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Address for correspondence: Loïc Josseran, French National Institute for Public Health Surveillance, Institut de Veille Sanitaire, 12, Rue du Val d'Osne, 94415 Saint Maurice CEDEX, France; email: l.josseran@invs.sante.fr

Assay to Detect H5N1 Oseltamivir Resistance

To the Editor: Oseltamivir is a neuraminidase inhibitor approved for treatment and prevention of influenza virus infection. Oseltamivir resistance caused by a single amino acid substitution from histidine (H) to tyrosine (Y) at position 274 of the neuraminidase active site has been reported in persons infected both experimentally and naturally with influenza A virus subtype H5N1 (*1*,*2*). Evidence suggests that using lower doses of oseltamivir or shorter-than-recommended treatment periods can contribute to emergence of viral resistance (*1*,*3*) Currently, oseltamivir is being used in several countries that may be affected by epidemics of H5N1. Therefore, monitoring for oseltamivir-resistant strains of H5N1 during oseltamivir administration is essential for outbreak management and prevention.

Although real-time PCR or pyrosequencing is more rapid than high-throughput assays for mutation detection (*4*,*5*), the conventional PCR technique can be applied to detect drug-resistant mutation (*6*) in areas lacking real-time PCR or pyrosequencing capabilities. Therefore, to discriminate between oseltamivir-sensitive and oseltamivir-resistant strains, we developed a simple method, based on PCR, which takes advantage of the H274Y substitution. The forward primer was designed from the conserved region common to both wild-type and mutant strains; the reverse primers were designed specifically for wild-type and mutant strains, respectively, derived from the 3′ terminal base of each primer. The primers consisted of a forward primer N1f (nt 517-534: 5′-GGGGCTGTGGCTGTATTG-3′) and reverse primer H274r (nt 759-784: 5′-GGATAACAGGAGCAYTCCTCATAGTG -3′) for wild-type strain detection or Y274r (nt 759-784: 5′-GGATAACAGGAGCAYTCCTCATAGTA-3′) for mutant strain detection. (Note: Underlined letters represent differences in nucleotides between plus vs. minus primers.) Both strains yielded products of ≈267 bp; hence, the assay consisted of 2 separate reactions for detecting wild-type and mutant strains, respectively.

For each reaction, 1.0 μL cDNA was combined with a reaction mixture that contained 10 μL 2.5× MasterMix (Eppendorf, Hamburg, Germany), forward and reverse primers at a final concentration of 0.15 μM, and nuclease-free water to a final volume of 20 μL. Thermocycling conditions comprised initial denaturation at 94°C for 3 min and 35 cycles of amplification including denaturation (94°C, 30 s), annealing (65°C, 50 s), extension (72°C, 45 s), and final extension (72°C, 7 min). Subsequently, 10 μL of the amplified products was analyzed by using 2% agarose gel electrophoresis.

To optimize the assay, we performed PCR-based H274Y mutagenesis of the N1 fragment of the H5N1 virus (primers on request). The resulting mutagenic and wild-type products were cloned into the pGEM-T Easy Vectors (Promega, Madison, WI, USA), confirmed by direct sequencing, and then used as positive controls. Preliminary results showed that the wild-type primer was specific for the oseltamivir-sensitive strain, whereas the mutant primer can be used to detect the oseltamivir-resistant strain exclusively because no significant cross-amplification had been observed.

To establish sensitivity, serial 10-fold dilutions of the standard N1 plasmids (wild-type and mutant) ranging from 10^9 to 10^1 copies/μL were used as a template. The threshold concentrations for detection of wild-types and mutants were 10^3 copies/μL. To provide semiquantitative data to detect subpopulations of the resistant variants, the 2 control plasmids were mixed at wild-type:variant ratios of 10^8:10^2, 10^7:10^3, 10^6:10^4, 10^5:10^5, 10^4:10^6, 10^3:10^7, and 10^2:10^8. The result showed that the density of the expected bands depended on the amount of DNA templates (Figure B). However, the mixing experiments indicated that the predominant mixtures of wild-type:resistant variant were 80:20, which is the lowest ratio of resistant variants that the assay can reliably detect (data not shown). To assess specificity, human DNA and viral cDNA extracted from other subtypes of influenza A virus (N2–N9) were subjected to this assay. No cross-reaction occurred with human DNA or other subtypes of influenza A virus.

We further validated the assay by testing 3 specimens from hosts treated with oseltamivir and 17 specimens from untreated hosts; infection with H5N1 was detected by using multiplex real-time PCR (*7*). The specimens from oseltamivir recipients were isolated from a Vietnamese patient on the third day after he received a prophylactic dose (*1*) and from 2 tigers (CU-T7 and KU-11) (*8*). The specimens from untreated hosts were isolated from the plasma of an H5N1-infected human (*9*) and from virus-containing allantoic fluid isolated from infected chickens, ducks,

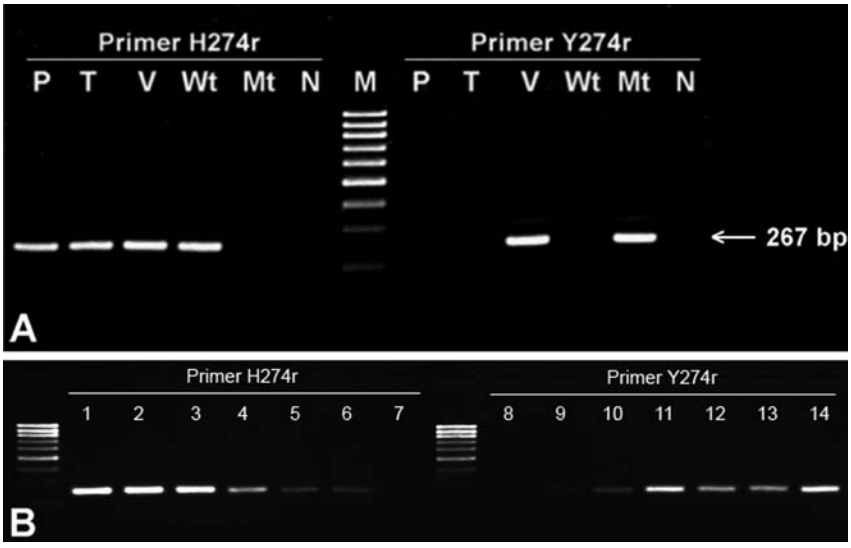

Figure. A) Representative result from conventional PCR that used H274r primer for oseltamivir-sensitive and Y274r primer for oseltamivir-resistant detection in samples isolated from human plasma (P), tiger (T), and Vietnamese patient (V). Plasmids containing N1 fragments obtained from PCR-based mutagenesis for wild-type H274 (Wt) and mutant Y274 (Mt) were used as positive controls in each reaction. (N, no template control; M, 100-bp molecular marker.) B) Semiquantitative data on the ability of the assay to detect subpopulations of the resistant variants. The 2 control plasmids were mixed at wild-type:variant ratios of 10^8:10^2 (lanes 1 and 8), 10^7:10^3 (lanes 2 and 9), 10^6:10^4 (lanes 3 and 10), 10^5:10^5 (lanes 4 and 11), 10^4:10^6 (lanes 5 and 12), 10^3:10^7 (lanes 6 and 13), and 10^2:10^8 (lanes 7 and 14).

and cats (n = 16) during a 2005 outbreak in Thailand. The specimen isolated from the Vietnamese patient yielded detectable bands after amplification by wild-type and mutant primer sets, which indicates that this specimen contains mixed populations of wild-type and resistant strains (Figure, A). The result was confirmed by cloning the amplicon of the Vietnamese strain into the pGEM-T EASY vector (Promega). Ten colonies were randomly picked and sequenced; 9 clones were mutant, and 1 was wild type. The other specimens produced a visible positive band only on amplification using the wild-type primer set, which indicates that samples containing these strains were oseltamivir sensitive.

The assay described here provides an accurate, cost-effective, and highly efficient approach to detecting oseltamivir-sensitive and oseltamivir-resistant H5N1 strains; it is based on conventional PCR and takes advantage of the H274Y substitution within the neuraminidase gene. This simple technique can be applied in all laboratories that lack more advanced and expensive instruments; thus, it provides a valuable tool for managing and preventing influenza A H5N1 outbreaks. Concerning the spread of mutant viruses, however, the fitness of the viruses needs further investigation.

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Kamol Suwannakarn,*
Salin Chutinimitkul,*
Sunchai Payungporn,*
Thaweesak Chieochansin,*
Apiradee Theamboonlers,*
Alongkorn Amonsin,*
Sudarat Damrongwatanapokin,†
Le Quynh Mai,‡
Nguyen Hong Hanh,‡
and Yong Poovorawan*

*Chulalongkorn University, Bangkok, Thailand; †National Institute of Animal Health, Bangkok; and ‡National Institute of Hygiene and Epidemiology, Hanoi, Vietnam

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Address for correspondence: Yong Poovorawan, Center of Excellence in Viral Hepatitis Research, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand; email: yong.p@chula.ac.th

Laboratory Exposure to Influenza A H2N2, Germany, 2004–2005

To the Editor: From November 2004 to February 2005, a company contracted by the College of American Pathologists (CAP) sent influenza quality assurance samples containing live influenza A H2N2 viruses (A/Japan/305/57) to 3,748 international laboratories (*1*,*2*). Of these, 3,686 (98%) were located in Canada or the United States. In Germany, 6 laboratories received at least 1 of 3 samples, 2 for virus antigen detection and 1 for virus culture; all contained live virus and were formatted identically. No information on infectivity or virulence of the samples was available. Because of the absence of human-to-human influenza A H2N2 virus transmission since 1968, this situation provided the rare opportunity to investigate whether infections with this strain had occurred in any of the laboratories.

We used a standardized questionnaire to obtain from the laboratories information on when the CAP samples had been received, which of the 3 quality assurance specimens they contained, and how many employees had been involved in their handling. A second questionnaire was distributed to personnel in microbiology and virology departments. This elicited information regarding routine laboratory activities, contact with CAP samples, tasks performed in conjunction with handling of the samples, and any influenzalike symptoms (sudden onset of fever, cough, headache, and muscle pain) within the respective time frame. Persons who had worked in a receiving laboratory from September 1, 2004, to April 15, 2005, and had performed routine procedures in virology (defined as transport of samples, preparation of samples for diagnostic testing, antigen testing, nucleic acid amplification testing, and virus isolation) were eligible for the study. From May 4 to May 19, 2005, we visited the laboratories to interview supervisory personnel regarding routine work-up of samples and to collect blood from study participants for serologic testing.

The national reference laboratory for influenza at Robert Koch Institute performed serologic testing for antibodies against A/Singapore/1/57 (H2N2) virus by hemagglutination inhibition. We compared antibody titers of laboratory workers who worked with a CAP sample with those who did not. However, this comparison might not show a difference if (silent) virus transmission had occurred among laboratory staff. To exclude this possibility, we also compared titers of workers born before 1969 with those in a group of volunteers from Robert Koch Institute also born before 1969. Titers <10 were assigned a value of 1.

Of 47 laboratory workers, 18 either declined to participate or were excluded because they did not perform any of the defined routine procedures during the defined period. Thus, 29 (62%) workers were included in the study, of whom 14 (48%) reported having worked with CAP samples. Of these 14 workers, 12 (2 exclusively) transported samples and 11 (2 exclusively) prepared the samples; 9 (1 exclusively) performed antigen testing, 2 (0) performed nucleic acid amplification tests and 4 (0) performed virus isolation. Fourteen workers (48%) reported no contact with the samples, and 1 (3%) was unsure. None of the 29 participants reported any event that could have led to release of infectious material during the respective time frame, such as broken test tubes or dropped culture plates. Participating laboratories reported that all procedures were performed under appropriate hygienic and safety precautions. No person had ≥3 symptoms typical for influenzalike illness in the 4 days after having worked with a CAP sample.

Specific influenza A H2N2 antibody titers were determined in 25 study participants. None had a titer >80, two (8%) had a titer of 80, three (12%) had titers of 40, two (8%) had titers of 20, and the remaining 18 (72%) had titers ≤10. Three (21%) of 14 workers exposed to CAP samples and 4 (40%) of 10 who denied exposure had titers ≥20. All 7 were born before 1969. The geometric mean of titers of all participating workers born before 1969 did not differ significantly from that of the Robert Koch Institute employees ($p = 0.28$; Figure).

In summary, no evidence was found for laboratory infections with the influenza A H2N2 virus. The risk for laboratory-acquired influenza infections is unknown. Severe acute respiratory syndrome coronavirus and *Mycobacterium tuberculosis* are infectious agents whose transmission characteristics are similar to those of influenza. For severe acute respiratory syndrome coronavirus, laboratory-acquired infections are well documented (*3*,*4*). For *M. tuberculosis*, there are strong indications that they occur (*5*–*7*). From a public health perspective and in view of the current importance given to influenza and a possible pandemic, accurate characterization of the risk for influenza infections in laboratory settings is needed. The small number of persons included in this study limits the conclusions that can be drawn. Potentially exposed workers were