Surveillance of Childhood Influenza Virus Infection: What Is the Best Diagnostic Method To Use for Archival Samples?

Brent Frisbie,¹ Yi-Wei Tang,^{2,3} Marie Griffin,^{3,4} Katherine Poehling,⁵
Peter F. Wright,⁵ Kathy Holland,⁵ and Kathryn M. Edwards^{5*}

*Department of Pathology,² Department of Medicine,³ Department of Preventive Medicine,⁴
and Department of Pediatrics,⁵ Division of Infectious Disease, School of Medicine,¹
Vanderbilt University, Nashville, Tennessee*

Received 6 September 2002/Returned for modification 29 November 2002/Accepted 17 November 2003

Despite the clinical importance of influenza virus in pediatric respiratory infections, the optimal set of diagnostic tests to use when conducting studies using archival samples is not clear. In this study, we compared diagnostic tests for influenza virus in 75 children younger than 5 years of age who presented with symptomatic respiratory infection during one of four influenza seasons, had negative viral cultures for other respiratory pathogens, and had both an archival nasal aspirate obtained at the time of illness and serology spanning that influenza season. For all eligible children, we compared the results of viral culture performed at the time of collection with serology and PCR of archival nasal aspirates. Using real-time viral culture as the “gold standard,” the test characteristics of PCR of archival nasal aspirates (sensitivity, 82%; specificity, 100%) and serology (sensitivity, 82%; specificity, 87%) were similar. The relatively low sensitivity of PCR of archival nasal samples in this study compared to that of PCR of fresh samples in a previous study suggests that RNA degradation occurred despite storage of the specimens at -70°C . RNA degradation would also explain why only 11 (52%) of 21 archival nasal samples that had positive influenza virus cultures at the time of collection had positive repeat cultures in the summer of 2000. Thus, in archival specimens stored at -70°C , PCR was more sensitive than viral culture. However, testing of fresh specimens had the highest yield in this study. Studies of optimal methods for specimen storage are needed.

Influenza virus is an important cause of febrile respiratory infections in children. Large population-based studies using administrative databases indicate that rates of hospitalization, antibiotic use, and outpatient visits for children consistently increase when influenza virus is circulating (8, 10). Prospective studies of children in inpatient and outpatient settings using viral culture alone or in combination with serology indicate that a high proportion of respiratory illness during the winter is caused by influenza virus (1, 3, 5, 6, 11, 21). However, estimates of the medical care burden of influenza virus in children have been uncertain, due in part to the lack of large population-based studies that have used state-of-the-art viral diagnostic tests. In fact, the optimal set of diagnostic tests to detect influenza virus infections is debatable, particularly when retrospective epidemiologic studies are conducted using archival samples.

The Centers for Disease Control and Prevention (CDC) recently established a new population-based surveillance network, the New Vaccine Surveillance Network (NVSN), to determine the burden of viral respiratory illnesses in hospitalized children younger than 5 years of age. In a previous NVSN study, the results of a rapid diagnostic test for influenza virus were compared to those of viral culture and/or PCR (13). In that study a fully validated and Clinical Laboratory Improvement Amendments-approved PCR of freshly collected samples detected 60% more influenza virus infections than culture

alone. Similarly, other investigators have reported that PCR increases the detection rate of influenza virus compared to viral culture by 3 to 40% (4, 5, 7, 12, 14–16, 19–21). Using our repository of archival nasal-wash samples and paired sera bracketing influenza seasons from young children followed in our clinic, we assessed symptomatic acute respiratory illnesses during four consecutive influenza seasons. The results from real-time viral culture were compared with acute- and convalescent-phase sera and with influenza virus PCR analyses and viral culture of frozen archival nasal samples.

MATERIALS AND METHODS

Population. Children who were recruited as newborns and received primary medical care, including routine immunizations, sick visits, and follow-up visits, in the Vanderbilt Vaccine Clinic (VVC) comprised the study population. To determine the epidemiology of viral illnesses in children, informed consent was obtained for all children enrolled in the VVC to provide nasal aspirates during sick visits and periodic serum samples during well visits. All children younger than 5 years of age who had archival nasal aspirates obtained during a respiratory illness and serum samples from ≤ 3 months before and after that illness during four influenza seasons spanning 1996 to 2000 were included in the study. Children with another viral pathogen cultured during the influenza season were excluded from the study. Eligible children who received either inactivated influenza vaccine or experimental live attenuated influenza vaccine during that influenza season were also excluded from the analysis.

Specimen collection. Nasal aspirates were obtained using 15 ml of 0.9% saline solution instilled in the nares with a bulb syringe. The solution was then collected in a cup and transferred to a vial containing penicillin-gentamicin and gelatin. An aliquot was used for viral testing at the time of collection, and the remainder of the nasal aspirate was frozen at -70°C . Serum specimens were collected, separated after 30 min at room temperature, and stored at -40°C .

Viral culture. Results from nasal-wash samples obtained from ill children presenting to the VVC between October 1996 and April 2000 are included in this report. Aliquots of the nasal samples (0.2 ml/tube) were inoculated onto rhesus

* Corresponding author. Mailing address: CCC-5323 MCN, Pediatric Clinical Research Office, Vanderbilt University School of Medicine, Nashville, TN 37232-2573. Phone: (615) 322-3078. Fax: (615) 322-2733. E-mail: kathryn.edwards@vanderbilt.edu.

TABLE 1. Influenza season, strain identified, and number of children with acute respiratory infection and appropriate specimens

Yr	Strain	No. of children tested	No. of positive cultures
1996–1997	A/Nanchang (H3N2)	31	6
	B/Harbin		1
1997–1998	A/Sydney (H3N2)	9	3
1998–1999	A/Sydney (H3N2)	31	8
	B/Beijing		2
1999–2000	A/Sydney (H3N2)	4	2
Total		75	22

monkey kidney (RMK) (Diagnostic Hybrids, Inc., Athens, Ohio) and Madin-Darby canine kidney (MDCK) (Aviron, Mountain View, Calif.) cell culture lines. The cultures underwent stationary incubation at 34°C and were observed for cytopathic effects three times per week. On day 5, one of two tubes of RMK and MDCK cells were hemadsorbed with 0.1% guinea pig red blood cells. If negative, they were washed and the medium was replaced. On day 10, both tubes of each cell line were hemadsorbed with 0.1% guinea pig red blood cells. All hemadsorbing isolates were confirmed using indirect fluorescent antibody procedures (Trinity Biotech PLC, Co. Wicklow, Ireland). To evaluate whether viable influenza virus degraded in archival nasal aspirates, a second viral culture was performed in the summer of 2000 on archival aspirates that were influenza virus culture positive at the time of collection.

Serology. Hemagglutination inhibition assays were performed on pre- and postseason archival serum samples using a standard assay protocol with reagents provided by the CDC (9). All sera were pretreated with receptor-destroying enzyme (*Vibrio cholerae* abnormal type 558 strain RDE; product code 340122; Denka Seiken Co., Ogdensburg, N.Y.) to eliminate nonspecific inhibitors. Seropositivity was defined as a fourfold or greater rise between pre- and postseason antibody titers for circulating influenza virus strains.

RT-PCR. The influenza virus PCR protocol has been described (13). Briefly, nucleic acids from 100 µl of the thawed archival nasal samples were extracted with RNAzol B (Leedo Laboratories, Inc., Houston, Tex.) according to the manufacturer's instructions. The extracted RNA was resuspended in 50 µl of 1× EN buffer (Applied Biosystems, Foster City, Calif.). A colorimetric microtiter plate PCR system was used to detect influenza virus A and B RNAs as previously described (17, 18). The PCR mixture (50 µl) contained the following: 1× EN buffer; 18% glycerol; 300 µM dATP, dCTP, and dGTP; 285 µM dUTP; 15 µM digoxigenin-11-dUTP (Roche Diagnostics, Indianapolis, Ind.); 0.5 µM each primer; 0.01 U of uracil-*N*-glycosylase (UNG) (Epicentre Technologies, Madison, Wis.)/µl; 0.15 U of *Tth* polymerase (Applied Biosystems)/µl; and 25 µl of specimen extract. The reaction mixture was placed in an ABI 9700 thermal cycler programmed for a one-step reverse transcription (RT)-PCR procedure. The procedure included (i) an initial UNG activation, RT, and UNG inactivation-denaturation of 5 min at 50°C, 30 min at 65°C, and 3 min at 94°C; (ii) 5 cycles of 15 s at 94°C and 30 s at 60°C; (iii) 45 cycles of 15 s at 90°C and 30 s at 60°C; and (iv) a 10-min extension at 72°C. Primer sets for influenza virus A (FluA01 [5'-CTT CTR ACC GAR GTC GAA ACG-3', where R = A or G] and FluA02 [5'-GAC AAA GCG TCT ACG CTG CAG-3']) and influenza virus B (BHA-188 [5'-AGA CCA GAG GGA AAC TAT GCC C-3'] and BHA-347 [5'-CTG TCG TGC ATT ATA GGA AAG CAC-3']) were designed to target the influenza virus A matrix gene (22) and the influenza virus B hemagglutinin gene (16), respectively. The output signal was measured at an optical density of 450 nm (OD₄₅₀). A positive result was defined as an OD₄₅₀-OD₄₉₀ value of ≥0.1. The test sensitivity was 0.01 PFU per ml for both of these PCR assays, which were developed and validated at Vanderbilt University Medical Center Clinical Laboratory under the 1988 Clinical Laboratory Improvement Amendments regulations.

RESULTS

Local strains of influenza virus that circulated during each of the four study years and the number of children enrolled in each season are shown in Table 1. Each year, influenza virus A H3N2 strains circulated: A/Nanchang in 1996–1997 and A/Sydney strain in 1997–1998, 1998–1999, and 1999–2000. Influenza virus B cocirculated with influenza virus A H3N2 during the

TABLE 2. Influenza virus detection: PCR of archival samples compared with culture at time of illness and acute and convalescent archival serology

PCR/serology results ^a	No. culture positive	No. culture negative
+/+	14	0
+/-	4	0
-/+	4	7
-/-	0	46
Total	22	53

^a +, positive; -, negative.

1996–1997 and 1998–1999 influenza seasons. We identified 75 VVC children with symptomatic illness during four influenza seasons with a viral culture performed at the time of illness, archival nasal aspirates available for PCR analysis, and archival paired sera bracketing each influenza season.

Twenty-two of the 75 children (29%) had culture-confirmed influenza virus A or B infection. Of the 22 culture-positive samples, 18 were also positive for influenza virus A or B by PCR (Table 2). All 53 of the culture-negative samples were negative by PCR. Using viral culture at the time of collection as the “gold standard,” the sensitivity, specificity, positive predictive value, and negative predictive value of PCR on archival specimens were 82, 100, 100, and 93%, respectively.

Because storage might have contributed to the reduced yield in the PCR, we repeated viral cultures of 21 of 22 (95%) archival nasal aspirates with positive cultures at the time of collection; one sample had inadequate residual volume for repeat culture. Among these 21 archival nasal aspirates, 11 (52%) had a positive repeat culture in the summer of 2000. The PCR was positive for all of the samples that were culture positive when repeated and for none of the samples that were negative on repeat culture.

Fourfold serologic rises were noted in 25 of 75 children (33%) with symptomatic illness during the study period. Of the 22 children with positive influenza virus cultures, 18 were also positive by serologic criteria (Table 2). In addition, another 7 of the 53 culture-negative children had fourfold titer rises to circulating influenza virus strains, and four of the viral-culture-positive subjects had no serologic response to the infection. Thus, using viral culture at the time of collection as the gold standard, the sensitivity, specificity, positive predictive value, and negative predictive value of serology on paired archival specimens were 82, 87, 72, and 92%, respectively.

Because viral culture does not detect all influenza virus infections (13), we compared the PCR of archival nasal aspirates to viral culture performed at the time of collection and to serology. In this analysis, a positive viral culture or positive serology was considered to indicate an influenza virus infection. Using culture and/or serology as the gold standard, PCR of archival nasal aspirates had a sensitivity of 62%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 81%.

DISCUSSION

Because influenza virus is commonly associated with significant morbidity and mortality in the elderly population, wide-

spread influenza vaccination in this age group has been strongly encouraged for many years. In contrast, widespread immunization of healthy children has not been generally recommended. However, two recent studies reported that the influenza virus-attributable hospitalization rate among children aged <2 years was similar to that of persons aged ≥ 65 years (8, 10). Hence, in March 2002, the Advisory Committee on Immunization Practices recommended that influenza vaccine be encouraged for all children between the ages of 6 and 23 months (2). The wider use of inactivated vaccine, the licensure of a live attenuated intranasal vaccine for children >5 years of age, and the need to determine the impact of influenza virus infections before and after vaccination programs will be measured in the CDC-funded NVSN. These initiatives prompted an earlier analysis (13) and the present study to determine the best methods to detect influenza virus infection retrospectively in archival specimens from children presenting with respiratory symptoms during influenza season.

Viral culture, the traditional gold standard for influenza virus detection, is an important and useful surveillance tool because it allows scientists to track and record the antigenic drifts and shifts of the influenza virus. However, the clinical utility of viral cultures is hampered by the several days to weeks it takes before definitive results are known. Comparing cultures performed at the time of illness with serology and PCR performed on archival samples, we made several observations. First, the majority of samples (82%) that were culture positive for influenza virus at the time of acute illness retained sufficient RNA to be detectable by PCR after several years of storage. However, not all culture-positive cases were detected by PCR, and no additional culture-negative cases were detected by PCR. These results contrast sharply with those of a previous study using the same validated PCR of fresh samples in which the PCR sensitivity was 92% (11 of 12 culture-positive specimens were identified by PCR) and seven culture-negative samples were PCR positive (13). The lower rate of detection of influenza virus by PCR of archival nasal specimens compared to PCR of fresh specimens is probably attributable to RNA degradation even when specimens are stored at -70°C . This explanation is supported by the higher sensitivity of PCR among samples that were positive than among negative samples on repeat culture.

Seroconversion in the face of negative influenza virus culture or PCR confirmation is more difficult to explain. This situation could represent asymptomatic infection, mildly symptomatic infection for which medical attention was not sought, symptomatic illness where inadequate samples were obtained, symptomatic illness in which cultures were obtained so late in the clinical illness that they were no longer positive, or undocumented influenza vaccination. The finding that a number of children with culture-confirmed influenza virus infection did not demonstrate a serologic response is also problematic. Seroconversion studies require acute- and convalescent-phase sera, results cannot be determined for at least a month, and blood sampling is often a disincentive for surveillance participation. In contrast, if properly frozen, archival serum does retain antibody titers and can be used many years after it was initially obtained.

PCR was shown to be very useful in an earlier study when performed on freshly obtained samples. However, whether

the PCR may be positive in the absence of clinical disease or for prolonged periods after resolution of symptoms has not been determined and is the subject of an ongoing investigation in the NVSN. The present study did show an advantage for PCR testing over culture of frozen specimens. Further, it demonstrated the limitations of PCR of archival samples and highlighted the need for further investigation of the best methods to preserve samples for retrospective epidemiologic studies.

ACKNOWLEDGMENTS

Funding was provided by contracts with the CDC (U38/CCU417958 and 99116) and by a summer scholarship from the Amos Christie Pediatric Foundation, Vanderbilt University Medical Center (B.F.).

REFERENCES

1. Aymard, M., J. J. Chomel, J. P. Allard, et al. 1994. Epidemiology of viral infections and evaluation of the potential benefit of OM-85 BV on the virologic status of children attending day-care centers. *Respiration* **61**:24–31.
2. Bridges, C. B., K. Fukuda, T. M. Uyeki, N. J. Cox, and J. A. Singleton. 2002. Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *Morb. Mortal. Wkly. Rep. Recomm. Rep.* **51**:1–31.
3. Carman, W. F., L. A. Wallace, J. Walker, et al. 2000. Rapid virological surveillance of community influenza infection in general practice. *BMJ* **321**:736–737.
4. Cherian T., L. Bobo, M. C. Steinhoff, R. A. Karron, and R. H. Yolken. 1994. Use of PCR-enzyme immunoassay for identification of influenza A virus matrix RNA in clinical samples negative for cultivable virus. *J. Clin. Microbiol.* **32**:623–628.
5. Esteban, R. E., A. M. Jimenez, A. L. Orozco, I. G. Girones, P. G. Sanchez, and F. S. Garcia. 1996. Etiology of acute respiratory infections in 87 hospitalized children. *Rev. Clin. Esp.* **196**:82–86.
6. Frank, A. L., L. H. Taber, C. R. Wells, J. M. Wells, W. P. Glezen, and A. Paredes. 1981. Patterns of shedding of myxoviruses and paramyxoviruses in children. *J. Infect. Dis.* **144**:433–441.
7. Hermann, B., C. Larsson, and B. W. Zwegberg. 2001. Simultaneous detection and typing of influenza viruses A and B by a nested reverse transcription-PCR: comparison to virus isolation and antigen detection by immunofluorescence and optical immunoassay. *J. Clin. Microbiol.* **39**:134–138.
8. Izureta, H. S., W. W. Thompson, P. Kramarz, et al. 2000. Influenza and the rates of hospitalization for respiratory disease among infants and young children. *N. Engl. J. Med.* **342**:231–239.
9. Lennette, E. H., and N. J. Schmidt. 1995. General principles for laboratory diagnoses of viral, rickettsial, and chlamydial infections, p. 7–14. In E. H. Lennette, D. A. Lennette, and E. T. Lennette (ed.), *Diagnostic procedures for viral, rickettsial, and chlamydial infections*, 7th ed. American Public Health Association, Washington, D.C.
10. Neuzil, K. M., B. G. Mellen, P. F. Wright, E. F. Mitchel, Jr., and M. R. Griffin. 2000. The effect of influenza on hospitalizations, outpatient visits, and courses of antibiotics in children. *N. Engl. J. Med.* **342**:225–231.
11. Neuzil, K. M., Y. Zhu, M. R. Griffin, et al. 2002. Burden of inter pandemic influenza in children younger than 5 years: a 25-year prospective study. *J. Infect. Dis.* **185**:147–152.
12. Plakokefalos, E., P. Markoulatos, E. Ktenas, N. Spyrou, and N. 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.
13. Poehling, K. A., M. R. Griffin, R. S. Dittus, Y. W. Tang, K. Holland, H. Li, and K. M. Edwards. 2002. Bedside diagnosis of influenza virus infections in hospitalized children. *Pediatrics* **110**:83–88.
14. 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.
15. Rebelo-de-Andrade, H., and M. C. Zambon. 2000. Different diagnostic methods for detection of influenza epidemics. *Epidemiol. Infect.* **124**:515–522.
16. 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.
17. Tang, Y. W., P. J. Heimgartner, S. J. Tollefson, T. J. Berg, P. N. Rys, H. Li, T. F. Smith, D. H. Persing, and P. F. Wright. 1999. A colorimetric microtiter plate PCR system detects respiratory syncytial virus in nasal aspirates and discriminates subtypes A and B. *Diagn. Microbiol. Infect. Dis.* **34**:333–337.
18. Tang, Y. W., P. N. Rys, B. J. Rutledge, P. S. Mitchell, T. F. Smith, and D. H. Persing. 1998. Comparative evaluation of colorimetric microtiter plate systems for detection of herpes simplex virus in cerebrospinal fluid. *J. Clin. Microbiol.* **36**:2714–2717.
19. Vabret, A., G. Sapin, B. Lezin, A. Mosnier, J. Cohen, L. Burnouf, J.

- Petitjean, S. Gouarin, M. Campet, and F. Freymuth. 2000. Comparison of three non-nested RT-PCR for the detection of influenza A viruses. *J. Clin. Virol.* **17**:167–175.
20. van Elden, L. J. R., M. Nihuis, 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.
21. Wallace, L. A., K. A. McAulay, J. D. Douglas, A. G. Elder, D. J. Stott, W. F. Carman, et al. 1999. Influenza diagnosis: from dark isolation into the molecular light. *J. Infect.* **39**:221–226.
22. Winter, G., and S. Fields. 1980. Cloning of influenza cDNA into M13: the sequence of the RNA segment encoding the A/PR/8/34 matrix protein. *Nucleic Acids Res.* **8**:1965–1974.