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Protocols

Early detection of influenza A(H5) viruses with affinity for the human sialic acid receptor by MALDI-TOF mass spectrometry based mutation detection

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Highly pathogenic avian influenza (HPAI) A(H5N1) strains have been causing sporadic cases of disease in South East Asia and Africa for many years. These cases are associated with a high fatality rate, and it is feared that the virus could evolve into a strain capable of causing a pandemic.

It is likely that a requirement for a A(H5) pandemic to occur is a switch in the receptor affinity of the virus. Candidate mutations in the hemagglutinin glycoprotein have been identified in the literature, and their emergence in circulating viruses would be an ominous development.

This study describes a method to identify the presence of these mutations, even within a quasispecies, using RT-PCR followed by *in vitro* translation and peptide characterization by MALDI-TOF mass spectrometry.

1. Introduction

The highly pathogenic strains of avian influenza A(H5N1) currently circulating in Asia and Africa (Li et al., 2004; WHO, 2005) continue to raise concerns about its emergence into a pandemic strain. Although this virus remains poorly transmissible between humans, as of November 19, 2010 there have been 508 human cases, associated with a very high mortality rate of approximately 59% (WHO, 2010). To date, the majority of infections have been acquired by exposure to infected birds which are known to shed the viruses in abundance. The mutations required for the HPAI A(H5N1) virus to become fully adapted to human hosts and trigger a pandemic are not known with certainty. There is however a broad agreement that a key requirement for the virus to become a pandemic hazard would be enhanced transmissibility between humans.

Currently, HPAI A(H5N1) has retained the receptor affinity that is typical of avian influenza viruses, namely an affinity for a terminal sialic acid (SA) linked to a galactose by an α-2,3 glycosidic bond (α-2,3 SA). The ability of the virus to cause a lower respiratory tract (LRT) infection in humans has been explained by the demonstration of a small number of cells in the LRT, characterized as pneumocytes type II, that carry the α-2,3 SA receptor (Shinya et al., 2006; van Riel et al., 2006). It is postulated that in order to become a pandemic threat, a necessary requirement for the HPAI A(H5N1) virus will be a switch in receptor affinity from α-2,3 SA to α-2,6 SA, based on the much greater availability of the α-2,6 SA receptor on respiratory epithelial cells in both the upper respiratory tract and the lower respiratory tract. The requirement for receptor affinity switching is further supported by the empirical observation that the seasonal influenza strains and the pandemic strains of 1918, 1957 and 1968 utilized the α-2,6 SA receptor (Harvey et al., 2004; Kuiken et al., 2006; Stevens et al., 2006b; Vines et al., 1998).

While the mutations required for A(H5) viruses to switch their receptor affinity are not known with certainty, several lines of evidence point toward specific mutations as likely candidates. Three structural elements can be identified in the receptor binding domain (RBD) of the HA glycoprotein: the 190 α-helix; the 130-loop; and the 220-loop (Stevens et al., 2006b). Within the 220-loop the amino acids (a.a.) at positions 226 and 228 (numbering as per the A(H3) subtype) appear to be critical for receptor specificity in

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subtypes A(H2) and A(H3): Leu 226 and Ser 228 favor the α-2,6 SA, Gln 226 and Gly 228 the α-2,3 SA (Stevens et al., 2006a; Vines et al., 1998). Site-directed mutagenesis of human A(H3) that reinstated the a.a. found in avian A(H3) isolates resulted in the expected switch back to affinity with α-2,3 SA (Vines et al., 1998). Because the HA glycoprotein of A(H5) belongs to the same clade as A(H2), these mutations constitute a logical candidate for an affinity switch in A(H5). This hypothesis is supported by X-ray crystallography studies of the A(H5N1) HA bound to receptor analogs (Ha et al., 2001) as well as by data from hemadsorption assays using the native A(H5N1) HA and a modified HA obtained by site directed mutagenesis (Harvey et al., 2004), and by studies of the binding of the HA glycoprotein on glycan arrays (Stevens et al., 2006b), which confirmed the specificity of the native A(H5N1) HA for α-2,3 SA and showed that a modified HA with Q226L and G228S had decreased affinity for α-2,3 SA. The modified HA did not bind to the classical α-2,6 SA receptor but did bind to a naturally occurring branched α-2,6 SA biantennary glycan; some human influenza virus strains are known to bind to this biantennary SA receptor (Stevens et al., 2006b). In a more recent study, Ayora-Talavera et al. (2009) further confirmed that an engineered HA double mutant Q226L and G228S of A(H5) displayed a binding pattern to epithelial cells of human tracheal tissue sections strikingly similar to that of human A(H3) subtype virus, and in fact even the single mutant G228S showed a modified enlarged binding pattern encompassing both ciliated and non ciliated cells. Stevens et al. (2006b) commented that the single mutant G228S acquired binding to the biantennary α-2,6 SA but noted that all natural isolates of human influenza harbor the double mutation and speculated that Q226L may be required for the stability of the HA glycoprotein.

Auewarakul et al. (2007) identified recently another pair of mutations within or close to the 130-loop of the RBD that, when present together, are predicted to alter the binding specificity of the A(H5N1) HA from α-2,3 SA to α-2,6 SA, based on molecular modeling studies. These mutations, L129V and A134V, were demonstrated in an isolate of HPAI A(H5N1) from a fatal case, and the predicted receptor affinity switch corroborated by hemagglutination assays (Auewarakul et al., 2007).

In summary, two pairs of mutations have been identified as candidates that facilitate the switch of the SA binding specificity of the HPAI A(H5N1) toward the human receptor. For each candidate pair it appears that both a.a. changes must be present. It is more probable that these mutations would appear sequentially rather than simultaneously (Ayora-Talavera et al., 2009), and consequently occurrence of single or double mutants may well be present in only a fraction of the viral population in a host, i.e. as a viral quasispecies. Indeed, in the isolate analyzed by Auewarakul et al. (2007) the mutant was detected as part of a quasispecies.

In order to better monitor for the emergence of these mutants with modified binding receptor affinity, this study proposes the use of a technique previously reported for the monitoring of hepatitis C virus (HCV) quasispecies, which used *in vitro* translation of the amplicons obtained by amplification of a viral genome region of interest, followed by MALDI-TOF mass spectrometry analysis (Ayers et al., 2002; Yea et al., 2007b, 2009).

2. Materials and methods

2.1. Viral RNA

Influenza A(H5N1) RNA was obtained from A(H5N1) Vietnam 2004, as described previously (Yea et al., 2007a).

2.2. Primers

Primers for the amplification and cloning of full length cDNA for the RNA segment coding for the H gene of A/1957(H2N2) were modified from universal primers for the HA segment (Hoffmann et al., 2001) to include restriction enzyme sites and a T7 core promoter to transcribe negative-strand RNA from the amplicon. The primer sequences were: BamHA-1: 5′-TAT TGG ATC CAG CAA AAG CAG GGG-3′; HindT7HA-2: 5′-ATC TCG AAG CTT TAA TAC GAC TCA CTA TAA GTA GAA ACA AGG GTG TTT T-3′.

Primers H5RBD1 and H5RBD2 were used as the outer pair in a nested PCR bracketing a region within the 220-loop of the RBD. The sequences of the primers were: H5RBD1: 5′-ATG TTT CCG TTG GAA CAT CAA CAC T-3′; H5RBD2: 5′-TTC TTG ACA ATT TTG TAC GCA TAT TC-3′.

Primers H5RBD3 and H5RBD4 constitute the inner pair of the nested PCR and also contained sequences to generate peptides in a coupled *in vitro* transcription translation reaction, including a core T7 promoter, Kozak consensus sequence and FLAG epitope coding sequence; they were modeled after the primers HCFLAGALA and HCFLAG-4 used for MALDI-TOF mutation detection of HCV quasispecies (Ayers et al., 2002). The sequences of the primers were: H5RBD3: 5′-CAC GAA TTC TAA TAC GAC TCA CTA TAG GGA CAG CCT GTG TTG CCA TGG CTG ACT ACA AGG ACG ACG ATG ACA AGA CAT CAA CAC TAA ACC AGA G-3′; H5RBD4: 5′-TTT TTT TTT TTT TTT ACT TGT CAT CGT CGT CCT TGT AGT CAG CAA TGA AAT TCC CAT TAC T-3′.

Similarly, for the region in the 130-loop of the RBD we designed primers for a nested RT-PCR. The sequences for the outer pair, H5RBD5 and H5RBD6, were as follows: H5RBD5: 5′-GCA GAA TAA ACC ATT TTG AGA AAA TTC AG-3′; H5RBD6: 5′-GTT GGG TAT GTA CTG TTC TTT TTG ATA AGC C-3′.

For the inner pair, the primers were also modified from (Ayers et al., 2002) in order to enable peptide synthesis: H5RBD7: 5′-CAC GAA TTC TAA TAC GAC TCA CTA TAG GGA CAG CCT GTG TTG CCA TGG CTG ACT ACA AGG ACG ACG ATG ACA AGG AGA AAA TTC AGA TCA TCC C-3′; H5RBD8: 5′-TTT TTT TTT TTT TTT ACT TGT CAT CGT CGT CCT TGT AGT CCT TTT TGA TAA GCC ATA CCA C-3′.

2.3. Long RT-PCR amplification and cloning of the full-length cDNA of the HA segment

2 μl of primer BamHA-1 (10 μM stock) and 1 μl of dNTP mix (10 mM each) were added to 10 μl of RNA and the mixture incubated at 65 °C for 5 min and put on ice. Then, 7 μl of a master mix containing 4 μl of Superscript III RT buffer 5× (Invitrogen), 1 μl of DTT (100 mM; Invitrogen), 1 μl of Superscript III reverse transcriptase (Invitrogen) and 1 μl of DNase and RNase free molecular grade double distilled water (ddH_2O; Invitrogen). The reaction was incubated at 47 °C for 1 h; finally, 1 μl of RNase H (Invitrogen) was added and the reaction incubated at 37 °C for 20 min. For the long PCR, 2 μl of the RT mix were added to 48 μl of a master mix containing 5 μl of 10× Advantage 2 buffer (Clontech), 1.25 μl of dNTP mix (10 mM each), 1 μl each of primers BamHA-1 and HindT7HA-2, 1 μl of Advantage 2 DNA polymerase (Clontech) and 38.75 μl of molecular grade double distilled water (Invitrogen). The reaction was performed on a Robocycler 40 (Stratagene) with the following parameters: denaturation at 99 °C × 35 s, annealing at 53 °C × 30 s, elongation at 68 °C × 3 min for the first 30 cycles, followed by denaturation at 99 °C × 35 s, annealing at 53 °C × 30 s, and elongation at 68 °C × 4 min for the last 10 cycles.

The full length amplicon was then digested with restriction enzymes Bam HI and Hind III, and cloned into the vector pGEM-7zf(+) (Promega) using standard methods.