

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: de Jong MD, Van Cam B, Qui PT, et al. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. *N Engl J Med* 2005;352:686-91.

Web-only supplementary information

Influenza A real-time reverse transcriptase (RT) PCR

Influenza A viral RNA was detected in clinical specimens or culture supernatant by real-time RT-PCR that used primers targeted at a conserved region of the matrix gene, INFA-165s 5'GACAAGACCAATCCTGTCACYTCTG3' and INFA-216r 5'AAGCGTCTACGCTGCAGTCC3', and the probe 5'FAM-TTCACGCTCACCGTGCCCAGTGAGC-TAMRA3'. Nucleic acids were extracted from 200 µl of CSF or serum, 100 µl of viral transport medium, or 20 µl of culture supernatant, using the method described by Boom et al.^{1,2}. Synthesis of cDNA was performed as described previously.³ For amplification, 1 µl of cDNA was used in a total reaction volume of 25 µl containing 1 unit of HotstarTaq DNA polymerase (Qiagen), 2.5 µl of 10x HotStarTaq PCR buffer, 5 mM of magnesium chloride, 400 nM of deoxynucleoside triphosphates (Roche Diagnostics), 400 nM of each of the two primers, and 40 nM of the TaqMan probe. Thermal cycling was performed in the iCycler iQ Real-Time PCR System (Biorad) under the following conditions: 95°C for 15 minutes, followed by 45 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds.

For estimation of the viral load in clinical specimens, PCR products were cloned into a TA cloning vector (pCR4, Invitrogen) to serve as an external standard for real-time quantitation. After purification, plasmid DNA was quantified by UV absorption at 260 nm. Confirmation of copy numbers was done by limiting dilution followed by PCR.⁴ Serial 10-fold dilutions (10^8 to 10^2 copies per ml) of linearized plasmid were used as standards for real-time quantitation. Viral load was expressed as the number of cDNA copies per milliliter of specimen.

References

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3. Tran TH, Nguyen TL, Nguyen TD, et al. Avian influenza A (H5N1) in 10 patients in Vietnam. *N Engl J Med* 2004; 350:1179-88.
4. Diaco R. Practical considerations for the design of quantitative PCR assays. In: Innis MA, Gelfand DA, Sninsky JJ, eds. *PCR strategies*. New York, N.Y.: Academic Press, Inc., 1995:84-108.