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Use of PCR-Enzyme Immunoassay for Identification of Influenza A Virus Matrix RNA in Clinical Samples Negative for Cultivable Virus

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Influenza A virus infections are a major cause of morbidity and mortality worldwide. Standard diagnostic methods either are not efficient in identifying infected individuals in a timely manner or lack sensitivity. We developed a PCR-enzyme immunoassay (PCR-EIA) for the detection of influenza A virus RNA in respiratory secretions. A reverse transcription PCR was performed with oligonucleotide primers directed at a highly conserved area of the influenza A matrix gene. Amplified DNA was identified by hybridization in solution to a nested biotinylated RNA probe and quantitated in an EIA. PCR-EIA detected small quantities of RNA from the three prevalent subtypes of human influenza A virus. Influenza B and C, parainfluenza, measles, mumps, and respiratory syncytial viruses tested negative. The potential efficiency of PCR-EIA for use in clinical diagnosis was determined by testing 90 nasal wash specimens obtained daily over a 10-day period from nine human volunteers infected with influenza A virus. Thirty-seven of the postinfection samples had detectable influenza A virus RNA by PCR-EIA, whereas only 26 postinfection samples were positive by culture. PCR-EIA was particularly efficient for the identification of influenza A virus in samples obtained more than 4 days after infection. Seventeen of 45 such samples were positive, whereas virus was cultivated from 4 samples ($P < 0.00005$). All preinfection samples from volunteers subsequently infected with influenza A virus were negative by PCR-EIA, as were samples from a volunteer infected with parainfluenza virus type 3. Nucleic acid amplification techniques represent important tools for the timely and sensitive diagnosis of influenza A virus infections and, therefore, their management and control.

Influenza A virus is a major etiological agent of respiratory disease in the United States and many other areas of the world. Diseases resulting from influenza virus infection range from mild upper respiratory symptoms in healthy adults to life-threatening respiratory failure in high-risk individuals (14). Influenza, which typically occurs in yearly epidemics worldwide, annually results in approximately 30,000 excess deaths in the United States (16).

The control of influenza transmission in a high-risk population is dependent upon immunizations, the rapid identification of cases, and the institution of infection control measures. In addition, antiviral drugs have been shown to modulate the course of infection if administered prior to exposure or early in the course of acute infection (8). However, the implementation of prophylactic and therapeutic regimens has been hampered by limitations inherent in the methods available for the timely diagnosis of influenza A virus infections. The standard laboratory methods for the detection of influenza A virus are based on the isolation of virus in tissue culture (10, 17). While cultivation methods can detect small numbers of viable virions, the time required for the identification of virus precludes its use for the early administration of antiviral chemotherapy. In addition, quantitative studies of viral shedding have indicated that the concentration of viable virus in nasopharyngeal secretions diminishes rapidly after the first days of infection and that virus can be difficult to recover late in the course of infection (13).

For these reasons, additional assays have been devised for the practical diagnosis of infections caused by influenza A virus. For example, viral antigens can be rapidly detected in nasal secretions by immunofluorescence or enzyme immunoassay (EIA) techniques. However, these assays are less sensitive than culture for the diagnosis of influenza A virus infections (6, 7). The measurement of antiviral antibodies provides a sensitive means for the retrospective identification of infected individuals. However, serological methods are of limited usefulness for the diagnosis of acute infections.

The recent development of methods for the amplification of nucleic acids provides a diagnostic methodology which has the potential of detecting low levels of viral nucleic acids in clinical samples (9, 12). We have recently developed a modified reverse transcription PCR technique which allows for the efficient amplification of viral RNA from clinical samples (25). We have also adapted EIA techniques to the rapid, quantitative detection of nucleic acids amplified from clinical specimens (PCR-EIA) (2, 5). We used these methods to examine the shedding of viral RNA in sequential respiratory samples obtained from human volunteers infected with a variety of strains of influenza A virus.

MATERIALS AND METHODS

Virus strains. The following virus strains were obtained from the American Type Culture Collection (Rockville, Md.): influenza A2/Japan/305/57 (H2N2), influenza A/swine/76/31,

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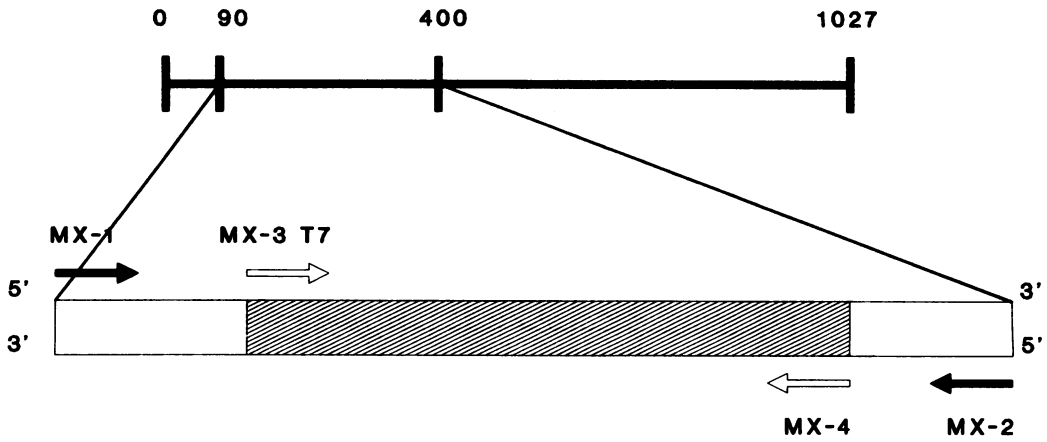

FIG. 1. Locations of the outer and nested sets of primers and the segment used for preparation of the biotinylated probe (hatched) of gene segment 7 of influenza A virus. Solid arrows are outer primers MX-1 (base pairs 90 to 115) and MX-2 (base pairs 377 to 400). Open arrows are nested primers MX-3 (base pairs 160 to 177) and MX-4 (base pairs 313 to 337). The hatched region is the RNA probe.

influenza C/Taylor/1233/47, parainfluenza type 1 (HA-2) strain C-35, parainfluenza type 4A strain M-25, parainfluenza type 2, mumps virus, respiratory syncytial virus Long strain, and the Edmonston strain of measles virus. Virus strains were also obtained from the Center for Immunization Research, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Md. These included influenza A wild-type viruses influenza A/Kawasaki/9/86 (H1N1), influenza A/Los Angeles/2/87 (H3N2), influenza A/Puerto Rico/8/34 (H1N1), and influenza A/Texas/1/85 (H1N1); live attenuated cold-adapted strains containing the internal gene segments from cold-adapted influenza A/Ann Arbor/6/60 and hemagglutinin (H) and neuraminidase (N) genes from influenza A/Korea/1/82 (H3N2) and influenza A/Bethesda/85 (H3N2); influenza B/Ann Arbor/1/86; influenza B/Victoria; and the JS strain of parainfluenza type 3 virus.

Specimens from volunteers. Serial daily nasal wash specimens collected from nine human volunteers prior to and following inoculation with the influenza A virus strains were also obtained from the Center for Immunization Research. These included four volunteers inoculated with wild-type influenza A/Texas/1/85 (H1N1), four with wild-type influenza A/Kawasaki/9/86 (H1N1), and one with wild-type influenza A/Los Angeles/2/87 (H3N2). In addition, samples from one volunteer infected with parainfluenza type 3 virus were also included. The details of these studies have been described previously (4, 11, 23). These studies were approved by the Clinical Research Subpanel of the National Institutes of Allergy and Infectious Diseases, the Human Volunteer Research Committee at the University of Maryland School of Medicine, and the Joint Committee on Clinical Investigation of the Johns Hopkins Medical Institutions.

Following collection, the nasal wash specimens were cultured for virus and the quantity of virus present was titrated as described previously (18). Briefly, 0.1 ml of specimen was inoculated into each of four Madin-Darby canine kidney (MDCK) cell monolayer cultures. The presence of virus was detected by the hemadsorption test performed when a cytopathic effect was noted or, in the absence of a cytopathic effect, on days 7 and 14, respectively, by using chicken erythrocytes. The virus was identified by immunofluorescence. In positive specimens, the virus was titrated with frozen aliquots of the original specimen. Subjects were considered to have been infected with influenza A virus if they shed cultivable virus or had a fourfold or greater rise in hemagglutination-inhibition antibody titer (18).

Frozen aliquots of these specimens were tested by PCR-EIA, under code, without knowledge of the culture results.

Primers. A 310-bp segment of the matrix protein gene, conserved for influenza A virus but substantially different from the matrix protein genes of influenza B and C viruses and the other paramixoviruses, was selected for amplification (Fig. 1). The primers and their sequences were as follows: MX-1, 5′-CCGAGATCGCGCAGAGACTTCAAGAT-3′ (base pairs 90 to 115), and MX-2, 5′-GGCAAGTGCACCAGCAGAATA ACT-3′ (base pairs 377 to 400).

An inner set of primers was used to amplify a 178-bp nested segment of the viral genome, which was then used to prepare a biotinylated RNA probe as described below. The two primers and their nucleotide sequences were as follows: MX-3T7, 5′-TTAATACGACTCACTATAGGTGCTAAAGACAAGA CCAATCCT-3′ (base pairs 160 to 177; the T7 promoter sequence is underlined), and MX-4, 5′-CCTAAGTTTTCTA TACAGTTTAACT-3′ (base pairs 313 to 337).

RNA extraction and reverse transcription. Viral RNA was extracted from a 100-μl volume of specimen by using the acid guanidinium and phenol method of Chomczynski and Sacchi (3), with the exception that 95% cold ethanol instead of isopropanol was used for reprecipitation of the RNA and the RNA was suspended in 10 μl of diethyl pyrocarbonate (DEPC)-treated water prior to reverse transcription.

Two microliters of the viral RNA was reverse transcribed with a standard mixture, in addition to random hexamers ($pd[N]_6$; Pharmacia LKB Biotechnology, Piscataway, N.J.) and Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, Md.), for 1 h at 37°C.

PCR. Five microliters of viral cDNA was amplified in a 100-μl total volume with 0.5 μM primers (MX-1 and MX-2), 0.2 mM deoxynucleoside triphosphates, 1× PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin), and 2.5 U of *Taq* polymerase (Boehringer GmbH, Mannheim, Germany). The thermofile parameters were 30 cycles of 94, 68, and 72°C for 1 min, 30 s, and 1 min, respectively.

Following amplification, electrophoresis was performed through a 7.5% polyacrylamide gel at 53 mA, and the bands were visualized after silver nitrate staining.

Preparation of biotinylated probe. A biotinylated probe was prepared by previously described methods (2, 5). Briefly, 5 ng

of the amplified DNA from influenza A/Los Angeles/2/87 (H3N2) (MX-1–MX-2) was reamplified by using the nested primers (MX-3T7 and MX-4), and 300 ng of the amplified product was transcribed by using biotin-11 UTP (Enzo, New York, N.Y.). The DNA template was digested with RQ 1 DNase (Promega, Madison, Wis.). The biotinylated probe was purified by Sephadex G-25 chromatography on NAP-5 columns (Pharmacia LKB Biotechnology, Piscataway, N.J.) by using DEPC-treated water with 0.5% sodium dodecyl sulfate as the equilibration and elution buffer. The probe was divided into aliquots and was stored at −70°C.

Solution hybridization and EIA for DNA-RNA hybrids. The nonisotopic solution hybridization reaction and EIA for the detection of the amplified DNA were performed as described previously (2, 5). Briefly, the PCR product was denatured by boiling, and the denatured product was hybridized in solution with 2 μl of biotinylated RNA probe per ml at 80°C for 1 h. Triton X-100 (to 1%) was then added to the cooled hybrids, and the mixtures were reacted, in duplicate, with anti-biotin-coated wells of a microtiter plate. A monoclonal antibody–β-galactosidase conjugate to DNA-RNA hybrids was reacted in a sandwich EIA, and the fluorescent galactoside-umbelliferone cleavage product was measured.

Reagent blanks were included in each batch and in all steps of the assay, namely, RNA extraction, reverse transcription, and PCR. A sample was considered to be positive if it generated a fluorescence activity which was three standard deviations greater than the mean fluorescence activity of the negative control samples assayed in the same test run. The fluorescence generated by the reagent blanks and uninfected MDCK cells was used to calculate the cutoff value for a positive test in the assays of virus in tissue culture. For volunteer nasal wash specimens, the fluorescence generated by the reagent blanks was used to calculate cutoff values for preinfection samples. The fluorescence generated by the preinfection nasal wash samples was then included in calculating fresh cutoff values for evaluating postinfection samples. Positive samples were further quantified by calculating a specific activity computed by subtracting the cutoff value from the mean fluorescence activity generated by the sample. Influenza A virus strains or nasal wash specimens known to be positive for influenza A virus by both culture and PCR-EIA were run as positive controls in each batch.

The McNemar test was used to estimate the statistical significance of the differences in detection rates of virus by culture and PCR-EIA.

RESULTS

Amplification of RNA from stock viruses. The reactivities of the oligonucleotide primers directed at the influenza A virus matrix gene were initially evaluated for the amplification of RNA from defined viral strains. We tested seven strains of influenza A containing greater than $10^{4.5}$ 50% tissue culture infective doses ($TCID_{50}$s) per ml of virus. We also tested equivalent concentrations of influenza B virus, influenza C virus, parainfluenza virus types 1, 2, 3, and 4, respiratory syncytial virus, measles virus, and mumps virus. The amplified products were then evaluated for hybridization to the biotinylated RNA probe directed at influenza A virus matrix gene sequences internal to the target sites of the oligonucleotide primer (Fig. 1). As depicted in Fig. 2, all of the influenza A virus strains had specific activities greater than 260 fluorescence units, whereas none of the other viruses gave positive reactions.

The sensitivity of the PCR-EIA system was further delineated by testing serial fivefold dilutions of RNA extracted from three prevalent subtypes of human influenza A virus with known titers (Fig. 3). The minimal titers of virus for which an appropriate band (310 bp) was visualized by polyacrylamide gel electrophoresis and silver staining were 3×10^2, $3 \times 10^{2.5}$, and $8 \times 10^{0.75}$ $TCID_{50}$s in the tested volume of sample for influenza A/Los Angeles/2/87 (H3N2), influenza A/Kawasaki/9/86 (H1N1), and influenza A/Japan/305/57 (H2N2), respectively. By PCR-EIA, the minimum viral titers detectable were 6.4×10, $0.5 \times 10^{0.5}$, and $1 \times 10^{0.25}$ $TCID_{50}$s in the tested volume of sample, respectively, for the same three strains.

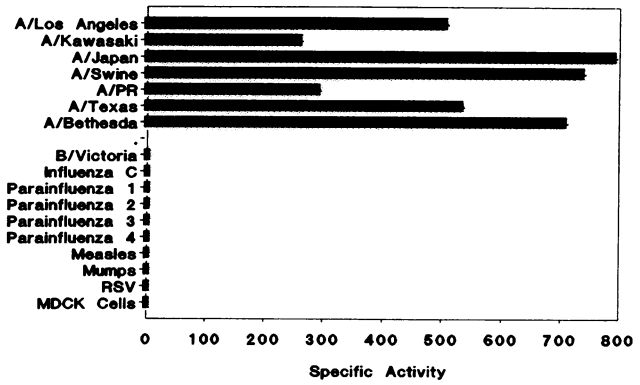

FIG. 2. Specific activity (fluorescence value) by PCR-EIA of the tested influenza A virus strains and influenza B virus, influenza C virus, parainfluenza virus types 1, 2, 3, and 4, respiratory syncytial virus (RSV), and measles and mumps viruses.

Amplification of RNA from nasal wash specimens from human volunteers infected with influenza A virus. The results of PCR-EIA and culture for the detection of virus are depicted in Fig. 4. Overall, 21 samples were positive by both culture and PCR-EIA, 5 samples were positive by culture alone, and 16 samples were positive by PCR-EIA alone. The five culture-positive PCR-EIA-negative samples had virus titers ranging from $10^{-0.2}$ to $10^{0.8}$ $TCID_{50}$s in the tested volume of sample. During the first 4 days postinfection, PCR-EIA and virus culture displayed similar levels of sensitivity (20 of 36 samples versus 22 of 36 samples that were positive; $P > 0.35$), whereas PCR-EIA was substantially more sensitive in samples obtained more than 4 days following infection (17 of 45 samples versus 4 of 45 samples that were positive; $P < 0.00005$). At least one sample from individuals infected with influenza A virus contained viral RNA. For one volunteer with detectable viral RNA, there were no samples from which virus could be cultivated, but the volunteer demonstrated an increase in influenza A virus hemagglutination-inhibition antibody titer (1:8 to 1:128). All samples from a volunteer infected with parainfluenza virus type 3 were negative for influenza A virus RNA.

In order to check for sample inhibition in three of five PCR-EIA-negative, culture-positive samples, 10^4 $TCID_{50}$s of influenza A/Texas/1/85 (H1N1) were added to each sample and to water controls. Repeat PCR-EIA was performed to compare the spiked samples from volunteers with spiked water and unspiked nasal wash samples. The unspiked samples from volunteers remained negative. The fluorescence generated by two of the three spiked samples was within two standard deviations of the fluorescence generated by the water control; the remaining spiked sample generated fluorescence which was greater than two standard deviations of the fluorescence

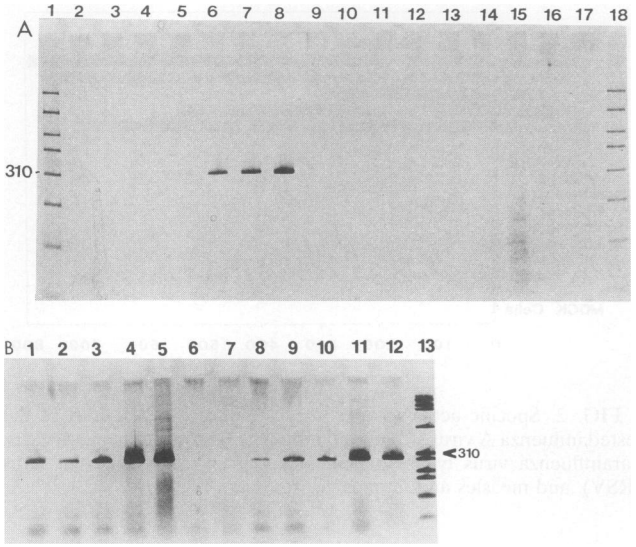

FIG. 3. (A) Polyacrylamide gel electrophoresis and silver staining of PCR products from influenza A2/Japan/305/57 (H2N2) at virus titers of $1 \times 10^{-0.4}$ (lane 2), $1 \times 10^{0.25}$ (lane 3), $1.6 \times 10^{0.75}$ (lane 4), $8 \times 10^{0.75}$ (lane 5), $4 \times 10^{1.75}$ (lane 6), $2 \times 10^{2.75}$ (lane 7), and $10^{3.75}$ (lane 8) $TCID_{50}$s per volume of sample tested; respiratory syncytial virus (lane 9); mumps virus (lane 10); measles virus (lane 11); parainfluenza virus types 4 (lane 12), 3 (lane 13), 2 (lane 14), and 1 (lane 15); influenza C virus (lane 16); influenza B virus (lane 17); and gel markers (GelMarker I, Research Genetics, Huntsville, Ala.) (lanes 1 and 18). (B) Polyacrylamide gel electrophoresis and silver staining of PCR products from influenza A/Los Angeles/2/87 (H3N2) at virus titers of 3×10^2 (lane 1), 1.6×10^3 (lane 2), 8×10^3 (lane 3), 4×10^4 (lane 4), and 2×10^5 (lane 5) $TCID_{50}$s per test; water blanks (lanes 6 and 7); PCR products of influenza A/Kawasaki/9/86 (H1N1) at virus titers of $3 \times 10^{2.5}$ (lane 8), $1.6 \times 10^{3.5}$ (lane 9), $8 \times 10^{3.5}$ (lane 10), $4 \times 10^{4.5}$ (lane 11), and $2 \times 10^{5.5}$ (lane 12) $TCID_{50}$s per test; ϕX174 replicative form *Hae*III fragment DNAs (GIBCO BRL) (lane 13).

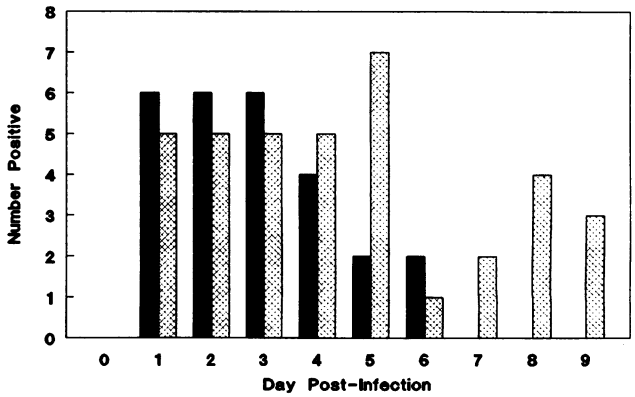

FIG. 4. Detection of influenza A virus by culture (■) and PCR-EIA (▩) in serial nasal wash specimens from volunteers infected with virus. One specimen from each of nine volunteers was tested by culture as well as PCR-EIA on each of the indicated days.

generated by the control, indicating that inhibitors were not present in the samples.

Symptoms of influenza, including fever with a temperature of ≥100°F (38°C), myalgia, rhinorrhea, pharyngitis, or otitis media, occurred in six volunteers. Fever lasting 1 to 3 days was present in four volunteers; the remaining two had no fever but had myalgia and rhinorrhea. Of the 33 days when any symptom was present in the six symptomatic volunteers, virus was isolated by culture on 19 days and detected by PCR-EIA on 23 days ($P > 0.1$). Of the 20 person-days of rhinorrhea in symptomatic patients, virus was detectable by culture on 14 days, whereas it was detectable by PCR-EIA on 17 days ($P = 0.037$). The one volunteer in whom influenza A virus RNA was detected in the absence of cultivable virus was also symptomatic, with myalgia, otitis media, and sinusitis.

DISCUSSION

Our study documents that PCR-EIA is a sensitive and specific method for the detection of influenza A virus infection. This method used oligonucleotide primers directed at the gene encoding the highly conserved matrix protein, thus allowing detection of all influenza A virus strains (19). In addition, the PCR-EIA method, which makes use of an EIA to detect DNA amplified by PCR, was substantially more sensitive than identification of the amplified DNA by gel techniques alone.

One problem inherent in the application of sensitive assays for clinical diagnosis is the interpretation of results in which the more sensitive assay detects an analyte in a sample negative by standard assay systems. Previous studies have documented that nucleic acid amplification techniques can detect viral RNA in situations in which virus cannot be cultivated by standard methods (20, 25). However, the diagnostic significance of samples which are reactive by nucleic acid amplification techniques but which are negative by standard virological methods has been difficult to determine with certainty. The evaluation of respiratory samples from human volunteers infected with influenza A virus under controlled conditions allowed us to examine the relative sensitivities of PCR-EIA and viral cultivation at defined points during the course of infection.

We found that culture and PCR-EIA had equivalent degrees of sensitivity during the first 4 days following infection. On the other hand, PCR-EIA was substantially more sensitive than culture later in the course of infection. From days 5 through 9, PCR-EIA could detect influenza A virus RNA in 17 of 45 samples, while only 4 of 45 samples were culture positive during that period of time ($P < 0.00005$). The five samples which were positive by culture but negative by PCR-EIA had $10^{0.8}$ $TCID_{50}$s or less virus in the volume of sample which was tested and, thus, would not have been expected to be positive. We were unable to find evidence for the presence of inhibitors in the culture-positive, PCR-EIA-negative samples. The testing of larger volumes might enhance the detection of influenza A virus RNA in these situations. The specificity of the PCR-EIA was documented by the fact that viral RNA was not detected in any sample obtained prior to viral challenge. In addition, samples obtained from the volunteer infected with parainfluenza virus were consistently negative in the assay. It should be noted that the low rate of culture positivity late in the course of illness occurred even though the samples were collected and processed under carefully controlled conditions and the tissue culture system was optimized for the detection of the challenge strain of virus.

Our findings demonstrate that clinical symptoms and the shedding of viral RNA can persist after virus becomes undetectable by cultivation methods. These data are consistent with those from previous studies which documented that many patients with respiratory symptoms have a serological response to influenza A virus antigens in the absence of the isolation of virus from respiratory secretions (15). It is of note in this

regard that influenza A virus was not cultivated from any of the daily respiratory samples of one study subject, even though that RNA was detectable by PCR-EIA and he displayed a seroresponse to influenza A virus hemagglutinin.

The results of the study indicate that cell culture techniques may fail to detect influenza A virus infection in patients who are brought to medical attention late in the course of their illness. This group includes many individuals with the most serious complications of influenza A virus infection. For example, virus is rarely isolated from patients with secondary complications of influenza A virus infection such as bacterial pneumonia, even in cases in which the diagnosis of influenza A virus infection was indicated by serological evaluations (1). Virus is rarely isolated from any body site of patients with neurological complications, especially Guillian-Barré syndrome (24). The availability of efficient methods for the diagnosis of infections caused by influenza A virus throughout the course of illness would allow for the accurate identification of influenza A virus infection in patients with these late complications of infection. This identification would be important not only for the management of infected patients but also for the development of strategies for the prevention of disease in high-risk populations. It should be noted in this regard that, while our assay focused on the detection of the matrix gene shared by all strains of influenza A virus, other nucleic acid amplification assays have been devised to characterize neutralization epitopes on the hemagglutinin gene and genes that encode resistance to host defenses (21, 26). The application of these assays would provide additional information concerning the epidemiology of the infecting strain within a population.

The present study did not address the question of the potential infectivity of symptomatic patients who shed viral RNA in the absence of cultivable virus. In light of the high concentration of RNases present in the respiratory microflora, it is unlikely that naked single-stranded RNA would persist without the capsid in respiratory secretions (22). On the other hand, it is possible that the viral RNA is shed in the form of defective particles or is otherwise protected from digestion by RNase. In such situations, the patient would not be expected to transmit the infection to other individuals. However, it is also possible that the PCR-EIA is capable of detecting viral particles which do not efficiently replicate in tissue culture systems but which are still potentially infectious for humans. For example, it is possible that proteases in nasal secretions can activate virus and remove complexed immunoglobulins, rendering the virus infectious following inhalation. The performance of additional studies should be directed at determining the infectivity of viral RNA amplified from the fluids of infected individuals in whom virus has not been detected by standard culture systems.

Our study documents that nucleic acid amplification techniques can detect viral nucleic acids in defined samples which do not contain virus detected by a "gold standard" assay under conditions which document assay specificity. It is also likely that similar nucleic acid amplification will be capable of the specific detection of additional species of viral DNA or RNA in samples from which the corresponding virus cannot be cultivated, provided that care is taken to maximize sensitivity and avoid false-positive reactions caused by sample contamination. Nucleic acid amplification assays for the detection of other viruses should be evaluated under conditions which allow for accurate definitions of assay reactivity. Nucleic acid amplification procedures which have documented sensitivity and specificity have a great deal of potential for the identification of patients with viral infections and for the rational application of antiviral therapies.

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