JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2010, p. 3817–3819 Vol. 48, No. 10
0095-1137/10/$12.00 doi:10.1128/JCM.01237-10

Fast-Track Communication

Evidence for Amino Acid Changes in a 57-Base-Pair Region of the Highly Conserved Matrix Gene of Pandemic (H1N1) 2009 Influenza A Virus▽

Received 18 June 2010/Returned for modification 6 July 2010/Accepted 22 July 2010

Pandemic (H1N1) 2009 influenza A virus originated due to triple reassortment of human, avian, and swine viruses (6). The pandemic epicenter was in Mexico, where the first human case was reported on 12 April 2009 (1). Thereafter, 16,813 pandemic H1N1 influenza A-related deaths have been reported to the World Health Organization (WHO) by 213 countries between April 2009 and March 2010 (8). From a laboratory perspective, detection and discrimination of pandemic H1N1 from seasonal and swine H1N1 is crucial for accurate diagnosis and disease surveillance.

We previously described a laboratory-developed influenza A virus real-time reverse transcription-PCR (rRT-PCR) assay (Mayo FLU A) for simultaneous identification and subtype discrimination of influenza A virus RNA using melting temperature (T_m) analysis (3, 9). As discussed in the paper by Dhiman et al., loss of subtype discriminatory ability occurred in just 3 months of testing for the pandemic virus, due to multiple point mutations in the targeted 242-base region of the highly conserved matrix (M) gene as determined on a subset of 12 isolates (3). In the current paper, we have extended our analysis to a significantly larger data set to conduct mutational and amino acid analysis and characterize the genetic changes in the M gene of the pandemic H1N1 influenza A virus.

Between 1 May 2009 and 31 December 2009, we detected 1,731 influenza A virus clinical isolates out of 9,614 isolates tested (18% positivity rate) using the Mayo FLU A assay; the majority were identified as the pandemic H1N1 subtype based on predefined T_m criteria (50.5°C to 53.2°C). However, between 14 August and 31 December 2009, 48 clinical samples were identified with T_ms outside the validated range for pandemic H1N1 (48 of 1,579 isolates [3.0%] during this period), with an overall lower mean T_m of 46.71 ± 1.73°C. Only one specimen, from Ann Arbor, MI, had a higher T_m of 55.5°C, which is unexpected, given that mutations typically cause a decrease rather than an increase in T_m. The significance of this result is unknown, as we did not have additional specimens from that geographical area to confirm if this result was an actual trend or an outlier.

All atypical T_m samples were confirmed as pandemic H1N1 using the CDC swine flu assay. We sequenced the 242-base oligonucleotide amplicon in 37 isolates to identify the mutations responsible for the atypical T_ms. Mutational analysis revealed multiple point mutations in the 57-bp region detected by the probes compared to the original pandemic H1N1 gene sequence published by the WHO on 28 April 2009 (2) (Table 1). Six of these were silent mutations, resulting in nucleotide changes GAG→GAA, ATC→ATT, GCG→GCA, AGA→AGG, CTG→TTG, and AAC→AAT at amino acid positions 23, 24, 25, 27, 28, and 36, respectively, compared to the original pandemic H1N1 gene sequence. Of interest, two additional nonsynonymous mutations, CTG→ATG and ACA→ATA, resulted in downstream changes in amino acid sequence at positions Leu28Met and Thr37Ile, respectively (Fig. 1). We also observed geographic clustering among the mutations in these analyses (Table 1).

Since the emergence of pandemic H1N1, several studies have tried to characterize the mutational trends of this new virus (4, 5, 7). Pan et al. described the emergence of a signature residue at the position of nucleoprotein 100 (NP-100) (valine to isoleucine) that exhibited a dominant change in as many as 93% of isolates by the later phases of the pandemic. In addition, four nonsignature residues, at positions neuraminidase 91 (NA-91), NA-233, hemagglutinin 206 (HA-206), and nonstructural protein 1 (NS1) to NS123, were observed during a short period of time within the epidemic (5). All of these mutant residues were characterized in the viral functional domains, suggesting potent roles in the human adaption and virulence. Mutations were also observed in M1/M2 genes, but they were of low frequency with an unknown role. Potdar et al. reported several mutations throughout the viral genome, including a predominant D225G mutation in the H gene present in the receptor binding domain of Indian novel H1N1 isolates (7). Nelson et al. conducted a whole-genome phylogenetic analysis on 290 isolates of pandemic H1N1 influenza virus collected globally and identified seven major clades that have cocirculated worldwide since April 2009 (4). Several amino acid changes were also identified, predominantly in the HA, NA, and NP genes. However, no mutations were observed in the matrix gene in the latter two studies.

In our study, we found eight mutations in a very short sequence span of only 57 bp of the influenza A virus matrix gene, of which two were nonsynonymous (Leu28Met and Thr37Ile). This is an unexpected finding, as the influenza A virus matrix gene is relatively conserved and is not thought to be under the same genetic selective pressures as other genes, such as HA and NA, that have very high mutability rates. The implications of these findings for viral adaptation and virulence have yet to be determined. However, from a diagnostic perspective, these mutations resulted in the loss of viral subtype discriminatory ability using T_m analysis within just 3 months of the pandemic and hindered laboratory diagnosis. Evidence for mutability in the highly conserved M gene of influenza virus calls for pandemic as well as routine *in silico* monitoring of primers and probes for optimal target coverage.

TABLE 1. Sequence alignment in the discriminatory region of the amplified matrix gene from 37 clinical isolates with atypical T_ms using the Mayo FLU A assay

Sequence source or patient no.	Mayo FLU A T_m (°C)	State[a]	Sequence of discriminatory region of M gene[d]		
			Fluorescein probe	CT[b]	Red 640 probe
WHO sequence			CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
1	55.5	MI	CGA**A**ATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
2	47.8	PA	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
3[c]	47.4	PA	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
4[c]	47.4	PA	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
5[c]	47.5	PA	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
6[c]	47.6	PA	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
7	47.9	PA	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
8	47.6	PA	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
9[c]	41.6	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**TAT**AGATCTTGAGGC
10	41.8	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**TAT**AGATCTTGAGGC
11[c]	41.9	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**TAT**AGATCTTGAGGC
12	47.6	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
13	47.6	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
14	47.7	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
15	47.5	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
16	48.0	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
17	47.6	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
18	47.6	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
19	47.7	OH	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
20	46.1	OH	CGAGATCGC**A**CAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
21	45.6	OH	CGAGATCGC**A**CAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
22	45.4	OH	CGAGATCGCGCAGAGA**TT**GGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
23	45.1	MN	CGAGATCGCGCAGAGA**AT**GGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
24	45.1	MN	CGAGATCGCGCAGAGA**AT**GGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
25	46.2	MN	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
26[c]	47.4	MN	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACA**T**AGATCTTGAGGC
27[c]	47.3	MN	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACA**T**AGATCTTGAGGC
28[c]	48.1	MN	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
29	45.3	MA	CGAGATCGCGCAGAG**G**CTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
30	45.1	MA	CGAGATCGCGCAGAG**G**CTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
31	45.5	MA	CGAGATCGCGCAGAG**G**CTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
32	45.5	MA	CGAGATCGCGCAGAG**G**CTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
33[c]	45.7	AZ	CGAGATCGC**A**CAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
34[c]	46.0	AZ	CGAGATCGC**A**CAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
35	46.0	AZ	CGAGATCGC**A**CAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC
36	47.3	Kuwait	CGAGATCGCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAA**T**ACAGATCTTGAGGC
37	44.4	NY	CGAGAT**T**GCGCAGAGACTGGAAAGTGT	CT	TTGCAGGAAAGAACACAGATCTTGAGGC

[a] State where patient resided, specifically Ann Arbor, MI; Sewickley, PA; Beaver, PA; Cleveland, OH; Owatonna, MN; Racine, MN; Rochester, MN; Spring Valley, MN; Northampton, MA; Phoenix, AZ; Salusai, Kuwait; and Utica, NY.

[b] CT is a two-base-pair sequence between the fluorescein probe and the red 640 probe.

[c] Previously published sequences (3). One previously published sequence was not included in the final data due to inconsistent results.

[d] All mutations are shown in bold.

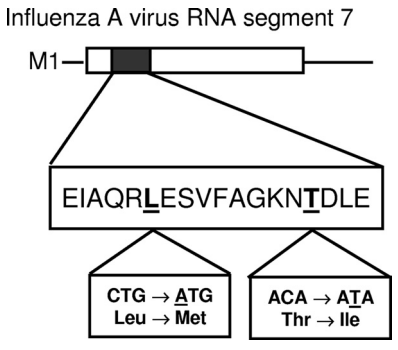

FIG. 1. Amino acid sequence of the 57-bp discriminatory region of pandemic (H1N1) 2009 influenza A virus.

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▽ Published ahead of print on 4 August 2010.