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Genotyping of a nosocomial outbreak of pandemic influenza A/H1N1 2009

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ABSTRACT

Background: Epidemiological surveys have revealed outbreaks of pandemic influenza A (H1N1) 2009 in several different contexts. Molecular characterization of the influenza virus could help to provide a more accurate description of these outbreaks.
Objective: To genotype pandemic influenza A (H1N1) 2009 isolates from an epidemiologically defined nosocomial outbreak.
Study design: We sequenced the neuraminidase (NA) and hemagglutinin (HA) influenza A (H1N1) 2009 genes from ten HIV-positive patients involved in an epidemiologically defined outbreak in the Clinical Microbiology and Infectious Diseases (CMID) Department. Sequences were aligned to search for specific genetic features of the involved strain. We also analyzed 37 unrelated influenza A (H1N1) 2009 cases from other hospital departments. All the sequences were used to obtain phylogenetic trees.
Results: Identical genotypic features were shared by nine of the 10 cases initially considered to be involved in the outbreak, but not by the remaining case. These features involved two silent mutations at N385 and V407 in the NA gene and three amino acid substitutions in the HA gene (D225E, A189T, and P300S). Searching for these substitutions in patients with influenza A (H1N1) 2009 hospitalized in other departments during the same period allowed us to identify an additional unsuspected immunocompetent case. The five outbreak-specific substitutions were absent in the remaining 36 unrelated controls. One of the substitutions (P300S) rendered detection of this variant by the CDC protocol inefficient. The other outbreak-specific substitutions (D225E and A189T) were identified at codons that have been analyzed in the context of virulence.
Conclusions: Genotyping is essential to ensure a more accurate description of pandemic influenza A (H1N1) 2009 outbreaks.

1. Background

Since the emergence of the pandemic influenza A (H1N1) 2009 variant, genotyping has been applied to monitor antiviral resistance and virulence mutations.[1–4] In addition, genotyping and molecular phylogenetic analysis can provide a more accurate description of epidemiologically suspected transmission events involving the new variant.[5,6]

We used these molecular strategies to characterize an epidemiologically defined nosocomial outbreak of influenza A/H1N1v detected during 19th–25th October, 2009.[7] Identification of specific genetic markers for the variant involved allowed us to refine the description of the outbreak. We discuss the involvement of some of these mutations in RT-PCR-based diagnosis and transmissibility.

2. Objective

To use genotyping and molecular phylogenetic methods to analyze the influenza A (H1N1) 2009-positive isolates involved in an epidemiologically defined nosocomial outbreak.

3. Study design

3.1. Patients

We performed a retrospective study of 49 patients with confirmed influenza A (H1N1) 2009 infection (12 from the HIV

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hospitalization unit of the Clinical Microbiology and Infectious Diseases [CMID] Department and 37 patients from other hospital units). Among the 37 controls from other hospital units, 23 isolates were from specimens taken during the outbreak period (±1 week); one sample was taken 20 days before, 7 were obtained between 1 day and 10 days later, and 6 between 18 days and 45 days later.

3.2. Molecular analysis

RNA was extracted from nasopharyngeal swabs. The presence of influenza A (H1N1) 2009 was detected using real-time RT-PCR according to the WHO/CDC protocol (http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf). The neuraminidase (NA) and hemagglutinin (HA) genes were sequenced using the primers previously reported by the WHO/CDC. NA and HA mutant sequences were submitted to GenBank (accession numbers HQ121411, HQ121412, and HQ121413).

All the RT-PCR and sequencing reactions were performed twice to confirm the results and, when available, in consecutive specimens from the same patient.

3.3. Phylogenetic analysis

Partial HA and NA sequences from this study (accessions HQ121411, HQ121412 and HQ12413) and 2 controls were aligned and phylogenetic trees were obtained using MEGA 5.05[8] and the maximum likelihood approach. The most appropriate evolutionary model for maximum likelihood reconstruction was determined by selecting the model with the lowest Bayesian information criterion (BIC) value for each gene. The reliability of the inferred clades was evaluated by bootstrapping based on 1000 pseudo-random replicates.

4. Results

At admission, the 12 HIV-infected cases (median CD4 count, 149 cells/μL) involved in the outbreak[7] in the CMID department were symptom-free in relation to influenza; median time to onset of influenza symptoms after admission was 20 days (IQR, 11.5–43.2). Patients were hospitalized for several reasons: cryptococcal meningitis (one patient), non-Hodgkin lymphoma (1 patient), soft tissue infection (1 patient), right foot ischemia in a patient with known peripheral vascular disease, pulmonary tuberculosis (2 patients), hepatic decompensation (2 patients), acute chronic obstructive pulmonary disease (2 patients), septic arthritis (1 patient), and pneumococcal pneumonia (1 patient).

Viral RNA was available from ten of the 12 HIV-infected patients with influenza A (H1N1) 2009 infection (CMID1–CMID10). The complete amplified sequence for the HA and NA regions was analyzed to identify specific genotypic features. Five outbreak-specific substitutions were identified for all but one of the ten cases. Therefore, this case (CMID10) was not considered as being involved in the outbreak. Of the 5 substitutions which characterized the outbreak, we identified 3 amino acid substitutions at codons 189 (A189T), 225 (D225E), and 300 (P300S) in the HA gene and two silent mutations at N385 (from AAT to AAC) and V407 (from GTT to GTC) in the NA gene.

The 5 outbreak-specific substitutions were analyzed in 37 additional cases of influenza A (H1N1) 2009 infection from hospital units other than the CMID department (23 cases temporally related, within ±7 days), and in 34 cases, none of the substitutions were found, thus reinforcing the clonal robustness of the outbreak. In two cases (GMH2 and GMH3), only D225E and P300S were detected, and in the remaining case (GMH1), hospitalized in a department other than CMID, we identified all five outbreak-specific substitutions.

Maximum likelihood phylogenetic trees were obtained with gamma models for NA[9] and HA,[10] as determined by their lowest BIC values among 24 alternative models tested with MEGA. In both trees (data not shown), all the sequences from the CMID unit (with the exception of CMID10) clustered in a distinct, well-supported clade that also included the sequence from the patient from a different department (GMH1). To better reveal the distinctiveness of the outbreak clade, we obtained a concatenated sequence with the partial HA and NA sequences of each isolate, and the corresponding multiple alignment was used to derive the maximum likelihood tree (Fig. 1) using the Tamura92 + gamma model. The major features of this tree were consistent with those obtained for each partial gene separately, and bootstrap support values were higher. The clade encompassing outbreak-derived sequences was highly supported (BS = 89%), its value being the highest for non-identical sequences in the analysis. As described, it included nine sequences from the CMID (all except CMID10) and that of the patient from a different hospital department (GMH1).

Case GMH1 was a 79-year-old immunocompetent woman hospitalized in a different department to the CMID. Her clinical history revealed that she had been admitted one month earlier for scheduled surgery and that her diagnosis of influenza was made during the outbreak, thus fulfilling the criteria for nosocomial acquisition.

One of the substitutions mentioned (P300S) overlaps with the CDC swineH1 probe, which is used to diagnose influenza A (H1N1) 2009 infection. Identification of influenza A (H1N1) 2009 following WHO/CDC protocols in the specimens involved in the outbreak was rendered inefficient by this mismatch. These specimens displayed an abnormal RT-PCR-positive amplification signal for swH1 (low fluorescence and high Ct), which did not correlate with the proper amplification signal detected for InfA. In these cases, the RealTime ready Inf A/H1N1 Detection Set (Roche Diagnostics, Mannheim, Germany) was applied. Genetic sequencing is an easy and useful way to monitor changes in the virus that might affect molecular diagnosis. Finally, the specific NA gene mutation associated with oseltamivir resistance (H275Y) was not found in the outbreak strain. Virulence mutations in the HA gene (D225G and D190E) were not found either; however, other substitutions at codon 225 (D225E) and at a codon adjacent to 190 (A189T) were identified.

5. Discussion

Several reports have focused on transmission events involving influenza A (H1N1) 2009,[11–13] and even on events involving high-risk resistant and/or virulent influenza A (H1N1) 2009 variants.[5,14,15]

In addition to the interest in identifying the transmission of high-risk variants, the survey of transmission events by the new pandemic variant is relevant from an epidemiological point of view.[16,17] In our institution, epidemiologic analysis revealed an outbreak involving HIV-positive patients hospitalized in the CMID department. During the outbreak, strict respiratory isolation measures were observed for all patients with suspected or confirmed influenza A (H1N1) 2009 infection, and communal areas of the unit were closed down. These measures prevented additional spread.

Application of molecular tools helped to refine the description of the outbreak. The finding of five outbreak-specific substitutions allowed us to identify a case that was initially suspected of being involved but that was later revealed to be infected by a different strain and a case hospitalized in another department whose viral variant had the same substitutions as the outbreak variant. The latter finding was especially relevant, as it led us to include an immunocompetent case from an independent department in an outbreak that had initially been restricted to immunosuppressed cases and to a single department. It seems that there was a potential

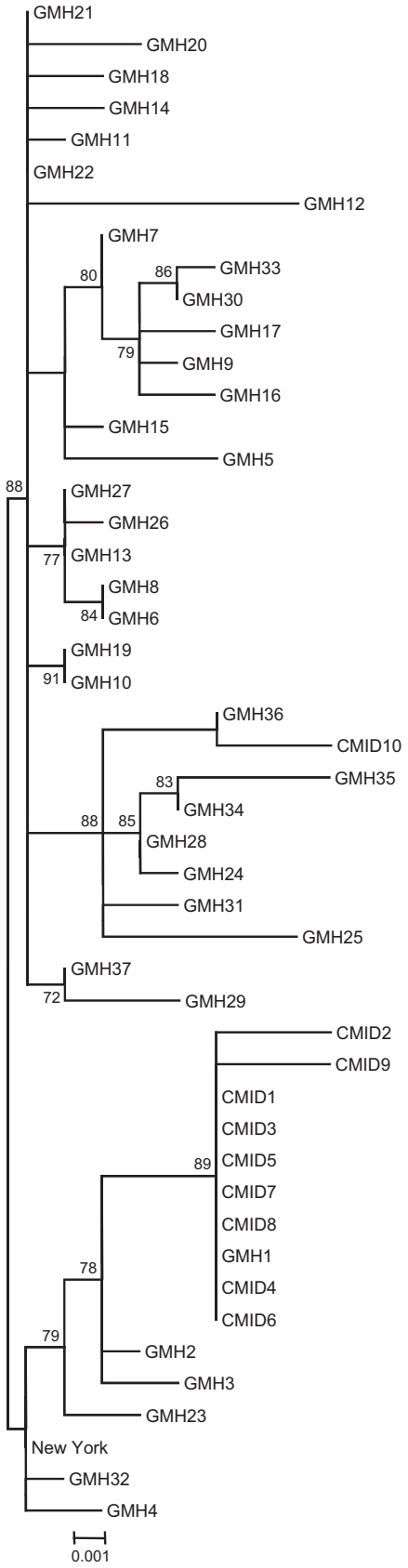

Fig. 1. Maximum likelihood phylogenetic tree obtained with the concatenated alignment of HA and NA sequences of pandemic H1N1 2009 viruses from outbreak isolates and controls, using the Tamura92+gamma model of evolution. Bootstrap support values after 1000 pseudo-random replicates higher than 70% are shown in the corresponding nodes.

epidemiological link between the patients in the CMID department and the immunocompetent case, since a physician from the department where the immunocompetent woman was attended had been requested (through interdepartmental consultation) to examine a patient affected by the outbreak in the HIV unit immediately before the immunocompetent woman's diagnosis of influenza was confirmed.

One of the substitutions identified in the influenza A (H1N1) 2009 strain involved in the outbreak—P300S in the HA gene—rendered detection by the CDC protocol inefficient, and this may have masked other infected cases. P300S caused a mismatch in the hybridization sequence for the CDC swH1 probe. Therefore, our findings highlight the need for further studies on influenza A cases in which altered influenza A (H1N1) 2009 amplification is detected. Such studies could help to refine the molecular tools used to detect influenza A (H1N1) 2009. Other authors have reported mismatches in the binding regions targeted by CDC primers and swH1probes that affect the sensitivity of the CDC protocol in detecting influenza A (H1N1) 2009.[18,19] Our finding indicates that periodical DNA sequencing can be used to monitor changes in the virus that might affect molecular diagnosis.

Also noteworthy is the finding of the substitutions D225E and A189T, which map in positions that either coincide with or are adjacent to codons where other changes have been considered to be involved with severity[1] and virulence[20]. The simultaneous presence of D225G and D190E (due to expansion of viral receptor specificity) has been considered relevant for the acquisition of a hypervirulent phenotype during the 1918 influenza pandemic.[21] While the role of D225E in virulence has been ruled out,[22,23] the combination of the D225E mutation and the non-conservative substitution at the adjacent codon 189 (A to T) described in this outbreak could be a variant of those found during the 1918 pandemic and, therefore, could have a potential role in increased transmissibility. However, we only found four severe cases among those sharing these genetic features, with the result that this hypothesis has yet to be proved. A forthcoming survey of this variant in contexts where molecular protocols efficiently targeted the accompanying substitutions could help to clarify the role of these substitutions, not necessarily in severity, but in transmissibility.

We describe the genetic characterization of a nosocomial outbreak of pandemic influenza A (H1N1) 2009 infection. Molecular fingerprinting enabled us to confirm the epidemiologically suspected outbreak and to identify cases that had been either wrongly included or not suspected of being involved. Genotyping extended the geographic limits of the outbreak and the clinical profile of the cases involved. Some outbreak-specific genetic substitutions had additional relevance. One led to inefficient detection of the outbreak variant by the CDC protocol. A further two corresponded to codons that may play a role in virulence or transmissibility.

Conflict of interest

This study does not present any conflicts of interest for its authors.

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