations during a Phase II evaluation (Marty et al., 2014). However no increased hepatotoxicity was noted in the IV zanamivir groups in the phase III trial in adults (Marty et al., 2017) and in the phase II trial in children (Bradley et al., 2017). In this child's history, the 14-day interval between IV zanamivir treatment discontinuation and appearance of liver injury signs suggests that

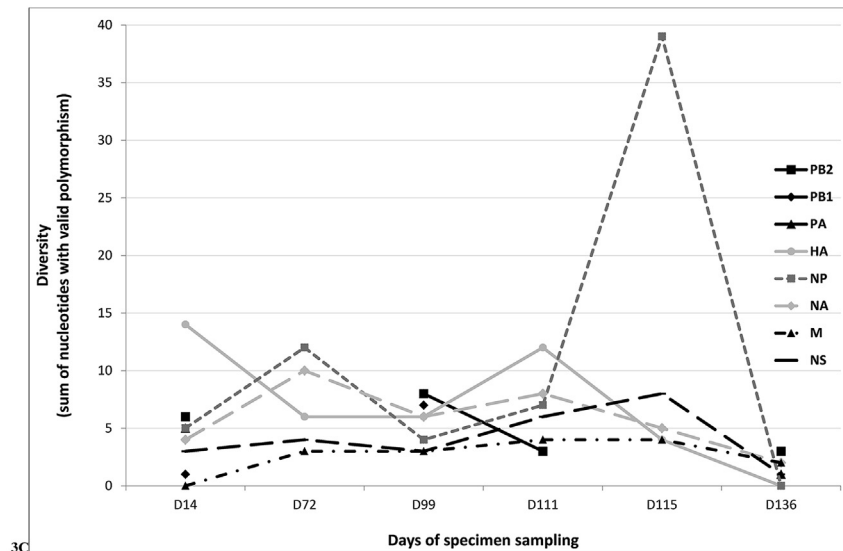
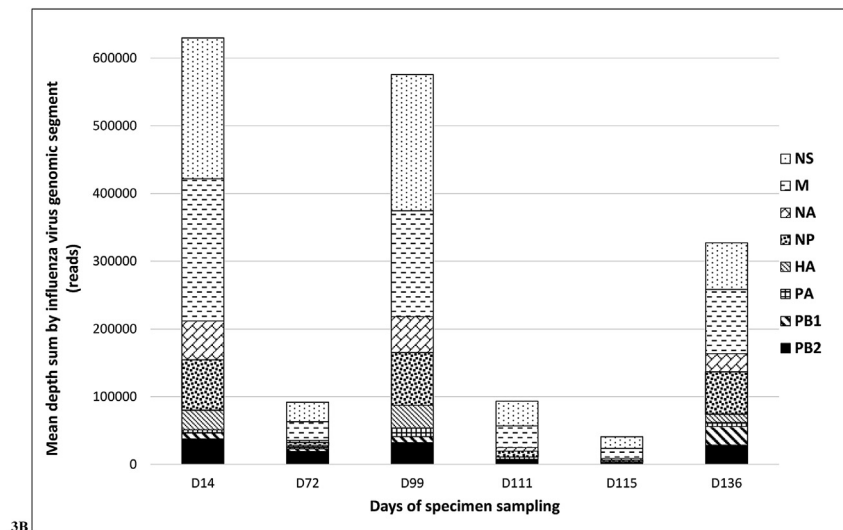
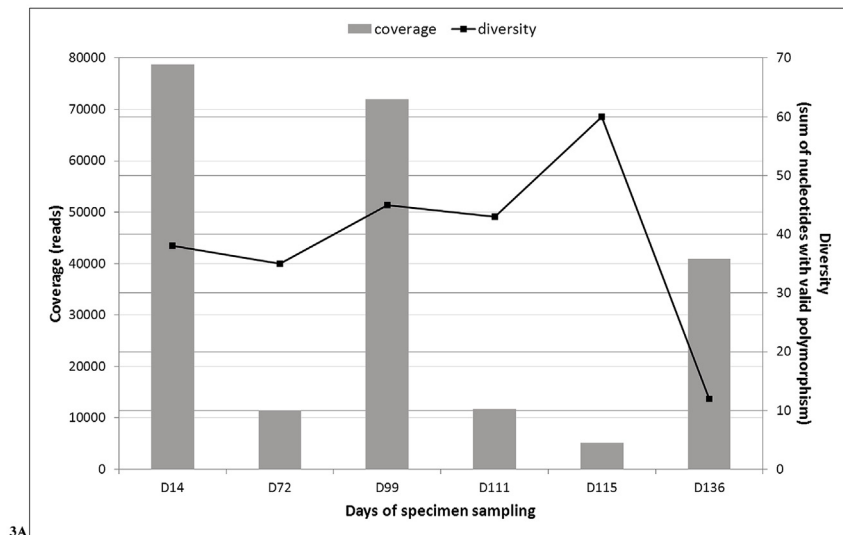


Fig. 2. Influenza viral sequence coverage and genomic diversity. (2A) Influenza viral sequence coverage and genomic diversity: viral sequence coverage corresponds to the mean number of reads obtained for influenza virus genome segments for each specimen, and global genomic diversity is the sum of nucleotides identified for all the segments with a mean depth > 2,000X. (2B) Mean depth by influenza viral genomic segment. (2C) Viral genomic diversity according to segment: viral genomic diversity is the sum of nucleotides identified with a valid polymorphism on each genome segment. Only the results of diversity obtained when mean depth was > 2,000X are presented. Due to insufficient coverage, data for specimens at D40 and D121 were not presented. Results for PB2, PB1 and PA segments at D72 and D115 and for PB1 segment at D111 are not shown. Day of specimen sampling correspond to the day after beginning of influenza symptoms.

hepatotoxicity resulted from GvHD and/or from immunosuppressive drugs toxicity.

As only few antivirals were available, conventional virological techniques were sufficient to allow the influenza infection follow-up

and antiviral adaptation. However, we only had very partial information thus we performed retrospectively an NGS analysis to characterize viral genomic diversity along the course of this prolonged influenza episode. NGS allowed the study of influenza viral quasispecies, under

Table 2

Amino acid homology between the patient's first specimen and the A/South Africa/3626/2013 reference strain sequences.

Protein	Substitutions (variant %) ^a in first specimen at D14 by comparison with the A/SouthAfrica/3626/2013
PB2	M28K (7.7%), E72D (8.9%), T102N, T129I [§] (12.7%)
PB1	R687Q
PA	R356K, D529N, K716N
HA	K119R (14.6%), E127D, H138Q, A139D (7.6%), K142R (12.6%), N162S, S183P (44.5%), A186T (11.5%), D187G (8.4%), S190I (8.6%), I216K (58.8%), E224V (36.9%), V524L
NP	G17E (37.9%), I41T [§] (8.1%), N101D, V119I (45.8%)
NA	L40I, V81I (5.3%), L85I, M242I (18.3%), G249R, N307K
M1	None
M2	None
NS1	M6R (57.5%), T76A, V178I (15.4%)
NS2	M6R (57.5%)

The A/South Africa/3626/2013 strain (GISAID sequence n° 175880) was used as a reference. For HA, the numbering system was based on the mature HA sequence, without the 17 amino-acid residues of the signal peptide.

Variant populations detected by Illumina sequencing only are indicated with a §.

^a When a mix of wild type and variant amino acids was detected, the proportion (%) of variant substitution was indicated between parentheses.

antiviral and altered-immune pressures, crosschecked on two different platforms, which support the validity of results reported. However, differences in viral populations could be influenced by the sampling site as it was shown for NGS analysis of bronchoalveolar lavages and NS collected the same day (Trebien et al., 2017). Moreover, the longer segments (PB2, PB1, and PA) presented a lower coverage in both techniques. This technical limitation led to an absence of conclusion as to whether large deletions were due to a technical problem or truly reflected the presence of defective interfering particles (Dimmock and Easton, 2014; Saira et al., 2013). Similarly to RSV studies, genomic diversity was shown unequally distributed along the viral genome with highly-conserved regions and highly-varying regions (Do et al., 2015). In this study, we observed variation in genomic diversity among segments, with most of the variation occurring in proteins exposed to antibody neutralization (HA or NA) but also in NP and less in M or PA segments. The high genomic diversity detected in NP may reflect a bias of selection as the coverage was the lowest for this D115 specimen (Fig. 2A). Viral genomic diversity seemed to increase concomitantly with selection by immunity and lymphocytes recovery after cord blood allograft or by antiviral treatment, as previously reported for RSV infection in an SCID patient (Grad et al., 2014). We did not detect the D222G substitution in HA known to be associated to illness severity (Casalegno et al., 2014; Goka et al., 2014). Severe respiratory distress was concomitant with partial immune reconstitution leading to inflammation; viral clearance was observed only when lymphocytes increased (Gooskens et al., 2016).

NGS allowed a better characterization of influenza variants selected by antiviral treatment. The NA-H275Y substitution is the most frequently encountered in N1, under oseltamivir pressure, despite lowering viral fitness (Abed et al., 2014). These data are consistent with the present study where NA 275Y variants were repeatedly detected only as a minority under antiviral pressure; corresponding influenza virus isolates were oseltamivir-sensitive suggesting that NA 275Y variants were outcompeted by wild-type viruses. Our results were consistent for specimens tested both with RT-qPCR and NGS. Our results are consistent with previous studies demonstrating that 275Y variants were present at concentration below the threshold value in first specimens and were selected by oseltamivir treatment (Ghedini et al., 2011, 2012). *In vitro* studies suggested that oseltamivir could expand diversification of influenza virus by facilitating accumulation of virions in intercellular spaces (Mori et al., 2015). This accumulation allows cell-to-cell transmission and co-infection between defective and helper viruses (Mori

et al., 2015). In the case presented herein we could not ascertain whether oseltamivir treatment could increase viral genomic diversity, as coverage was insufficient for specimens at D40 and D121 (sampled just after the end or during oseltamivir treatment). However, as discussed above, oseltamivir was responsible for selection of oseltamivir-resistant variants and then for evolution of viral genomic diversity.

In the present case, NGS allowed E119A minor variants detection under zanamivir selection. The emergence of substitutions E119G and E119D in N1 have been described in two cases of very immunocompromised patients under IV zanamivir treatment (L'Huillier et al., 2015; Tamura et al., 2015). Mainly mixes of E119G and E119D substitution were detected but E119A was also detected in one specimen at a very low percent (0.5%) (L'Huillier et al., 2015). *In vitro*, seasonal A(H1N1) influenza viruses bearing a NA-E119A substitution were selected with laninamivir (Samson et al., 2014). A recombinant A(H1N1)pdm09 virus harboring a NA-E119A substitution exhibited a 90- and 17-fold increase in zanamivir and oseltamivir IC₅₀ respectively and a reduced NA activity (36% compared to wild type NA) (Samson et al., 2014).

5. Conclusions

To conclude, the present report demonstrated that NGS is particularly relevant in specific situations, as major immune defects, where minor viral variants could reveal a risk of antiviral efficacy loss. However, to modify clinical management of influenza infection in immunocompromised patients, the use of NGS would need implementation of bioinformatics pipelines and softwares to obtain results rapidly and the development of alternative efficient antiviral agents against influenza.

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Transparency declarations

(1) Conflict of interest

M.P. received a travel grant from Therdiag and Hologic to attend the ESCV 2017 and 2018 Conferences.

A.G. received a travel grant from Cobas to attend the RICAI 2017 Conference.

M.V. received a grant from Medical Wire to attend the ECCMID 2017 Congress.

B.L. is the co-Chair of the Global Influenza Initiative, a member of the scientific committee of the Global Hospital Influenza Surveillance Network, a member of the Foundation for Influenza. He received a travel grant from Alere to attend the RICAI 2017 Conference, and a travel Grant from Seegene to attend the ECCMID conference in 2017. All personal remuneration stopped in September 2010.

L.J. served on the French Advisory Board for SANOFI on the 'Quadrivalent Influenza Vaccine' in 2017.

V.E. received a travel grant from Roche Pharmaceuticals to attend the Fourth ESWI Conference and declares potential conflict of interests with Sanofi-Pasteur (intervention in symposia in 2013).

All other authors C.P., B.S., C.R., B.M., C.M., G.M., F.M.S., E.J., G.M. have no conflicts of interest to declare.

(2) This work has not been presented in any meeting.

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Table 3
Amino acid homology between the first and subsequent specimens sequences.

Protein	Substitutions (variant%) ^a in subsequent specimens compared to first specimen at D14					
	specimen at D72	specimen at D99	specimen at D111	specimen at D115	specimen at D136	
PB2	NI	T16P (7.0%)	T129I* (57.5%)	NI	V89M	
		T129I* (71.4%)				
		Y704H (12.9%)	L716R (5.3%)			D680G (13.4%)
		A722T (10.4%) L725P (30.3%)	L725P (6.7%)			
PBI	NI	E297D (17.7%)	NI	NI	K11X (56.6%) (2nt)	
PA HA	NI	E237K (41.6%)	E237K (59.0%)	NI	K744X (23.7%) (1nt) E237K	
NP	K142R* (54.0%) S183P* A186T* (55.0%)	V19L (11.1%) K142R* S183P* A186T*	K142R* (84.3%) S183P* (84.9%) A186T* (84.2%) I216K (86.2%) E224V (12.0%) V250A (26.0%)	K142R* S183P* A186T*	K142R* S183P* A186T*	
		V250A (7.3%)				
			G17E* (94.2%)	G17E* (74.1%)	I460V (8.0%) G17E* (76.6%) G34D (5.4%) I41T* (63.1%) I109V [§] (6.6%) V119I* (21.9%) A286S* (19.7%)	G17E*
			I41T* (6.7%)		R384G (19.0%) K400R (19.7%) I406T (6.9%)	
		V119I* (88.8%)	V119I* (11.1%) A286S* (25.4%)	A286S* (40.9%) R293K [§] (8.1%)	A286S* (19.7%)	A286S*
	S482N [§] (6.7%)					
	NA	G96E (7.2%)		N50S [§] (5.8%)		
		M242I* (19.3%)	M242I* (42.6%)	E119A (10.3%) M242I* (29.8%)	M242I* (81.5%)	N270K
		H275Y (29.1%)				
	M1	None	None	None	None	F62V (62.4%)
M2	V68M (5.1%)	None	None			
NS1	M6R* (30.6%)		M6R* (9.8%)	S82N [§] (6.8%) M6R* (66.7%) I90L [§] (5.7%)	S82N	
			M106V (15.6%)	Q109K (8.9%)		
			I111T (8.8%)			
NS2	V178I* (58.9%)	V157I (6.7%) V178I* (80.9%)	V178I* (81.1%)	V178I* (25.2%)	V178I*	
	M6R* (30.6%)	None	M6R* (9.8%)	M6R* (66.7%) S29N [§] (6.4%)		
	M83I (6.9%)				K64E (5.4%)	

We considered only the result obtained for segments with a mean depth > 2000 X. Due to insufficient coverage, data for specimens at D40 and D121 were not presented. For HA, the numbering system was based on the mature HA sequence, without the 17 amino-acid residues of the signal peptide.

Specimen at D72 was a NS. Other specimens at D99, D111, D115 and D136 were NPA.

Variants populations detected in first specimen at D14 by comparison with A/South Africa/3626/2013 reference strain but with various proportions in subsequent specimens are indicated with *.

Variants populations detected by Illumina sequencing only are indicated with a [§].

Deletions of two nucleotides and one nucleotide detected in PBI segment at D136 were responsible for amino acid deletions K11X and K744X respectively.

Abbreviations: D, Day; NI, not interpretable; NPA, nasopharyngeal aspirate.

^a The proportion (%) of variant substitution was indicated between parentheses.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://>

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