JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2005, p. 1768–1775 Vol. 43, No. 4
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.4.1768–1775.2005

Evaluation of PCR Testing of Ethanol-Fixed Nasal Swab Specimens as an Augmented Surveillance Strategy for Influenza Virus and Adenovirus Identification

A. E. Krafft,[1]* K. L. Russell,[2] A. W. Hawksworth,[2] S. McCall,[1] M. Irvine,[2] L. T. Daum,[3] J. L. Connoly,[4] A. H. Reid,[1] J. C. Gaydos,[5] and J. K. Taubenberger[1]

Department of Molecular Pathology, Armed Forces Institute of Pathology, Rockville, Maryland[1]; Department of Defense Center for Deployment Health Research, Naval Health Research Center, San Diego, California[2]; Molecular Virology Branch, Brooks City-Base, Texas[3]; Moncrief Army Hospital, Fort Jackson, South Carolina[4]; and The DoD Global Emerging Infections Surveillance and Response System, Walter Reed Army Institute of Research, Silver Spring, Maryland[5]

Received 17 August 2004/Returned for modification 1 October 2004/Accepted 24 November 2004

Viral culture isolation has been widely accepted as the "gold standard" for laboratory confirmation of viral infection; however, it requires ultralow temperature specimen storage. Storage of specimens in ethanol at room temperature could expand our ability to conduct active surveillance and retrospective screenings of viruses with rapid and inexpensive real-time PCR tests, including isolates from remote regions where freezing specimens for culture is not feasible. Molecular methods allow for rapid identification of viral pathogens without the need to maintain viability. We hypothesized that ethanol, while inactivating viruses, can preserve DNA and RNA for PCR-based methods. To evaluate the use of ethanol-stored specimens for augmenting surveillance for detection of influenza viruses A and B and adenoviruses (AdV), paired nasal swab specimens were collected from 384 recruits with febrile respiratory illness at Fort Jackson, S.C., in a 2-year study. One swab was stored at ambient temperature in 100% ethanol for up to 6 months, and the other swab was stored at −70°C in viral medium. For viral detection, frozen specimens were cultured for a variety of respiratory viruses, and ethanol-fixed specimens were tested with TaqMan (TM) probe and LightCycler SYBR green (SG) melting curve assays with at least two different PCR targets for each virus. The sensitivities of the TM and SG assays on specimens stored in ethanol for 1 month were 75% and 58% for influenza A, 89% and 67% for influenza B, and 93 to 98% and 57% for AdV, respectively. Lower specificities of the real-time assays corresponded to the increased detection of PCR-positive but culture-negative specimens. Influenza virus RNA was detected as well or better after 6 months of storage in ethanol.

Outbreaks of febrile respiratory illness (FRI), also known as influenza-like illness or acute respiratory disease, have the potential for epidemic spread to at-risk civilian and military populations and are of grave concern to public health scientists (34, 35). A myriad of common respiratory viral pathogens may cause large outbreaks of FRI, including adenoviruses (AdV) and influenza virus types A (FluA) and B (FluB). Adenovirus epidemics occur frequently in new U.S. military recruit populations undergoing the rigors of basic combat training (18, 34). Influenza A viruses cause global epidemics in humans and domestic animals and are a major public health concern because they can change rapidly as they circulate continually in human, swine, equine, and avian populations (50). Influenza B viruses can also cause yearly epidemics, primarily in schools and military camps, but they circulate almost solely in human populations (21, 26).

Among the group of viruses associated with FRI, influenza A viruses are the most significant, causing yearly outbreaks and occasional pandemics (10). The current system of influenza surveillance requires the culture of viral isolates, severely limiting the reach of the surveillance network because of the expense associated with the collection and transport of frozen specimens for culture and serologic characterization in reference laboratories (20). Respiratory tract swab specimens stored in ethanol could greatly simplify and expand our ability to conduct active surveillance and retrospective screenings of known and newly emerging RNA and DNA viruses with rapid and inexpensive real-time PCR tests, including influenza isolates from remote regions where the collection and processing of specimens for routine culture and analysis are not economically feasible.

Influenza A and B viruses are enveloped negative-strand RNA viruses with eight gene segments, each encoding one or two proteins (32). The major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), are primarily responsible for the antigenic variation seen in influenza viruses and serve as the basis for further subtyping of influenza type A viruses (51). The extreme genetic variability of influenza A viruses makes designing useful, rapid, molecular-based assays challenging (45), but several different approaches have been successfully utilized by targeting the highly conserved RNA gene segment encoding the internally expressed matrix 1 (M1) or nonstructural (NS) protein (16, 44). Reverse transcription-PCR (RT-PCR) is effective for initial influenza diagnosis and has greater sensitivity than other available rapid assays (4, 5, 7,

* Corresponding author. Mailing address: Department of Molecular Pathology, Armed Forces Institute of Pathology, 1413 Research Blvd., Rockville, MD 20850-3125. Phone: (301) 319-0289. Fax: (301) 295-9507. E-mail: Amy.Krafft@afip.osd.mil.

17, 36). Molecular assays have also been used to subtype influenza A isolates, and sequence analysis of the HA gene may greatly enhance surveillance studies and vaccine strain selection (45). There is a need for the development of rapid assays capable of detecting all influenza A variants, including novel antigenic subtypes, and the emergence of a new pandemic strain, should it arise (33, 50).

While influenza virus epidemics pose serious problems for both civilian and military populations, the majority of FRI outbreaks in U.S. military training centers in recent years have been caused by adenoviruses (3, 24). Adenoviruses comprise a large family of double-stranded DNA viruses found in mammals, amphibians, and birds. The 51 human adenovirus serotypes are divided into six subgroups (A through F) and cause a great variety of clinical syndromes (28). The serotypes historically associated with FRI are classified in subgroup B (serotypes 3, 7, 14, and 21) and subgroup E (serotype 4). Acute respiratory infections caused by AdV may result in severe lower respiratory tract disease, requiring hospitalization, and, rarely, fatal pneumonia (39). From 1971 to 1996, vaccines against AdV serotype 4 (AdV4) and AdV7 were routinely administered to military trainees and were highly effective in minimizing adenovirus epidemics at basic training centers (9, 23). However, vaccine production ended in 1996, and supplies ran out in 1999. The loss of AdV vaccines precipitated a wave of AdV4 infections beginning in 1997. AdV4 is now the predominant cause of large, frequent respiratory disease epidemics in military training centers nationwide, where reported AdV infection rates can range from 30 to 80% of new recruits (3, 15, 25, 27, 30, 40). Molecular tests for the DNA genome of AdV typically target the genes encoding the three coat proteins: hexon, penton base, and fiber. For universal AdV detection directly from clinical specimens and in fixed tissues, PCR methods target short conserved regions at the 5′ and 3′ ends of the hexon gene (13, 49).

There is a great need for rapid and inexpensive surveillance methodology to detect respiratory viral pathogens. Although viral culture has been the "gold standard" for laboratory diagnosis, it requires specimen storage and transport in viral media maintained at ultralow temperatures to optimally preserve infectious viral particles. Viral culture methods are also time-consuming, labor-intensive, and expensive, and they occasionally fail to grow viruses to detectable titers, thus delaying characterization. In contrast, universal nucleic acid sequence detection-based methods can be performed directly on primary specimens without the need to maintain viability for culture. We hypothesized that the storage of specimens in ethanol would inactivate viral infectivity yet preserve viral DNA and RNA for molecular characterization. Further, we reasoned that molecular assays would detect viruses that failed to grow well in traditional cell culture.

The Department of Defense (DoD) Global Emerging Infections Surveillance and Response System supports active FRI surveillance for influenza viruses, AdV, and other respiratory pathogens among U.S. military trainees, active duty military, and dependents at DoD sites (12). Viral pathogen identification is performed at the Naval Health Research Center (NHRC) in San Diego, Calif., and the Air Force Institute for Operational Health Virology Laboratory in Brooks City-Base, Tex. Epidemiological data and new influenza isolates gathered under the DoD Worldwide Influenza Surveillance Program are shared with the Centers for Disease Control and Prevention and the World Health Organization (6). In an effort to simplify and expand global virological surveillance to regions where culture-based techniques are not feasible, we conducted a 2-year pilot study at Fort Jackson, S.C., in the 2001-2002 and 2002-2003 influenza seasons to evaluate the potential for storage of collected respiratory samples in room temperature ethanol.

MATERIALS AND METHODS

Study design. Nasal swab specimens were collected from 384 trainees who presented for medical care with FRI at the U.S. Army basic training site at Fort Jackson, S.C., in winter 2001-2002 and 2002-2003. One hundred ninety-five specimens were collected between December and March in the first year, and 189 specimens were collected in the second year of the study. The case definition of FRI was an oral temperature of ≥100.5°C and cough or sore throat or any case of radiographically confirmed pneumonia. Two Dacron swabs were taken, one from each nostril. One swab was placed into a transport vial containing 2.5 ml of Multi-Microbe media (M4) for viral transport medium (VTM) (Remel, Lenexa, Kans.) and the other in 2.5 ml of 100% ethanol. The original VTM specimen was mixed on a vortex mixer and, after the swab was removed, was transferred to four cryogenic vials in volumes of 1.0, 0.5, 0.5, and 0.5 ml and frozen at −70°C. Two frozen VTM specimens (1.0 and 0.5 ml) were shipped on dry ice to the NHRC for viral culture. The ethanol specimen swab was broken off so that the tip remained in the vial. The ethanol specimens, stored at ambient temperature between 15 and 35°C, and two 0.5-ml aliquots of frozen VTM specimens stored on dry ice were batched and shipped monthly to the Armed Forces Institute of Pathology for molecular studies.

Viral culture. To maximize virus isolation, 1.0-ml frozen specimens were cultured on two cell lines: the A549 human lung carcinoma cell line and primary rhesus monkey kidney cells. Cells were followed for cytopathic effect (CPE) for 14 days, and CPE-positive cells were tested by immunofluorescence assay. Positive cells were passed again and characterized. Influenza was typed by hemagglutination inhibition. Rhesus monkey kidney tubes still CPE negative after 14 days were screened for influenza by hemadsorption.

Test comparison. Laboratory investigators were blind to molecular and culture results and reported data to an independent coordinator at the NHRC (A. W. Hawksworth) for linkage analysis. Because there were a significant number of culture-negative but PCR-positive specimens in winter 2001-2002 in the ethanol-fixed specimens, additional steps were taken in winter 2002-2003 to address these putative false-positive specimens. In the second year, two different PCR methods, designated primary and secondary PCRs, were used for the detection of each virus in the ethanol-fixed specimens. Because PCR-based methods have been shown to be more sensitive than culture for the detection of many viruses, a specimen with positive results for two different PCR targets was considered a "molecular true positive"—a second gold standard—for the comparison of assay performances (22, 37, 38, 41, 47, 48). Results were calculated based on defining a true-positive specimen by culture or two molecular tests. Sensitivities, specificities, and predictive values of a positive or a negative test were calculated. Specimens that were positive for influenza by any test during the initial screening were retested at 6 months on new extracts, and sensitivities were calculated. A small number of negative specimens were retested for control purposes.

Reference stocks, primers, and probes. FluA (A/Hong Kong/68), FluB (B/Lee/40), and AdV serotypes 1, 4, and 7 were purchased from the American Type Culture Collection (ATCC), Manassas, Va. The ATCC strains and 12 clinical strains of FluA (H3N2 and H1N1) and FluB isolated at the NHRC in 2000-2001 were tested by molecular methods at the Armed Forces Institute of Pathology to compare the analytical sensitivities of new molecular tests. Sequence alignment of recent strains available in GenBank was done with the DNA Star software (Madison, WI). Primers and TaqMan (TM) probes for FluB and AdV were designed using Primer Express 1.0 software (Applied Biosystems [ABI], Weiterstadt, Germany). Target genes for PCR assays were the M1 gene of FluA, the HA and NS genes of FluB, the hexon gene of AdV, and the human beta-2-microglobulin gene for an RNA amplification control (1). The following reporter dyes were attached to the 5′ ends of different dual-labeled probes: 6-carboxyfluorescein (FAM), tetrachlorofluorescein, hexachlorofluorescein, and VIC. The quencher dyes, 6-carboxytetramethyl-rhodamine (TAMRA) and the black hole quencher BH1, were attached to the 3′ ends of different TM probes. Oligonucleotide primer and probe sequences and locations of gene targets are listed in

TABLE 1. Primers and probes for PCR detection of adenovirus and influenza A and influenza B viruses by TaqMan and LightCycler SYBR green analyses[a]

Primer or probe	Sequence	Region (bp) or reference
AdV		
Primary SG and TM (subgroup E) hexon		
F	CGACAGAACCCGGTATTTCAG	1083–1103
R	TGGTTTTCAATAATGCGCACA	1140–1160
Probe	(FAM)-TCATAGCTGTCCACCGCCTGATTCCA-(TAMRA)	1108–1133 R
Primary SG and TM (subgroup B) hexon		
F	CCCATGCGCTGGACATGA	2932–2949
R	CTTCGAAAAGAAGATAAAGCAGGGT	2979–3003
Probe	(FAM)-TGAGGTGGATCCCATGGATGAGCC-(TAMRA)	2954–2977
Secondary TM (subgroups B and E) hexon		
F	GATGGCCACCCCATCGATGMTGC	−1–22
R	GCGAACTGCACCAGACCCGGAC	74–95
Probe	(VIC)-TACATGCACATCGCCGGACAGGAMGCTTCGGAGT-(TAMRA)	34–67 R
FluA		
Primary TM matrix		
F	AGCAAAAGCAGGTAGATRTT	1–20
R	TCGGCTTTGAGGGG	79–93
Probe	(FAM)-AMCCGAGGTCGAAACGTAYG-(TAMRA)	37–56
Secondary SG matrix		Reference 43
F	AGATGAGTCTTCTAACCGAGGTCG	24–47
R	TGCAAAAACATCTTCAAGTCTCTG	101–124
FluB		
Primary TM hemagglutinin		
F	AAATACGGTGGATTAAACAAAAGCAA	970–995
R	AGCTCCGAAGAAACCCCTTTCC	1110–1131
Probe	CACCCATATTGGGCAATTTCCTATGGC	1004–1030
Secondary SG nonstructural		
F	TCACTCTTCGAGCGTCTTAATGAA	768–791
R	GGATAAGACTCCCACCGCAG	842–941

[a] Abbreviations: F, forward; R, reverse.

Table 1. Primers and TM probes were synthesized by Integrated DNA Technologies, Coralville, Iowa, or ABI, Foster City, Calif.

DNA/RNA preparation. A single DNA/RNA preparation was made from each specimen. Specimens were mixed for 10 s, and the nasal swab was removed using an aseptic technique. Approximately one-half of the ethanol cell suspension was transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 10 min to pellet cells. Specimens were treated according to a standard phenol-chloroform extraction protocol for DNA/RNA preparation (31) and resuspended in 50 μl of molecular-grade water. A known culture-positive specimen and a negative control (water) were included in each run. RNA was purified from influenza stocks by the Trizol method (Invitrogen, Carlsbad, CA). The viral copy numbers were estimated using the A_{260}/A_{280} RNA/DNA concentration method. Genome sizes of 13.6 kb for influenza A and B and 36 kb for adenoviruses were used for copy number calculations.

Influenza A assay development. In preliminary studies to select an assay for FluA detection, we tested several published RT-PCR assays, including one-step TaqMan assays for M1, N1, and N2 targets (41, 47) and a ^{32}P incorporation assay for M1 (46) on culture fluid from 12 FluA strains isolated at the NHRC in 2000-2001. Although the TaqMan assays were reported to detect 10 to 13 FluA virus particles per assay, we found greatly reduced detection sensitivity in our hands (data not shown), which could have been due to mutations (genetic drift) in recent isolates that would cause mismatches and decreased binding efficiency with these published primer/probe sets (45). Furthermore, the larger expected amplicon sizes (188 bp and 246 bp) of the older assays could also result in less-efficient PCRs (41, 47). In the two-step RT-PCR ^{32}P incorporation assay for the M1 gene (46), 1 μl of RNA extract was used for cDNA synthesis with moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamers (first step) and 1 μl of cDNA was used for PCR (second step). Radiolabeled PCR products were separated by 6% denaturing polyacrylamide gel electrophoresis, and a band of 115 bp was the expected product size for a positive sample. The older M1 assays were not tested on 2002-2003 specimens.

Primers and probes for the primary FluA TM assay used to test specimens in both years of the study and the secondary SYBR green (43) assay tested on winter 2002-2003 specimens targeted different M1 gene sequences and were designed and validated previously as TaqMan assays on all 15 FluA HA subtypes. The following reaction conditions were used for the primary TM assay: a one-step RT-PCR system (ABI), 300 nM concentrations of each primer, 67 nM probe, and 1.5 μl of template in a total volume of 25 μl. Samples were analyzed on the ABI PRISM Sequence Detection System 7700 (ABI SDS 7700) programmed for 30-min RT at 48°C, 10 min at 95°C, and then 50 cycles of 15 s at 95°C and 1 min at 55°C. The secondary SG assay used the one-step LightCycler (LC) RNA amplification kit SYBR green 1 with two enzymes (avian myeloblastosis virus reverse transcriptase and *Taq* polymerase), 1.5 μM concentrations of each primer, 6 mM $MgCl_2$, and 2 μl of template in a total volume of 20 μl. The LightCycler (Roche Diagnostics Corporation, Indianapolis, IN) was programmed for 20-min RT at 55°C, 30-s denaturation at 95°C, and 45 cycles of 95°C for 5 s, 60°C for 8 s, and 72°C for 5 s, followed by standard melting curve analysis. Two specific FluA melting peaks with different melting temperatures (T_m) (82.15°C and 83.6 °C ± 1°C) were observed, corresponding to the different matrix gene sequences of H1N1 and H1N2 subtypes cocirculating in winter 2002-2003. The T_m of the controls used were 85.0°C for the A/H3N2/Hong Kong/68 control, 83.6°C for an H3N2 clinical specimen from winter 2000-2001, and 79.1°C for a nonspecific peak intermittently observed in no-template water controls and negative samples. A threshold cycle (C_T) cutoff of <39.6 was used.

Influenza B assay development. Primary and secondary FluB assays were tested on all 2002-2003 specimens. A 140-bp region of the FluB HA gene was amplified in the one-step RT-PCR primary TM assay. The primary FluB HA TM reaction used the one-step RT-PCR system (ABI), 150 nM concentrations of each primer, 67 nM probe, and 2 μl of template in a total volume of 25 μl. Samples were analyzed on the ABI SDS 7700, programmed for 30-min RT at 48°C, 10 min at 95°C, and then 50 cycles of 12 s at 95°C and 1 min at 56°C. A positive case had a C_T cutoff of <39.6. A 74-bp region of the NS gene was the target for the secondary SG assay. The secondary SG assay used the one-step LightCycler RNA amplification kit SYBR green 1, 500 nM concentrations of each primer, 6 mM $MgCl_2$, and 2 μl of template in a total volume of 20 μl. The LC was programmed for 30-min RT at 55°C, 30-s denaturation at 95°C, and 45

cycles of 95°C for 0 s, 55°C for 10 s, and 72°C for 13 s, followed by standard melting curve analysis. Positive clinical samples had a specific T_m of 80.8°C ± 1°C. The T_m of the B/Lee/40 strain and two clinical specimens from winter 2000 was 81.6°C. A nonspecific peak was occasionally observed in no-template water controls and negative samples at 78.4°C. A C_T cutoff of <39.6 was used.

AdV assay development. The AdV hexon gene was the target in all real-time assays. For initial AdV screening in winter 2001-2002, LightCycler SG melting curve analysis was used with two primer sets specific for subgroup B or E AdV (primary SG), and each sample was tested in duplicate (1 μl and 1 μl of a 1:5 dilution). Borderline positive or ambiguous SG results were confirmed with Southern blot analysis of products with radiolabeled subgroup B- and E-specific probes. In winter 2002-2003, a single dilution (1 μl of extract) of each sample was tested by primary SG for rapid screening. The SG assays contained a master mix developed in-house with 50 mM Tris (pH 8.3), 500 μg/ml of bovine serum albumin, 200 μM deoxynucleoside triphosphates, 0.34× SYBR green (Molecular Probes), 3 mM $MgCl_2$, 200 nM concentrations of each primer, and 1.25 U of FastStart (Roche) in a total volume of 20 μl. The LC was programmed at 95°C for 4 min enzyme activation, 45 PCR cycles of 95°C for 0 s, 60°C for 10 s, and 72°C for 6 s, followed by standard melting curve analysis. The T_m of the AdV4 positive control (ATCC) was 82.5°C ± 1°C. Sequence analysis of the AdV4 prototype strain and recently sequenced variants showed no sequence variation within the amplicon target, a short conserved region between hypervariable regions (HVR 6 and HVR 7) of the primary SG assay; however, two sharp melting peaks centered at 80.8°C and 81.8°C were observed in clinical specimens with the lower melting product outside the accepted limits (± 1°C) of SYBR green assays (11). Consequently, the two probes used for Southern blot confirmation in winter 2001-2002 were resynthesized as TaqMan probes and used to confirm SYBR green results on the ABI SDS 7700 (primary TM). Retesting of each specimen by both primary TM assays for subgroup B and E confirmed the two different melting peaks as AdV4 strains (subgroup E). A secondary TM assay using a single primer pair and two dual-labeled probes designed to bind to conserved sequences at the 5′ end of the hexon gene for adenovirus subgroups B (types 3, 7, 11, 16, 21, and 34) and E (type 4) detection was tested on each specimen. The expected amplicon is 95 bp. The TaqMan assays contained the TaqMan Universal PCR master mix (ABI), 200 nM concentrations of each primer, 67 nM probe, and 1 μl of template in a total volume of 25 μl. The ABI SDS 7700 was programmed for 2 min at 50°C to activate AmpErase uracil-*N*-glycosylase, 10 min at 95°C to activate AmpliTaq Gold DNA polymerase, and 45 cycles of 15 s at 95°C and 1 min at 60°C. For the AdV TM assays, a C_T cutoff of <37.6 was used to define positive samples.

PCR comparison of frozen and ethanol-fixed samples. Additional frozen aliquots from parallel nasal swabs were tested with the molecular assays during the 2002-2003 season; however, reduced yields of viral and cellular nucleic acid were observed in the majority of frozen VTM samples (data not shown). The VTM and ethanol-fixed samples were not directly comparable after the original 2.5-ml VTM samples were divided into four aliquots for culture and molecular testing; the extract prepared from a 0.5-ml frozen VTM (one-fifth of the original specimen) contained less detectable nucleic acid than an extract prepared from one-half of the parallel ethanol-fixed swab.

RESULTS

Analytical sensitivities of the TaqMan and SYBR green assays. Consistent amplification and detection were generally observed across 6 orders of magnitude with all TaqMan assays and over a narrower range (10^4 to 10^5) with the SG melting curve assays on the LC. The sensitivity of the primary FluA assay was evaluated using 10-fold serial dilutions of purified RNA from the A/H3N2/Hong Kong/68 strain run in triplicate reactions. The estimated limit of FluA detection was 390 fg of template RNA (50,716 copies) with the SG assay and 48 fg of template RNA (6,240 copies) with the TM assay. Serial 10-fold dilutions of purified RNA from the B/Lee/40 strain were used to determine the sensitivity of the FluB molecular assays. The estimated limits of FluB detection were 680 fg of template RNA (89,054 copies) with the SG assay and 840 fg of template RNA (111,317 copies) with the TM assay. Serial dilutions of purified AdV stocks were tested by the SG assay and both TM PCR assays in quadruplicate. The estimated limits of AdV detection were 24 fg of template DNA (625 copies) with the primary SG, 192 fg of template DNA (5 copies) with the primary TM, and 4.8 fg (125 copies) with the secondary TM assay.

Influenza A testing. In winter 2001-2002, influenza A was cultured in 27 of 195 (14%) specimens. All isolates were subtyped as H3N2 isolates. Twelve of the ethanol-fixed samples that were culture negative were positive by two molecular tests (primary TM and ^{32}P incorporation assays), increasing the number of FluA true-positive specimens to 39 of 195 (20%). The primary TM assay tested on extracts prepared from specimens stored in ethanol for 1 month detected 34 of 39 true-positive samples (87% sensitivity and 95% specificity). The TM assay detected eight culture-negative specimens that were not positive by the ^{32}P assay, giving a false-positive rate of 5.1% (8/156).

In winter 2002-2003, influenza A was cultured in 11 of 189 (6%) specimens (Table 2). All 11 culture-positive isolates were subtyped as H1 (New Caledonia-like strains). One ethanol-fixed sample that was culture negative was positive by two molecular tests (primary TM and secondary SG assays), increasing the number of FluA true-positive specimens to 12 of 189 (6.3%). PCR testing for N1 and N2 targets revealed that half of the isolates were H1N1 and half were H1N2. In the initial 1-month screening, the primary TM assay in ethanol-stored samples detected 9 of the 12 true positives (75% sensitivity and 98% specificity). The SG assay detected 7 of the 12 true positives (58% sensitivity and 100% specificity). The TM assay detected three culture-negative specimens, giving a false-positive rate of 1.7% (3/177), and the SG false-positive rate was 0. After 6 months of storage, new RNA extracts were prepared from 40 ethanol-fixed specimens. The TM assay and the SG assay detected 8 of 12 true-positive (67% sensitivity) specimens. The specificities of the assays were not determined for this subset of specimens.

Influenza B testing. In winter 2002-2003, influenza B was cultured from 7 of 189 (3.7%) specimens. All of the B isolates were B/Hong Kong/1434/02-like. In the screening of ethanol and frozen specimens at 1 month, two specimens that were culture negative were positive by two molecular tests (primary TM and secondary SG assays), increasing the number of FluB true-positive specimens to 9 of 189 (4.7%). In the initial 1-month screening of ethanol-fixed samples, the primary TM assay detected 8 of the 9 true positives (89% sensitivity and 100% specificity) and the secondary SG assay detected 6 of 9 true positives (67% sensitivity and 100% specificity). After 6 months of storage, new RNA extracts were prepared from 40 ethanol-fixed specimens. Both RT-PCR assays were highly sensitive for the detection of FluB RNA after 6 months, with TM detecting 8 out of 9 true-positive specimens and SG detecting 9 out of 9 true-positive specimens. The specificities of the assays were not determined for this subset of specimens.

Adenovirus testing. In winter 2001-2002, 62 of 195 (32%) nasal swab specimens were culture positive for AdV and 91 (47%) of the parallel ethanol-fixed swab samples were PCR positive by AdV SG testing (94% sensitivity and 75% specificity compared to culture alone). Subgroup-specific PCR and Southern blot confirmation determined that AdV4 predominated, with subgroup B found in only 2% of isolates. The large number of PCR-positive, culture-negative specimens prompted

TABLE 2. Detection of influenza A and influenza B viruses and adenovirus in 2002–2003 nasal swab specimens stored in ethanol at ambient temperature by TM and LightCycler SG PCR methods[a]

Detection method	Mo no.	Sensitivity		Specificity		PPV		NPV	
		No. of culture positive specimens/ total no. of specimens	% of specimens culture positive (95% CI)	No. of culture positive specimens/ total no. of specimens	% of specimens culture positive (95% CI)	No. of culture positive specimens/ total no. of specimens	% of specimens culture positive	No. of culture positive specimens/ total no. of specimens	% of specimens culture positive
Influenza A virus (12 TP)									
Viral culture	1	11/12	92 (76–100)	177/177	100	11/11	100	177/178	99
Primary TM	1	9/12	75 (51–100)	174/177	98 (96–100)	9/12	75	174/177	98
	6	8/12	67 (40–93)	nd		8/12	67	nd	
Secondary SG	1	7/12	58 (30–86)	177/177	100	7/7	100	177/182	97
	6	8/12	67 (40–93)	nd		8/8	100	nd	
Influenza B virus (9 TP)									
Viral culture	1	7/9	78 (51–100)	180/180	100	7/7	100	180/182	99
Primary TM	1	8/9	89 (68–100)	180/180	100	8/8	100	180/181	99
	6	8/9	89 (68–100)	nd		8/8	100	nd	
Secondary SG	1	6/9	67 (36–97)	180/180	100	6/6	100	180/183	98
	6	9/9	100	nd		9/9	100	nd	
Adenovirus (122 TP)									
Viral culture		63/122	52 (43–60)	67/67	100	63/63	100	67/126	53
Primary SG	1	69/122	57 (48–65)	64/67	96 (91–100)	69/72	96	64/117	55
Primary TM	1	119/122	98 (95–100)	47/67	70 (59–81)	119/139	86	47/50	94
Secondary TM	1	113/122	93 (88–97)	59/67	88 (80–96)	113/121	93	59/68	87

[a] TP, true positive specimen (positive by culture and/or two molecular tests); CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; nd, not determined. Specificity and PPV of viral culture are 100% by default, due to the definition of "true positive."

the use of a secondary PCR test the following season to better define the gold standard as "true positive."

In winter 2002-2003, AdV was cultured from 63 of 189 (33%) nasal swab specimens. Subgroup-specific PCR from these culture-positive samples found 94% to be AdV4 and 6% to be subgroup B. An analysis of the AdV culture-positive specimens stored in ethanol found that 58 of 63 (92%) of these specimens had positive results in two or more PCR assays. Of these specimens, 48 (76%) were positive in all three PCR assays, and 10 (16%) had positive results in two PCR assays. Of the 126 ethanol specimens that were culture negative for AdV, 59 (47%) had positive results in two or more molecular assays, increasing the number of true-positive specimens for AdV to 122 of 189 (64%). Of these 59 PCR-positive but culture-negative cases, 16 (27%) were positive for all three PCR assays and 43 (73%) for two PCR assays. The LC assay was positive for AdV in 69 of 122 (57% sensitivity and 96% specificity) true-positive specimens. The primary TM assay detected AdV in 119 of 122 (98% sensitivity and 70% specificity) true-positive specimens. Thirty-one culture-negative cases that were positive in only one PCR assay were considered false positives. The highest rate of false positives was found with the primary TM assay (30%, 20/67), followed by the secondary TM assay (12%, 8/67) and the primary SG assay (4%, 3/67).

A quantitative analysis of the TM assay C_T values was used to determine the relative amounts of viral DNA in the samples. When the AdV4 culture-positive specimens were tested with the primary AdV4 TM assay, an average mean C_T value of 24 ($n = 57$; median, 23; range, 16 to 37) was found. Similarly, when the AdV culture-positive specimens were tested with the secondary TM assay for both group B and E AdV, the average mean C_T value was 24 ($n = 59$; median, 23; range, 16 to 37). In contrast, the average mean C_T values seen with both AdV TM assays were significantly higher for the specimens negative by culture but determined to be true positives by two molecular tests. An average mean C_T value of 32.5 ($n = 59$; median, 33; range, 22 to 37) and C_T value of 34 ($n = 54$; median, 34; range, 23 to 37) were found with the primary and secondary TM assays, respectively. An average mean C_T value of 24 in culture-positive specimens reflects approximately 2^8-fold more starting template than an average mean C_T value of 32 in molecular true-positive but culture-negative samples, suggesting that PCR-positive but culture-negative samples contained approximately 2^8-fold less AdV DNA than culture-positive specimens.

DISCUSSION

In this study, we found that storage in 100% ethanol at ambient temperatures preserves viral RNA and DNA suitable for PCR analysis. In the FluA and FluB studies, RT-PCR could detect influenza RNA in nasal swab specimens stored for up to 6 months. Many recent studies have shown that real-time RT-PCR for influenza A and B virus detection by TM or fluorescence resonance energy transfer probe technology detects more positive specimens than viral isolation (29, 41, 42). PCR is expected to have an increased sensitivity of detection relative to culture because PCR detects short segments of viral RNA from viable as well as nonviable viruses; however, the reported rates of increased PCR detection over culture differ widely in different studies. Differences in the recovery rates of viable influenza viruses are frequently attributed to less than optimal specimen collection, storage, and transport of samples, RNA degradation during laboratory storage at 4°C and −20°C,

and multiple freeze-thaws of specimens before testing (22). Notably, FluA detection by TM assays was nearly double that detected by culture when the samples were collected and sent through the mail at room temperature or at 4°C and were in transit between 1 and 4 days (41, 47).

In winter 2001-2002, influenza activity was moderate, with only the H3N2 subtype of influenza A detected. The percentages of nasal swab specimens with positive viral culture results were 32% for AdV, 14% for FluA, and 0.5% for FluB. The sensitivity of detection for FluA was increased 6% when specimens negative by culture but positive by two independent RT-PCR methods were considered. In winter 2002-2003, influenza activity was mild, with FluA (HIN1 and H1N2 subtypes) and FluB detected (8). FluB caused approximately 40% of influenza nationwide in 2002-2003. A similar distribution of influenza types caused FRI at Fort Jackson (57% FluA and 43% FluB). The percentages of nasal swab specimens with positive viral culture results were 33% for AdV, 6% for FluA, and 4% for FluB. The sensitivity of detection for FluA and FluB was increased by only 1.6% when culture-negative specimens were considered positive by two independent RT-PCR methods. In contrast to previous studies (29, 41, 42, 47), in which TM assays detected large numbers of culture-negative samples, the marginal increase in the PCR detection of influenza viruses (6% for FluA in 2001-2002 and 1.6% for FluA and FluB combined in 2002-2003) could be attributed to optimal storage and transport of nasal swab specimens at −70°C in the current study.

The FluA M1 primers and probes for the primary TM assay and the primers for the secondary SG assays target highly conserved sequences at the 5′ ends of the M1 gene and gave small amplicon sizes of 93 bp and 99 bp, respectively, which are expected to be most efficiently amplified in real-time PCRs. Since these M1 gene targets are not subject to as much sequence variation as externally expressed gene segments (H and NA) and the primer/probe pairs had been previously validated on all 15 influenza A HA subtypes as TM assays, we expected to detect most FluA strains circulating at Fort Jackson during our study. However, the sensitivity of FluA detection from ethanol-fixed samples was 87% in 2001-2002 and 75% in 2002-2003 with our primary TM assay. It is possible that the FluA strains we studied had changed in the primer or probe binding site that would have reduced the sensitivity of the assay. The sensitivities of the RT-PCR assays directly on nasal swab specimens depend on several other factors, including the viral load, RNA degradation, the RNA extraction protocol, and proficiency of extraction. Sensitivity could be increased in future studies by using a larger volume of the original ethanol-fixed sample to prepare an RNA extract and further concentrating the final RNA extract. Although universal molecular assays for all FluA strains are achievable using internally expressed gene segments, such as M1 or NS1, annual sequence analysis of new strains is needed to detect any recent genetic variations that can affect the sensitivities of existing assays.

SYBR green melting curve analysis is widely used to identify and map single base mutations, such as single nucleotide polymorphisms, in gene studies and could conceivably be used to distinguish FluA subtypes. Interestingly, with our SG melting curve assay, two FluA subtypes cocirculating in 2002-2003 (H1N1 and H1N2) could be distinguished by melting temperature analysis, reflecting sequence variation in different matrix gene targets. The SG assay was adopted from a one-step RT-PCR TaqMan probe assay reported to detect approximately 10^3 matrix gene copies or 10 fg of in vitro-transcribed RNA (43) and the SG assay sensitivity was decreased about 10-fold compared to that of TaqMan. The benefits of the SG assay included providing a quick and inexpensive way to differentiate viral strain subtypes without requiring a probe binding detection strategy; however, further characterization studies are required.

The results of the AdV testing reflected the use of three assays with different sensitivities of detection. The sensitivities of the three assays with the primary SG, primary TM, and secondary TM assays were approximately 625, 5, and 125 copies of AdV4 per PCR, respectively. Overall, SG results were most similar to those of culture, but the two TM assays detected a large number of culture-negative specimens. In general, the primary TM assay detected more positives than the secondary TM assay, and the higher assay sensitivity corresponded to the apparent lower specificity when compared to culture. The majority of the culture-negative, PCR-positive specimens had low viral copy numbers (10 to 100 copies/PCR assay) detected by TaqMan quantitative analysis. Culture-positive specimens contained between 2^8 and 2^{10} more detectable AdV genomes than the PCR-positive but culture-negative specimens.

The detection of a large number of PCR-positive but culture-negative nasal swab specimens for AdV was unexpected and in contrast to previous studies. However, real-time TM assays on clinical specimens have not been reported until recently (19). Previously, PCR detected AdV in a large number of environmental (air filter) samples that were culture negative during an AdV-associated respiratory disease outbreak in November 1998 at Fort Jackson, S.C. (2, 14). The same generic PCR method (with detection of the 139-bp product by agarose gel electrophoresis and ethidium bromide staining) gave an excellent correlation with culture of throat swab specimens during the outbreak (15). The authors suggested that the amount of virus present in the air filters was insufficient for viral detection by culture, although the environmental inactivation of the viral particles, possibly by disinfectants, could not be excluded. The detection of low numbers of AdV with TM assays (viable or nonviable) was repeatedly observed in the current study.

A drawback of the strategy to collect specimens in 100% ethanol is that viruses cannot be cultured from ethanol-preserved samples; thus, cell culture would still be needed to provide viral isolates for antigenic characterization and future vaccine production. Nevertheless, because preservation in ethanol eliminates viral infectivity and preserves both DNA and RNA viruses, it may prove useful for rapid molecular testing of potential viral biothreat agents.

In summary, the real-time PCR tests on specimens stored in ethanol at room temperature performed well. Ethanol storage of samples collected in a remote setting where a cold chain cannot be maintained may augment future rapid molecular surveillance and could be a useful tool to perform sequence and phylogenetic analyses in retrospective epidemiological studies. As global attention is increasingly becoming focused on the potential for influenza to cause another worldwide

pandemic, every opportunity to increase global surveillance should be sought. Room temperature storage of clinical specimens in ethanol could augment future surveillance initiatives of not only influenza and adenovirus but potentially a host of other emerging infections.

ACKNOWLEDGMENTS

Partial support for this project was provided by DoD-Global Emerging Infections Surveillance and Response System.

The opinions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

REFERENCES

1. **Bijwaard, K. E., N. S. Aguilera, Y. Monczak, M. Trudel, J. K. Taubenberger, and J. H. Lichy.** 2001. Quantitative real-time reverse transcription-PCR assay for cyclin D1 expression: utility in the diagnosis of mantle cell lymphoma. Clin. Chem. **47:**195–201.
2. **Binn, L. N., J. L. Sanchez, F. Mitchell-Raymundo, S. A. Kolavic, C. Polyak, S. Cersovsky, and B. L. Innis.** 1999. Prospective study of adenoviral associated respiratory disease in male and female military recruits during basic training at Ft. Jackson, S.C. Fall 1998. Clin. Infect. Dis. **29:**1086.
3. **Blasiole, D. A., D. Metzgar, L. T. Daum, M. A. Ryan, J. Wu, C. Wills, C. T. Le, N. E. Freed, C. J. Hansen, G. C. Gray, and K. L. Russell.** 2004. Molecular analysis of adenovirus isolates from vaccinated and unvaccinated young adults. J. Clin. Microbiol. **42:**1686–1693.
4. **Boivin, G., S. Cote, P. Dery, G. De Serres, and M. G. Bergeron.** 2004. Multiplex real-time PCR assay for detection of influenza and human respiratory syncytial viruses. J. Clin. Microbiol. **42:**45–51.
5. **Boivin, G., I. Hardy, and A. Kress.** 2001. Evaluation of a rapid optical immunoassay for influenza viruses (FLU OIA test) in comparison with cell culture and reverse transcription-PCR. J. Clin. Microbiol. **39:**730–732.
6. **Canas, L. C., K. Lohman, J. A. Pavlin, T. Endy, D. L. Singh, P. Pandey, M. P. Shrestha, R. M. Scott, K. L. Russell, D. Watts, M. Hajdamowicz, I. Soriano, R. W. Douce, J. Neville, and J. C. Gaydos.** 2000. The Department of Defense laboratory-based global influenza surveillance system. Mil. Med. **165:**52–56.
7. **Carman, W. F., L. A. Wallace, J. Walker, S. McIntyre, A. Noone, P. Christie, J. Millar, and J. D. Douglas.** 2000. Rapid virological surveillance of community influenza infection in general practice. BMJ **321:**736–737.
8. **Centers for Disease Control and Prevention.** 2003. 2002-03 U.S. influenza season summary. [Online.] http://www.cdc.gov/flu/weekly/weeklyarchives 2002-2003/02-03summary.htm.
9. **Couch, R. B., R. M. Chanock, T. R. Cate, D. J. Lang, V. Knight, and R. J. Huebner.** 1963. Immunization with types 4 and 7 adenovirus by selective infection of the intestinal tract. Am. Rev. Respir. Dis. **88**(Suppl.)**:**394–403.
10. **Cox, N. J., and K. Subbarao.** 2000. Global epidemiology of influenza: past and present. Annu. Rev. Med. **51:**407–421.
11. **Crawford-Miksza, L. K., R. N. Nang, and D. P. Schnurr.** 1999. Strain variation in adenovirus serotypes 4 and 7a causing acute respiratory disease. J. Clin. Microbiol. **37:**1107–1112.
12. **Culpepper, R., and P. Kelley.** 2002. DOD-Global Emerging Infections Surveillance and Response System. Navy Med. **93:**10–14.
13. **Echavarria, M., M. Forman, J. Ticehurst, J. S. Dumler, and P. Charache.** 1998. PCR method for detection of adenovirus in urine of healthy and human immunodeficiency virus-infected individuals. J. Clin. Microbiol. **36:**3323–3326.
14. **Echavarria, M., S. A. Kolavic, S. Cersovsky, F. Mitchell, J. L. Sanchez, C. Polyak, B. L. Innis, and L. N. Binn.** 2000. Detection of adenoviruses (AdV) in culture-negative environmental samples by PCR during an AdV-associated respiratory disease outbreak. J. Clin. Microbiol. **38:**2982–2984.
15. **Echavarria, M., J. L. Sanchez, S. A. Kolavic-Gray, C. S. Polyak, F. Mitchell-Raymundo, B. L. Innis, D. Vaughn, R. Reynolds, and L. N. Binn.** 2003. Rapid detection of adenovirus in throat swab specimens by PCR during respiratory disease outbreaks among military recruits. J. Clin. Microbiol. **41:**810–812.
16. **Ellis, J., and M. Zambon.** 2002. Molecular diagnosis of influenza. Rev. Med. Virol. **12:**375–389.
17. **Ellis, J. S., D. M. Fleming, and M. C. Zambon.** 1997. Multiplex reverse transcription-PCR for surveillance of influenza A and B viruses in England and Wales in 1995 and 1996. J. Clin. Microbiol. **35:**2076–2082.
18. **Erdman, D. D., W. Xu, S. I. Gerber, G. C. Gray, D. Schnurr, A. E. Kajon, and L. J. Anderson.** 2002. Molecular epidemiology of adenovirus type 7 in the United States, 1966-2000. Emerg Infect. Dis. **8:**269–277.
19. **Faix, D. J., H. S. Houng, J. C. Gaydos, S. K. Liu, J. T. Connors, X. Brown, L. V. Asher, D. W. Vaughn, and L. N. Binn.** 2004. Evaluation of a rapid quantitative diagnostic test for adenovirus type 4. Clin. Infect. Dis. **38:**391–397.
20. **Fleming, D. M.** 2001. Influenza diagnosis and treatment: a view from clinical practice. Philos. Trans. R. Soc. Lond. B **356:**1933–1943.
21. **Frank, A. L., L. H. Taber, W. P. Glezen, E. A. Geyer, S. McIlwain, and A. Paredes.** 1983. Influenza B virus infections in the community and the family. The epidemics of 1976–1977 and 1979–1980 in Houston, Texas. Am. J. Epidemiol. **118:**313–325.
22. **Frisbie, B., Y. W. Tang, M. Griffin, K. Poehling, P. F. Wright, K. Holland, and K. M. Edwards.** 2004. Surveillance of childhood influenza virus infection: what is the best diagnostic method to use for archival samples? J. Clin. Microbiol. **42:**1181–1184.
23. **Gaydos, C. A., and J. C. Gaydos.** 1995. Adenovirus vaccines in the U.S. military. Mil. Med. **160:**300–304.
24. **Gray, G. C., J. D. Callahan, A. W. Hawksworth, C. A. Fisher, and J. C. Gaydos.** 1999. Respiratory diseases among U.S. military personnel: countering emerging threats. Emerg. Infect. Dis. **5:**379–385.
25. **Gray, G. C., P. R. Goswami, M. D. Malasig, A. W. Hawksworth, D. H. Trump, M. A. Ryan, D. P. Schnurr, et al.** 2000. Adult adenovirus infections: loss of orphaned vaccines precipitates military respiratory disease epidemics. Clin. Infect. Dis. **31:**663–670.
26. **Grotto, I., Y. Mandel, M. S. Green, N. Varsano, M. Gdalevich, I. Ashkenazi, and J. Shemer.** 1998. Influenza vaccine efficacy in young, healthy adults. Clin. Infect. Dis. **26:**913–917.
27. **Hendrix, R. M., J. L. Lindner, F. R. Benton, S. C. Monteith, M. A. Tuchscherer, G. C. Gray, and J. C. Gaydos.** 1999. Large, persistent epidemic of adenovirus type 4-associated acute respiratory disease in U.S. army trainees. Emerg. Infect. Dis. **5:**798–801.
28. **Horwitz, M.** 2001. Adenoviruses, p. 2301–2326. *In* D. Knipe and P. Howley (ed.), Fields virology. Lippincott Williams & Wilkins, Philadelphia, Pa.
29. **Koenig, M., S. Kosha, M. Hickman, D. Heath, S. Riddell, and W. Aldous.** 2003. Detection of influenza virus from throat and pharyngeal swabs with a nested duplex light cycler RT-PCR. Diagn. Microbiol. Infect. Dis. **46:**35–37.
30. **Kolavic-Gray, S. A., L. N. Binn, J. L. Sanchez, S. B. Cersovsky, C. S. Polyak, F. Mitchell-Raymundo, L. V. Asher, D. W. Vaughn, B. H. Feighner, and B. L. Innis.** 2002. Large epidemic of adenovirus type 4 infection among military trainees: epidemiological, clinical, and laboratory studies. Clin. Infect. Dis. **35:**808–818.
31. **Krafft, A. E., B. W. Duncan, K. E. Bijwaard, J. K. Taubenberger, and J. H. Lichy.** 1997. Optimization of the isolation and amplification of RNA from formalin-fixed, paraffin-embedded tissue: the Armed Forces Institute of Pathology experience and literature review. Mol. Diagn. **2:**217–230.
32. **Lamb, R., and R. Krug.** 2001. Orthomyxoviridae: the viruses and their replication, p. 1487–1531. *In* D. Knipe and P. Howley (ed.), Fields virology. Lippincott Williams & Wilkins, Philadelphia, Pa.
33. **Layne, S., T. Beugelsdijk, C. Patel, J. Taubenberger, N. Cox, I. Gust, A. Hay, M. Tashiro, and D. Lavanchy.** 2001. A global lab against influenza. Science **293:**1729.
34. **McNeill, K. M., F. R. Benton, S. C. Monteith, M. A. Tuchscherer, and J. C. Gaydos.** 2000. Epidemic spread of adenovirus type 4-associated acute respiratory disease between U.S. Army installations. Emerg. Infect. Dis. **6:**415–419.
35. **Monto, A. S.** 2004. Occurrence of respiratory virus: time, place and person. Pediatr. Infect. Dis. J. **23:**S58–S64.
36. **Plakokefalos, E., P. Markoulatos, E. Ktenas, N. Spyrou, and N. C. Vamvakopoulos.** 2000. A comparative study of immunocapture ELISA and RT-PCR for screening clinical samples from Southern Greece for human influenza virus types A and B. J. Med. Microbiol. **49:**1037–1041.
37. **Pregliasco, F., C. Mensi, L. Camorali, and G. Anselmi.** 1998. Comparison of RT-PCR with other diagnostic assays for rapid detection of influenza viruses. J. Med. Virol. **56:**168–173.
38. **Romero, J. R.** 1999. Reverse-transcription polymerase chain reaction detection of the enteroviruses. Arch. Pathol. Lab. Med. **123:**1161–1169.
39. **Ryan, M. A. K., G. C. Gray, M. D. Malasig, L. N. Binn, L. V. Asher, D. Cute, S. C. Kehl, B. E. Dunn, and A. Y. Yund.** 2001. Two fatal cases of adenovirus-related illness in previously healthy young adults-Illinois, 2000. Morb. Mortal. Wkly. Rep. **50:**553–555.
40. **Sanchez, J. L., L. N. Binn, B. L. Innis, R. D. Reynolds, T. Lee, F. Mitchell-Raymundo, S. C. Craig, J. P. Marquez, G. A. Shepherd, C. S. Polyak, J. Conolly, and K. F. Kohlhase.** 2001. Epidemic of adenovirus-induced respiratory illness among US military recruits: epidemiologic and immunologic risk factors in healthy, young adults. J. Med. Virol. **65:**710–718.
41. **Schweiger, B., I. Zadow, R. Heckler, H. Timm, and G. Pauli.** 2000. Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples. J. Clin. Microbiol. **38:**1552–1558.
42. **Smith, A. B., V. Mock, R. Melear, P. Colarusso, and D. E. Willis.** 2003. Rapid detection of influenza A and B viruses in clinical specimens by Light Cycler real time RT-PCR. J. Clin. Virol. **28:**51–58.
43. **Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum, and D. L. Suarez.** 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol. **40:**3256–3260.
44. **Syrmis, M. W., D. M. Whiley, M. Thomas, I. M. Mackay, J. Williamson, D. J. Siebert, M. D. Nissen, and T. P. Sloots.** 2004. A sensitive, specific, and

cost-effective multiplex reverse transcriptase-PCR assay for the detection of seven common respiratory viruses in respiratory samples. J. Mol. Diagn. **6:**125–131.
45. **Taubenberger, J. K., and S. P. Layne.** 2001. Diagnosis of influenza virus: coming to grips with the molecular era. Mol. Diagn. **6:**291–305.
46. **Taubenberger, J. K., A. H. Reid, A. E. Krafft, K. E. Bijwaard, and T. G. Fanning.** 1997. Initial genetic characterization of the 1918 "Spanish" influenza virus. Science **275:**1793–1796.
47. **van Elden, L. J., M. Nijhuis, P. Schipper, R. Schuurman, and A. M. van Loon.** 2001. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J. Clin. Microbiol. **39:**196–200.
48. **van Elden, L. J., M. G. van Kraaij, M. Nijhuis, K. A. Hendriksen, A. W. Dekker, M. Rozenberg-Arska, and A. M. van Loon.** 2002. Polymerase chain reaction is more sensitive than viral culture and antigen testing for the detection of respiratory viruses in adults with hematological cancer and pneumonia. Clin. Infect. Dis. **34:**177–183.
49. **Wadell, G., A. Allard, and H. Hierholzer.** 1999. Adenoviruses, p. 970–981. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, C. A. Tenover, and R. A. Yolken (ed.), Manual of clinical microbiology. ASM Press, Washington, D.C.
50. **Webby, R. J., and R. G. Webster.** 2003. Are we ready for pandemic influenza? Science **302:**1519–1522.
51. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. **56:**152–179.