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Virology

A molecular epidemiological study of human parainfluenza virus type 3 at a tertiary university hospital during 2013–2015 in Catalonia, Spain

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ABSTRACT

Human parainfluenza virus type 3 (HPIV-3) is one of the most common respiratory viruses particularly among young children and immunocompromised patients. The seasonality, prevalence and genetic diversity of HPIV-3 at a Spanish tertiary-hospital from 2013 to 2015 are reported. HPIV-3 infection was laboratory-confirmed in 102 patients (76%, under 5 years of age). Among <5 years-old patients, 9 (11.5%) were under any degree of immunosuppression, whereas this percentage was significantly higher (19; 79.2%) among patients older than 5 years. HPIV-3 was detected at varying levels, but mainly during spring and summer. All characterized HN/F sequences fell within C1b, C5 and in other two closely C3a-related groups. Furthermore, a new genetic lineage (C1c) was described. Genetic similarity and epidemiological data confirmed some nosocomial infections, highlighting the importance of the HPIV-3 surveillance, particularly in high-risk patients. This study provides valuable information on HPIV-3 diversity due to the scarce information in Europe.

1. Introduction

Human parainfluenza viruses (HPIVs) are an important cause of acute respiratory tract infections (ARI). Human parainfluenza virus type 3 (HPIV-3) is an enveloped non-segmented, negative, single-stranded RNA virus, which is classified as member of the genus *Respirovirus* of the family *Paramyxoviridae* (Henrickson, 2003). HPIV-3 infection in immunocompetent adults usually courses as mild and self-limited. However, HPIV-3 is the second cause of viral lower respiratory tract infection (LRTI) in young children, only preceded by respiratory syncytial virus (RSV) (Counihan et al., 2001; Henrickson, 2003; Iwane et al., 2004). Children under 5 years of age, immunocompromised individuals and the elderly are at high risk for severe or even fatal infection (Falsey, 2012; Henrickson, 2003; Liu et al., 2013; Reed et al., 1997).

Unlike other HPIVs, HPIV-3 usually causes seasonal outbreaks during spring and summer months in community; as well, it has been sometimes reported to cause nosocomial outbreaks, in particular among hematopoietic stem cell transplantation (HSCT) recipients (Cortez et al., 2001; Harvala et al., 2012; Jalal et al., 2007; Piralla et al., 2009; Ustun et al., 2012).

To date, neither antiviral drugs nor vaccines are approved for clinical use against HPIV-3 infection (Falsey, 2012; Guillon et al., 2014). In most severe cases the administration of ribavirin has been reported, but its efficacy and benefits have not still been demonstrated (Falsey, 2012). Regarding prophylaxis, two chimeric bovine/human PIV-3 vaccines based on haemagglutinin-neuraminidase (HN) and fusion (F) proteins were found to be well tolerated and immunogenic (Karron et al., 2012; Schmidt et al., 2011), but remained under study in clinical trials. HPIV-3, like other RNA viruses, has the capability to acquire random point mutations throughout its genome, mainly in the envelope glycoproteins (HN and F) that are under the selective pressure from human immune response. The rate of molecular evolution of HPIV-3 based on HN protein was estimated at 1.10×10^{-3} substitutions per site per year, similar to that of other virus genes such as the *G gene* of respiratory syncytial virus (Mizuta et al., 2014). The pattern of HPIV-3 evolution, based on the analysis of *F* gene sequences, it is quite similar to influenza virus B (Prinoski et al., 1992). Since the rest of the genome remains highly conserved, the non-coding region of *F* gene and the coding region of *HN* gene are often used to perform molecular epidemiological studies

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Table 1
Primers and PCR amplification and sequencing protocols of complete HN protein-coding region and partial *F* gene sequences. The M13 primer binding sites used for sequencing are underlined.

Gene (Fragment position*)	Primer name	Primer sequence (5′ - 3′)
PCR amplification protocol		
HN**	HN1-M13F	TGTAAAACGACGGCCAGTAACTTAGGAGTAAAGTTACRCA
(6708–7658)	HN1-M13R	CAGGAAACAGCTATGACCATCAAGTACAATTTCTTCTATGCC
HN**	HN2-M13F	TGTAAAACGACGGCCAGTCTGTAAAYTCAGAYTTGGTACCTG
(7474–8320)	HN2-M13R	CAGGAAACAGCTATGACCGCTGTTGAGTAAGTTATGACTGG
HN**	HN3-M13F	TGTAAAACGACGGCCAGTCATAATGTGCTATCAAGACCAGG
(8139–8572)	HN3-M13R	CAGGAAACAGCTATGACCCTGATTGCTGATTACTTATCATATACTTG
F	F-M13F	TGTAAAACGACGGCCAGTACTTAGGACAAAAGARGTCA
(4855–5243)	F-M13R	CAGGAAACAGCTATGACCACCACAAGAGTTAGARTCTTC
Thermal profile:	50 °C × 30 min – 95 °C × 15 min – 45 cycles (95 °C × 15 sec – 52 °C × 20 sec – 72 °C × 1 min) – 72 °C × 10 sec	
Sequencing protocol		
M13	M13F	TGTAAAACGACGGCCAGT
	M13R	CAGGAAACAGCTATGACC
PCR Thermal Profile	96 °C × 1 min – 30 cycles (96 °C × 10 sec – 50 °C × 5 sec – 60 °C × 4 min)	

* Nucleotide position relative to KF530250 sequence from GenBank.
** Primers as previously described [27], with minor modifications.

(Almajhdi, 2015; Côté et al., 1987; Mao et al., 2012; Prinoski et al., 1992; Storey et al., 1987).

The aim of this study was to describe the prevalence, the seasonality and the genetic diversity of HPIV-3 detected in respiratory specimens from patients attended at the Hospital Universitari Vall d'Hebron (HUVH) in Barcelona (Spain) from the 2013–2014 season to the 2014–2015 season.

2. Materials and Methods

2.1. Sample collection

A descriptive observational study was conducted from upper and lower respiratory tract specimens received to our laboratory for viral diagnosis, which were collected from patients, children and adults, attended at the emergency care unit or hospitalized in HUVH, a tertiary 1,200-bed university hospital, from week 40/2013 (2013–2014 season) to week 20/2015 (2014–2015 season), including the 2014 inter-seasonal period. In addition, demographic and clinical data from these patients were collected from the hospital clinical records. Institutional review board approval (PR_AG_156/2015) was previously obtained from the HUVH Clinical Research Ethics Committee.

2.2. Detection of HPIV-3 and RNA extraction

The detection of HPIV-3 and other respiratory viruses was routinely performed either by direct immunofluorescence antigen detection (D^3*Ultra* 8™ DFA Respiratory Virus Screening & Identification Kit Diagnostic HYBRIDS, USA) or by a multiplex real-time PCR assay (Anyplex II RV16 Detection Kit, Seegene, Korea). Total nucleic acids (NA) were purified by using NucliSENS® EasyMag® (BioMérieux, Marcy l'Etoile, France) from 400 μl of respiratory specimens and eluted to 100 μl according to the manufacturer's instructions. Eluted NAs were kept at −80 °C until use.

2.3. HN and F genes sequencing

From laboratory-confirmed HPIV-3 specimens, overlapping fragments that span the entire coding sequence of HN protein and partial *F* gene sequence were amplified by using a one-step RT-PCR-based

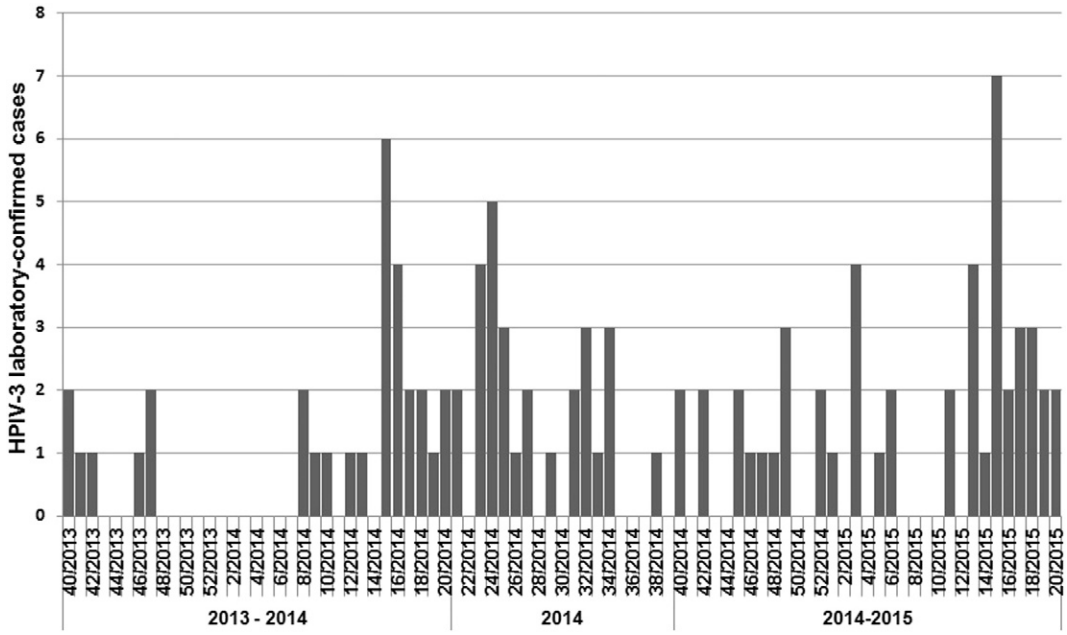

Fig. 1. Weekly distribution of laboratory-confirmed HPIV-3 cases detected from week 40/2013 (2013–2014 season) to week 20/2015 (2014–2015 season).

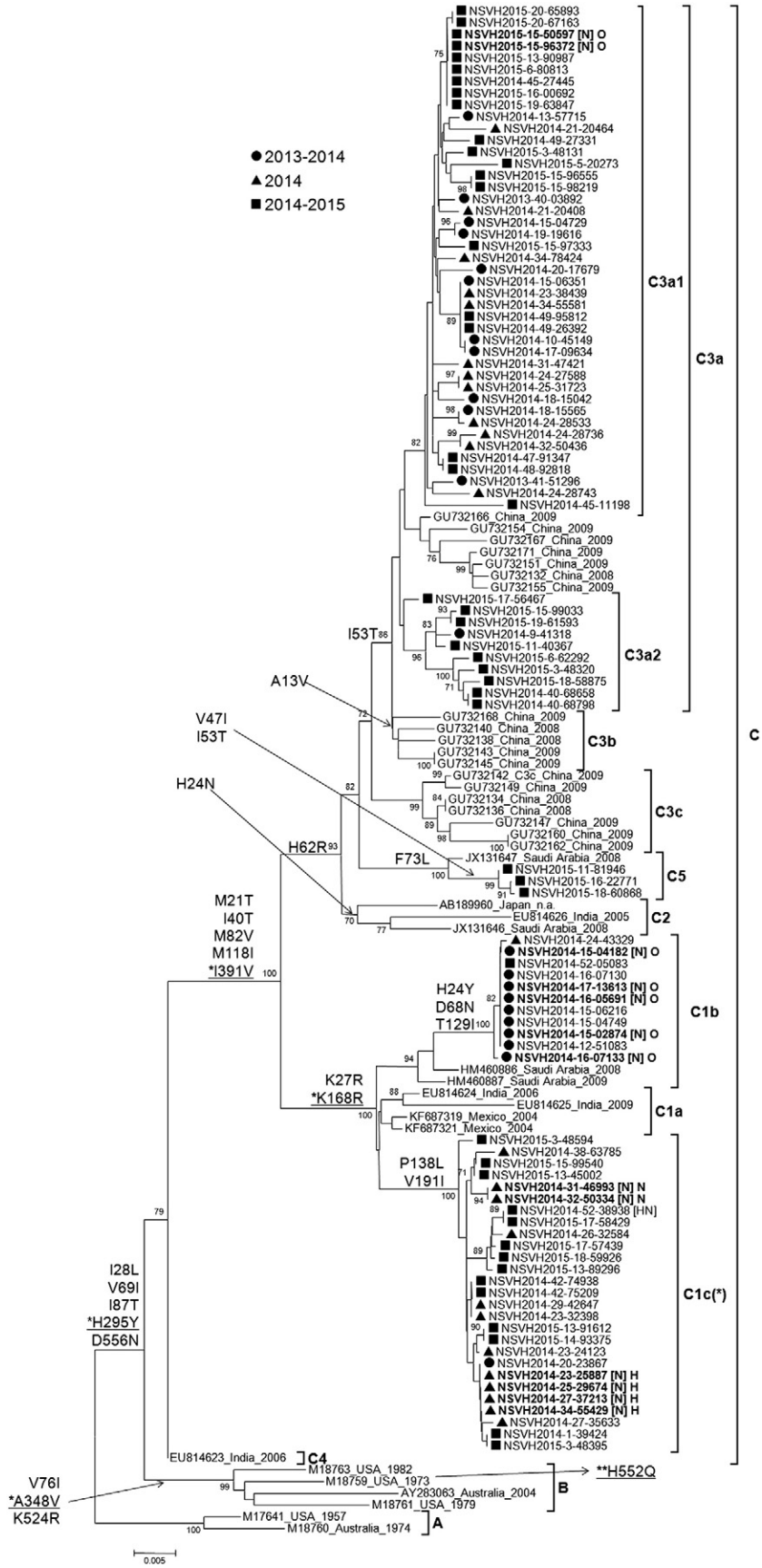

Fig. 2. Phylogenetic analysis based on the coding sequence of the HN protein (1719 nucleotides) from 94 HPIV-3 strains of the present study and other reference sequences. The evolutionary distances were computed using Tamura-Nei method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (0.28). Only bootstrap values higher than 70% are shown. Novel lineage described C1c (*) are also included. Strains associated with suspected nosocomial outbreaks are indicated as [N] followed by different letters relative to the affected hospital ward (H-hematology, O-oncohaematology, N-neonatology). Amino acid substitution within the antigenic epitopes (*) or the binding receptor site (**), are marked underlined.

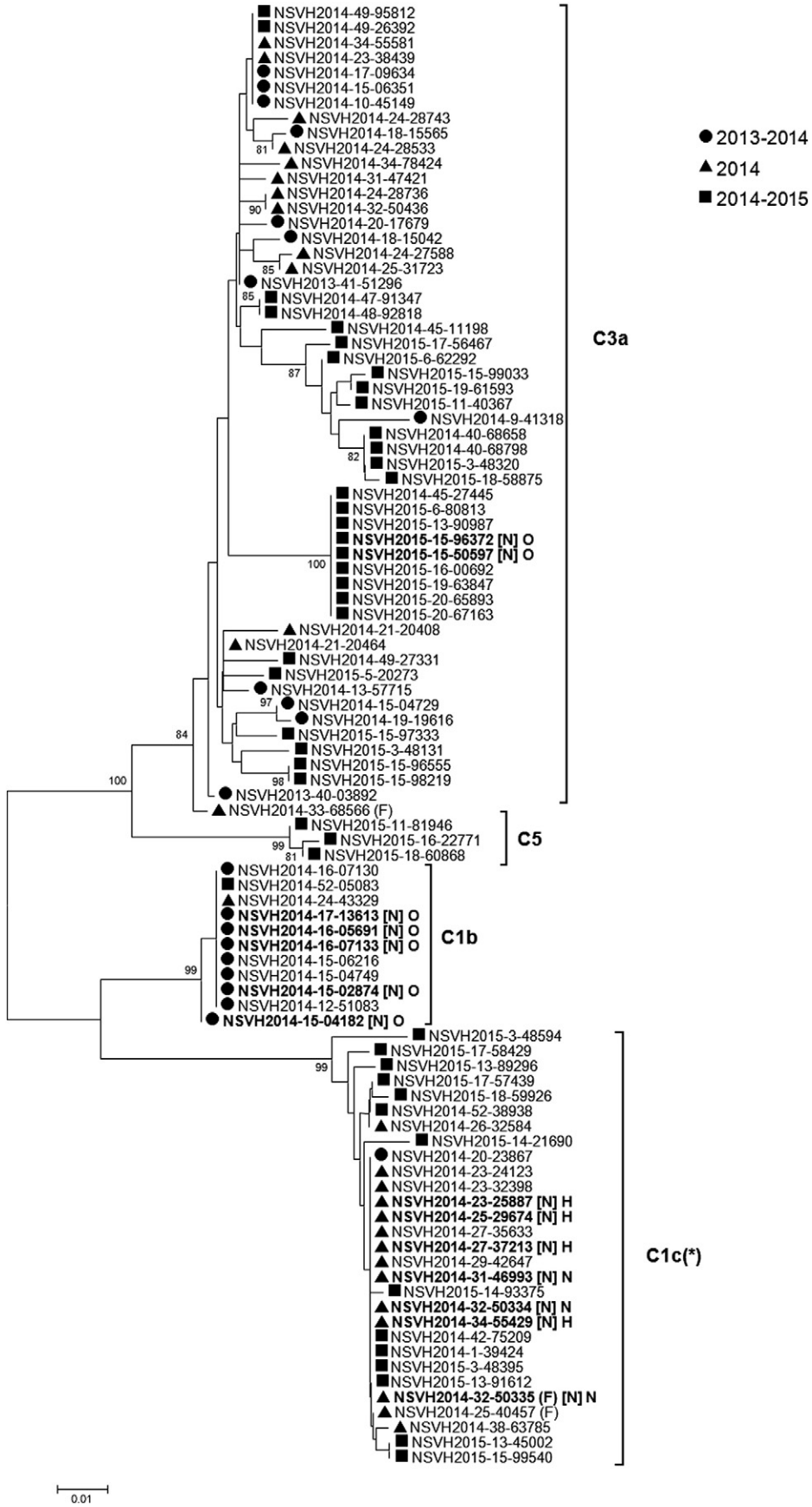

Fig. 3. Phylogenetic analysis based on the partial sequence of the *F* gene (390 nucleotides) from 97 HPIV-3 strains of the present study. The evolutionary distances were computed using Tamura-Nei method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (0,97). Only bootstrap values higher than 70% are shown. Novel lineage described C1c(*) are also included. Strains associated with suspected nosocomial outbreaks are indicated as [N] followed by different letters relative to the affected hospital ward (H-hematology, O-oncohaematology, N-neonatology). (F) Strains characterized only by the F protein.

assay with specific universal tailed primers (M13), as described in Table 1. PCR products were visualized as single bands by 1,5% agarose gel electrophoresis stained with SybrSafe® DNA Gel Stain (Invitrogen, USA). PCR products were subsequently purified using Exo-SAP-IT PCR Clean-up Kit (Affymetrix, USA) and sequenced by the ABI Prism Big Dye Terminator Cycle Sequencing Kit v3.1 in an ABI PRISM 3130XL sequencer (Applied Biosystems, USA), as shown in Table 1. Nucleotide sequences were assembled and edited using SeqScape v2.5 software (Applied Biosystems, USA).

2.4. Phylogenetic analysis

Phylogenetic analyses of HN and F sequences of the present study were performed together with homologous sequences from previous molecular epidemiological studies to be used as references, which were downloaded from NCBI GenBank database (Supplementary Table 1). Sequences were aligned using the MUSCLE program, implemented in MEGA v5.2 (Tamura et al., 2011). The molecular evolutionary models of nucleotide substitutions were fitted to the multiple sequence alignments using evolutionary analyses conducted in MEGA v5.2 (Tamura et al., 2011). The phylogenetic trees were reconstructed using neighbor-joining (NJ) distance method as implemented in MEGA v5.2 (Tamura et al., 2011) with the evolutionary model with the lowest Bayesian information criterion score. Reliability for the internal branch was assessed using the non-parametric bootstrap analysis with 1000 replicates.

2.5. Statistical analysis

Statistical analysis was performed using SPSS v17 (SPSS Inc., USA). Odd ratios (OR) and their 95% confidence intervals (CI) were calculated to assess associations between categorical variables. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Patient population

From week 40/2013 (October 2013) to week 20/2015 (May 2015), a total of 9,574 respiratory specimens from 5,888 patients (3,166 males, 54%; median age: 14.5 years; age range: <1 day – 97.8 years; interquartile range (IQR): 1.1–61.8 years) were received for respiratory virus detection, of which 108 (1%) specimens from 102 (2%) patients (56 males, 55%; median age: 1.5 years; age range: 13 days – 78.3 years; IQR: 5.1 months – 4.8 years) were HPIV-3 laboratory-confirmed. Among the 102 patients, 78 (76%) were children under 5 years of age and 3 (3%) were older than 65. Among patients younger than 5 years of age, 9 (11.5%) were under any degree of immunosuppression, whereas among patients over 5 years of age this percentage (19; 79.2%) was significantly higher (OR: 29.13; CI 95%: 8.73–97.25; $P< 0.0001$), many of whom (8; 42.1%) were HSCT recipients. Four (3.92%) patients died.

3.2. Co-detections with other respiratory viruses and seasonality

HPIV-3 co-detections were also found together with other respiratory viruses (7; 6,9%), such as rhinovirus (RV) (5; 71,4%), adenovirus (2; 28,6%) and enterovirus (2; 28,6%).

HPIV-3 detection was quite variable throughout the study period, but detection rates were higher during spring and summer months, showing maximum peaks at week 15 (spring) in both seasons (Fig. 1). A large number of detections were therefore reported during the 2014 inter-seasonal period.

3.3. Phylogenetic analyses

Phylogenetic analyses of HN (Fig. 2) and F (Fig. 3) sequences from 97 out of 102 confirmed cases revealed three phylogenetic groups (A, B and C), with subdivision of group C into different subgroups and lineages (C1a-b, C2, C3a-c, C4 and C5), as recently described by other authors (Almajhdi, 2015; Mao et al., 2012). All characterized HN (94/102) and F (97/102) sequences in the present study fell within C1b (11), C5 (3) and in other two closely C3a-related clusters (C3a1, 44; C3a2, 10), with bootstrap values higher than 80%. In addition, other 29 sequences clustered together within subgroup C with a 100% bootstrap value and a genetic distance (nucleotide differences per site) within group of 0.004. However, this genetic group showed a nucleotide divergence of 0.019 and 0.024 among C1a and C1b, respectively. According to Almajhdi (2015) this might be considered a new lineage, and was named in the present study as C1c following the current nomenclature (Almajhdi, 2015; Mao et al., 2012). A complete correspondence between the HN and F phylogenetic classifications was also observed. During the study period, viral variants belonging to different lineages were cocirculating, but showing variable rates among seasons (Supplementary Fig. 1). Sequences from five laboratory-confirmed specimens were partial or could not be obtained, due to the non-availability of the clinical sample for the sequencing, the low quality of the extracted NA, or the low viral load in the respiratory specimen.

Table 2
Predicted amino acid substitutions in antigenic epitopes of the HN glycoprotein previously characterized (Lawrence et al., 2004; Mizuta et al., 2014; van Wyke Coelingh et al., 1987).

Strain (Group, Subgroup or Lineage)	Amino acid substitutions*								
	K168	V289	N294	H295	A348	G354	V383	I391	T440
B				Y	V				
C1(a-c)	R			Y				V	
C2				Y				V	
C3a				Y				V	
NSVH2014–9-41318 (C3a1)	R								
NSVH2015–6-62292 (C3a1)	R								
NSVH2015–11-40367 (C3a1)	R								
NSVH2015–15-99033 (C3a1)	R								
NSVH2015–18-58875 (C3a1)	R								
NSVH2015–17-56467 (C3a1)	R	I					I		
NSVH2014–40-68658 (C3a1)	R					R			
NSVH2014–40-68798 (C3a1)	R					R			
NSVH2015–3-48320 (C3a1)	R								A
NSVH2014–18-15565 (C3a2)			S						
NSVH2014–24-28533 (C3a2)			S						
C3c				Y				V	
C4				Y					
C5				Y				V	

* Amino acid positions relative to prototype strain Wash/1957 (GenBank accession number M17641).

3.4. Amino acid sequence analysis of the HN protein

The comparison of the deduced HN amino acid sequences of the strains relative to the prototype strain (Wash/1957, GenBank accession number M17641) revealed some amino acid substitutions that define the different phylogenetic subgroups (Fig. 2). Furthermore, some amino acid substitutions fell within the previously characterized antigenic epitopes of the HN protein (Lawrence et al., 2004; Mizuta et al., 2014; van Wyke Coelingh et al., 1987), as shown in Table 2.

3.5. Epidemiologic investigation

The high similarity shown between some HN and F sequences (Figs. 2 and 3) suggested the probable nosocomial transmission among patients admitted in adult hematological, pediatric oncohaematological and neonatal intensive care units throughout the study period. Epidemiological investigation demonstrated, as shown in Fig. 4, that all suspected patients infected by C1c HPIV-3 and admitted in neonatal ICU, as well as, those infected by C3a HPIV-3 and admitted at the oncohaematological ward, coincided at some point in time and in space. However, nosocomial transmission in some C1b and C1c cases admitted at onco-hematology and hematology wards, respectively, could not be epidemiologically confirmed, but not discarded because of the lack of information.

All nucleotide sequences from the present study were submitted to GenBank database under accession numbers from KT796368 to KT796461 (complete HN sequences), and from KT796462 to KT796558 (partial F sequences).

4. Discussion

Although HPIV-3 is one of the most common respiratory viruses in particular among hospitalized patients, little and no recent information regarding genetic diversity of circulating strains is available in Europe.

During the study period, HPIV-3 prevalence in patients attended in our hospital was 2%, similar to the prevalence reported in the Catalan community (1–2%) through the PIDIRAC (Daily information on Acute Respiratory Illness Plan of Catalonia) Sentinel Surveillance Network, coordinated by the Health Public Agency of Catalan Government (http://grip.gencat.cat/ca/la_grip_professionals/documentacio). However, the studied cohorts of patients were different. The respiratory virus surveillance network in Catalonia is based on the laboratory-confirmation of respiratory specimens collected from outpatients with mild respiratory disease. Therefore, our results show a higher morbidity and mortality associated to this virus in our hospital, in particular in the youngest population or in patients with any degree of immunosuppression.

The results of this study showed that the population under 5 years of age has a greater susceptibility to HPIV-3 infection (Henrickson, 2003; Kim et al., 2013; Liu et al., 2013). But, since the HPIV-3 disease in immunocompetent adults usually courses as mild and self limited, non requiring hospital care attendance in most of cases, the number of adult HPIV-3 cases in our study might be underestimated. However, in the present study, the real clinical importance of HPIV-3 among patients older than 5 years with any degree of immunosuppression, mainly among HSCT recipients, is again highlighted (Cortez et al., 2001; Harvala et al., 2012; Jalal et al., 2007; Piralla et al., 2009).

The HPIV-3 co-infection rate (6.9%) with other respiratory viruses was similar to previous studies (5–27%) (Cilla et al., 2008; Pierangeli et al., 2007). Some studies also identified RV, as well as human coronavirus, as the most frequently detected viruses in co-detection with HPIV-3 (Mao et al., 2012). This finding is congruent because RV was one of the most prevalent respiratory viruses with a prolonged excretion period within the period of study in our hospital (data not shown), together with RSV and influenza viruses, that are the cause of seasonal epidemics every year. However, the HPIV-3 co-detection with RSV or with influenza viruses in the present study might not be surely well-represented, because these epidemic viruses are mostly detected by using point-of-care tests during the annual epidemics, and no further microbiological studies are usually performed to detect additional respiratory viruses. Nevertheless, a large number of co-detections should not be expected because, unlike RSV and influenza viruses that circulate during winter months, HPIV-3 usually did during the spring and summer months (inter-seasonal period), as previously described (Henrickson, 2003; Liu et al., 2013). These results also highlight the importance of the surveillance or detection of other respiratory viruses different from RSV and influenza viruses during the inter-seasonal period, particularly in patients with high risk of severe HPIV-3 infection.

In this study we provide valuable information about the genetic diversity of current circulating HPIV-3 strains due to the lack of recent knowledge in Europe. Different phylogenetic variants were simultaneously co-circulating, but showing variable prevalence among seasons. Phylogenetic group C remained as the most dynamic and widespread group worldwide (Almajhdi, 2015; Mao et al., 2012). Nucleotide sequences analyzed in comparison with different international strains demonstrated an association with some Chinese strains (C3a) (Mao et al., 2012) and Saudi Arabia strains (C1b and C5) (Almajhdi, 2015; Almajhdi et al., 2012), whereas no association with strains from America (Elango et al., 1986; van Wyke Coelingh et al., 1988), India (unpublished) or Australia (Lawrence et al., 2004; van Wyke Coelingh et al., 1988) was observed. In addition, a new lineage (C1c) was first described in the present study. It might have been circulating in previous seasons but the scarce molecular characterization of this virus in the European countries did not enable to discover it earlier.

The classifications by phylogenetic analyses of HN and F sequences showed a complete correspondence. Therefore, partial *F* gene sequencing might be used much more often for epidemiological studies than complete HN sequence, because the process by using a single PCR product is quite streamlined. Nevertheless, complete sequencing of the HN coding region, instead of the partial *F* gene, would be preferable to detect amino acid substitutions that might be related to changes in the functionality and antigenicity of the protein (Almajhdi, 2015; Almajhdi et al., 2012; Mao et al., 2012). Some amino acid substitutions within characterized antigenic sites were found in some strains. However, to acquire a better understanding of the importance of these mutations, further studies on the catalytic activities and antigenicity of the HPIV-3 HN protein are needed. In addition, recent knowledge about the genetic and antigenic features of current circulating strains should be relevant for vaccine development, and it is therefore

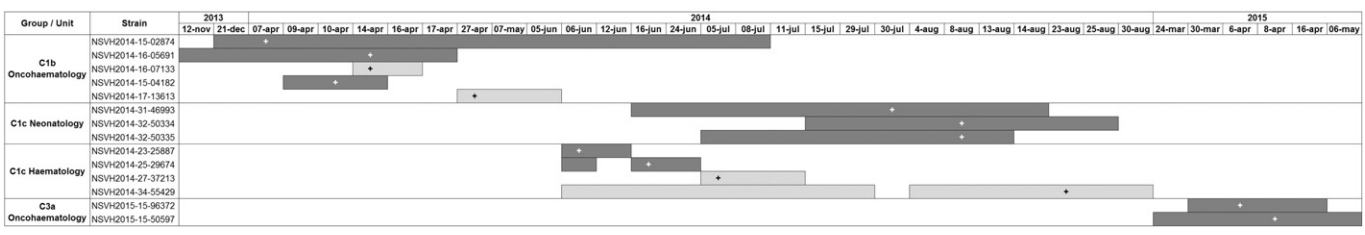

Fig. 4. Chronological appearance of the suspected HPIV-3 nosocomial clusters by phylogenetic analyses with (dark gray) or without (light gray) epidemiological confirmation. With a plus (+) the date of HPIV-3 laboratory-confirmation.

advisable to carry out a continuous viral surveillance to have an updated information.

Previous studies described nosocomial HPIV-3 outbreaks, especially in onco-hematological wards (Jalal et al., 2007; Lee et al., 2011; Piralla et al., 2009). In the present study phylogenetic analyses retrospectively revealed the high similarity between HN and F sequences of viruses from patients hospitalized or attended in the same unit for a short period of time, suggesting a possible nosocomial transmission, which was later confirmed by epidemiological investigation in some cases. In addition to rapid HPIV-3 laboratory-confirmation, the prospective implementation of molecular epidemiological analysis, especially in patients at high risk of severe infection, might provide the prompt recognition of nosocomial outbreaks, essential for the early implementation of spreading control measures (Berrueco et al., 2013; Lee et al., 2011).

In summary, valuable information about the genetic diversity of recent circulating HPIV-3 viruses in Europe is here reported. Continuous surveillance studies of HPIV-3 are needed to detect novel viral variants that might acquire genetic mutations related to changes of antigenic or tropism features, because they might become a challenge in the development and future use of vaccines. The suitability of sequencing to detect and to monitor prospectively nosocomial outbreaks is also remarked.

Ethical approval

Institutional review board approval (PR_AG_156/2015) was obtained from the Hospital Universitari Vall d'Hebron Clinical Research Ethics Committee.

Conflict of Interest

None.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.diagmicrobio.2016.07.023.

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