JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1993, p. 2218–2221
0095-1137/93/082218-04$02.00/0

Vol. 31, No. 8

Prospective Application of Reverse Transcriptase Polymerase Chain Reaction for Diagnosing Influenza Infections in Respiratory Samples from a Children's Hospital

E. C. J. CLAAS,[1]* A. J. VAN MILAAN,[1] M. J. W. SPRENGER,[1] M. RUITEN-STUIVER,[1] G. I. ARRON,[2] P. H. ROTHBARTH,[2,3] AND N. MASUREL[1,2,3]

Department of Virology, National Influenza Centre, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam,[1] Department of Virology, Sophia Children's Hospital, 3038 GE Rotterdam,[2] and Department of Virology, University Hospital Rotterdam, 3015 GD Rotterdam,[3] The Netherlands

Received 27 January 1993/Accepted 18 May 1993

A prospective clinical evaluation of the reverse transcriptase polymerase chain reaction (RNA PCR) for detection of influenza viruses was carried out with specimens from 342 patients of a children's hospital in The Netherlands. The RNA PCR, carried out directly on the specimens without an organic extraction, showed a sensitivity and specificity which are superior to those of direct immunofluorescence and comparable to those of cell culture combined with immunofluorescence (culture/IF). Negative results can be obtained within 2 days by the RNA PCR but may take up to 14 days by culture/IF. Because culturing is the standard technique for the detection of respiratory viruses, at this moment there are no strong arguments to replace culture/IF with RNA PCR for the detection of influenza A virus.

In acute respiratory infections, rapid diagnosis of the pathogen is important, e.g., for antiviral therapy, avoiding nosocomial spread of infections, or epidemiological measures. Influenza viruses are the most important causes of influenza-like illness in the adult, with considerable morbidity and mortality, mainly by secondary bacterial infections. In particular, elderly patients with underlying heart, lung, or immune disease or diabetes are at risk (1, 7, 18–20).

It is almost impossible to distinguish the pathogens of respiratory infections by their clinical presentation, and therefore they should be diagnosed by laboratory testing. Influenza viruses are generally detected by isolation of the virus from specimens of patients with acute respiratory disease or influenza-like illness on tissue culture cells. Faster results can be obtained by a short culture for 12 to 48 h with subsequent detection of the specific antigens by immunofluorescence (15). More-rapid diagnostic tests, such as antigen detection or molecular hybridization, have been carried out for detection of the virus as well (5, 8, 9, 16, 17, 21). The major drawback of these techniques is the limited sensitivity or specificity. Recently, several groups have carried out reverse transcriptase polymerase chain reaction (RNA PCR) for detection of influenza virus genomes (3, 14, 23). Theoretically, PCR is one of the most sensitive and specific techniques available at the moment. Many of its applications for detection of microbiological pathogens have been described (for a review, see reference 22), and although it is shown that the technique can be used for clinical samples, there is a lack of prospective clinical evaluations.

In a previous report, we described an RNA PCR for the type-specific detection of influenza viruses (6). In summary, the RNA PCR was shown to be a very specific and sensitive technique for detection of influenza virus RNA genomes in clinical specimens. In the study described here, the application of this technique to clinical specimens was prospectively evaluated.

* Corresponding author. Electronic mail address: claas@viro.fgg.eur.nl.

Clinical specimens were obtained from the University Hospital, Rotterdam, and from the Sophia Children's Hospital, Rotterdam. All samples were completed to 5 ml with Dulbecco's minimal essential medium (Flow Laboratories, Irvine, United Kingdom) containing antibiotics. For PCR analysis, 100 μl was put in a 1.5-ml reaction tube containing an equal volume of 50% (wt/vol) sucrose, and the samples were stored at −70°C until being tested.

For cell culture and direct immunofluorescence (DIF), the diluted specimen was centrifuged at 750 × *g* for 5 minutes. Six glass slides, covered with monolayers of tertiary Rhesus monkey kidney (MK) cells, were inoculated with 0.2 ml of supernatant by centrifugation at 840 × *g* for 1 h. The influenza viruses were grown in serum-free medium in the presence of trypsin (Gibco, Paisley, Scotland). After incubation for 12 to 24 h, the cells on two slides were fixed in acetone and stained with fluorescent monoclonal antibodies (Imagen; Dako Diagnostics, Copenhagen, Denmark) as described previously (15). In case of negative results, culturing of the other slides was continued. Immunofluorescence was repeated when cytopathologic changes (CPE) were observed or suspected. Cultures without any CPE were discarded after 14 days.

DIF was carried out on the cell pellet, which was resuspended in one drop of medium and subsequently smeared on multispot glass slides (Nutacon, Amsterdam, The Netherlands) and stained directly with monoclonal antibody.

To make the RNA PCR more fit for clinical diagnostic settings, the organic extraction of nucleic acids was omitted. To show the feasibility of this shorter pretreatment, initially a retrospective study was carried out on 22 nasopharyngeal aspirates, 2 bronchoalveolar lavages, and 6 throat swabs from the 1991–1992 season with known positive culture results. A hemagglutination inhibition assay (11) revealed that 18 influenza B, 10 influenza A/H1N1, and 2 influenza A/H3N2 virus strains were isolated from these specimens. For the RNA PCR, 25 μl of the clinical sample was centrifuged at 10,000 × *g* for 10 min. The pellet was resuspended in 25 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5),

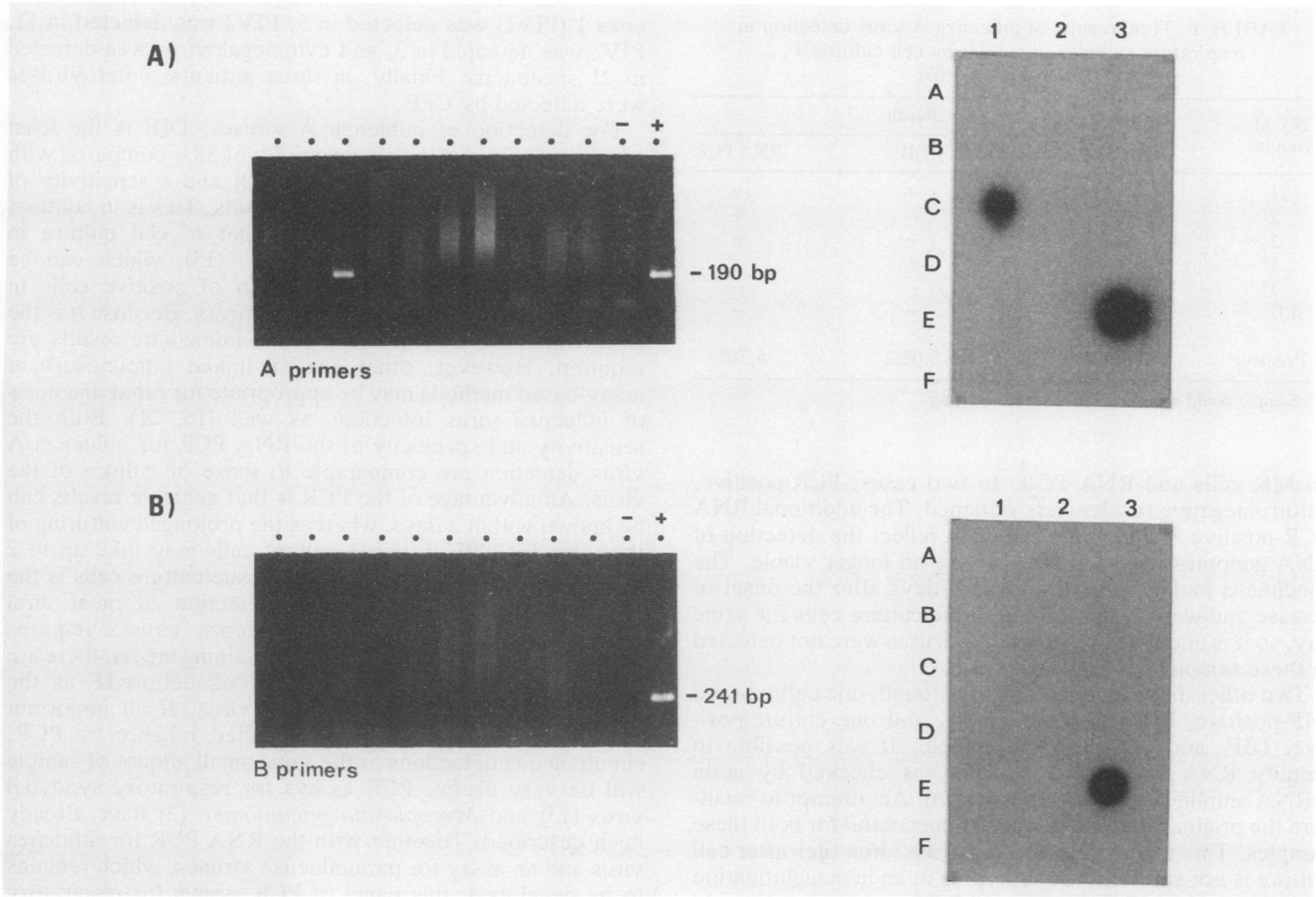

FIG. 1. Analysis of the PCR products on a 2% agarose gel after amplification with the influenza A (A) and the influenza B (B) virus primers. Besides the positive controls (+), one positive specimen (influenza A, lane 3) can be seen. This result is confirmed in the dot spot hybridization (C-1 on the exposed film). Positive controls are shown on spot E-3.

placed at 90°C for 5 min, and kept on ice. Then, 5 μl was used for each cDNA reaction and the cDNA was subjected to 5 cycles of amplification in the PCR at a 52°C annealing temperature and an additional 35 cycles at an annealing temperature of 46°C. The cDNA reaction and PCR were carried out as described previously (6) and according to a strict protocol with precautions to prevent contamination (10). Distilled water and MK-RNA were included as negative controls. For each sample, separate reactions were performed with the influenza A and influenza B virus primers (A-cDNA: AAGGGCTTTCACCGAAGAGG; A-rev: CCCA TTCTCATTACTGCTTC; B-cDNA: ATGGCCATCGGATC CTCAAC; B-rev: TGTCAGCTATTATGGAGCTG), which were derived from conserved NS gene sequences (6). The amplified products were analyzed by electrophoresis on a 2% agarose gel and by dot spot hybridization (A-probe: GTCCTCATCGGAGGACTTGAATGGAATGAT; B-probe: CCAATTTGGTCAAGAGCACCGATTATCACC) as previously described (6). The RNA PCR could confirm the presence of the influenza viruses in all of the 30 samples. In accordance with the virus subtypes, 18 influenza B and 12 influenza A viruses were detected.

The prospective evaluation was carried out with all clinical specimens, 430 nasopharyngeal aspirates and 4 bronchoalveolar lavages, which were submitted to the virologic laboratory of the Sophia Children's Hospital for testing on respiratory pathogens. A total of 434 samples from 342 patients were collected from 15 November 1991 until 15 April 1992. Typical RNA PCR results are shown in Fig. 1. All positive samples contained influenza A virus; no influenza B virus was detected. If a faint signal was found after hybridization although no signal could be observed on an agarose gel, RNA was extracted from the clinical sample using the guanidinium thiocyanate method of Chomczynski and Sacchi (4). This RNA was then subjected to the RNA PCR. As a consequence of this, a total of 12 samples were retested and only 1 was found positive.

Table 1 shows the overall results of the samples analyzed. Discrepancies were retested by culture and RNA PCR. Neither of the RNA PCR-negative, culture-positive samples could be cultured again. However, in one of these specimens the culture positivity was confirmed by DIF positivity and, in addition, a specific amplified product was generated by analyzing the supernatant of the cell culture with RNA PCR.

In Table 2 the sensitivities of the techniques are shown, compared with those of cell culture, RNA PCR, and the combined results. Cell culture and RNA PCR show similar sensitivities, whereas DIF showed the least sensitivity. The specificity for all three methods was >99%.

The results show that the RNA PCR was not clearly superior to isolation of the virus in tissue culture. Out of 342 patients, 23 (27 samples, all nasopharyngeal aspirates) were found positive for influenza A by one of the techniques. Twenty-three samples were found positive by both culturing

TABLE 1. Total results of influenza A virus detection in respiratory samples (n = 434) by cell culture/IF, DIF, and RNA PCR

No. of samples	Result		
	Culture/IF	DIF	RNA PCR
21	+	+	+
2	−	−	+
2	+	−	+
1[a]	+	+	−
1[a]	+	−	−
407	−	−	−
% Positive	5.76%	5.06%	5.76%

[a] Sample could not be cultured after retesting.

on MK cells and RNA PCR. In two cases, PCR-positive, culture-negative results were obtained. The additional RNA PCR-positive results most probably reflect the detection of RNA genomes from viruses that are no longer viable. The specimens had been taken within 4 days after the onset of disease and were inoculated on tissue culture cells the same day, so it is unclear why infectious viruses were not detected in these samples.

Two other discrepant results were found: one culture- and DIF-positive, PCR-negative sample, and one culture-positive, DIF- and PCR-negative sample. It was possible to amplify RNA from these samples, as checked by actin mRNA amplification (data not shown). An attempt to reculture the original specimens was not successful for both these samples. This reculturing is done if the virus titer after cell culture is not sufficient for subtyping in an hemagglutination inhibition assay. The culture- and DIF-positive result could be confirmed by performing RNA PCR on the supernatant of the culture. A possible explanation for this discrepancy is in a very small amount of infectious virus, which was found positive when immediately cultured but remained negative in the RNA PCR on the original specimen and upon retesting by culture. The result for the sample that was positive only by culture immunofluorescence (culture/IF) could not be confirmed in any way. Therefore, this was possibly a false-positive IF result, although this is impossible to prove. No serological data are available.

As shown from the results, only influenza A viruses, H1N1 as well as H3N2, but no influenza B virus circulated in the 1991–1992 season (12). Therefore, only the influenza A virus RNA PCR could be evaluated. The value of the influenza B virus PCR remains to be established.

The specimens were not tested for influenza A and B viruses only. From the 318 influenza A virus-negative patient specimens, respiratory syncytial virus was detected by cell culture/IF in 109, adenovirus was detected in 5, parainfluenza 1 (PIV1) was detected in 5, PIV2 was detected in 11, PIV3 was detected in 3, and cytomegalovirus was detected in 21 specimens. Finally, in three patients, enteroviruses were detected by CPE.

TABLE 2. Comparison of the sensitivities of cell culture/IF, RNA PCR, and DIF

Reference[a]	% Sensitivity		
	Culture/IF	DIF	RNA PCR
Cell culture/IF (n = 25)	100	88	92
RNA PCR (n = 25)	92	88	100
All (n = 27)	92.6	81.5	92.6

[a] Positive results by culture/IF, RNA PCR, and all methods are used for comparison.

For detection of influenza A viruses, DIF is the least sensitive technique with a sensitivity of 88% compared with culture/IF as well as with RNA PCR and a sensitivity of 81.5% compared with all positive results. This is in contrast with a sensitivity comparable to that of cell culture in respiratory syncytial virus diagnosis (15), which can be explained by the high concentration of positive cells in respiratory syncytial virus-positive smears. Because it is the fastest method, DIF is useful when immediate results are required. However, other enzyme-linked immunosorbent assay-based methods may be appropriate for rapid diagnosis of influenza virus infections as well (16, 21). Both the sensitivity and specificity of the RNA PCR for influenza A virus detection are comparable to those of culture of the virus. An advantage of the PCR is that negative results can be known within 2 days, whereas the prolonged culturing of the virus for CPE in tissue culture cells may take up to 2 weeks. Nevertheless, isolation on tissue culture cells is the standard method for diagnostic detection of most viral pathogens. Although culturing influenza viruses requires special treatment such as trypsin-containing media, there are no strong arguments for replacing cell culture/IF as the diagnostic method for influenza A virus. If all important respiratory pathogens can be detected reliably by PCR, simultaneous detections in the same small aliquot of sample will be very useful. PCR assays for respiratory syncytial virus (13) and *Mycoplasma pneumoniae* (2) have already been described. Together with the RNA PCR for influenza virus and an assay for parainfluenza viruses, which remains to be developed, this panel of PCR assays for respiratory pathogens may become a useful tool in respiratory surveillance.

This work was supported by the "Foundation for Respiratory Viruses, especially Influenza (SRVI)."

We thank Ruud van Beek for technical assistance.

REFERENCES

1. **Barker, W. H., and P. J. Mullooly.** 1982. Pneumonia and influenza deaths during epidemics. Arch. Intern. Med. **142**:85–89.
2. **Bernet, C., M. Garret, B. de Barbeyrac, C. Bebear, and J. Bonnet.** 1989. Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction. J. Clin. Microbiol. **27**:2492–2496.
3. **Bressoud, A., J. Whitcomb, C. Pourzand, O. Haller, and P. Cerutti.** 1990. Rapid detection of influenza virus H1 by the polymerase chain reaction. Biochem. Biophys. Res. Commun. **167**:425–430.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
5. **Chomel, J. J., D. Pardon, D. Thouvenot, J. P. Allard, and M. Aymard.** 1991. Comparison between three rapid methods for direct diagnosis of influenza and the conventional isolation procedure. Biologicals **19**:287–292.
6. **Claas, E. C. J., M. J. W. Sprenger, G. E. M. Kleter, R. van Beek, W. G. V. Quint, and N. Masurel.** 1992. Type-specific identification of influenza viruses A, B, and C by the polymerase chain reaction. J. Virol. Methods **39**:1–13.
7. **Diepersloot, R. J. A., K. P. Bouter, and J. B. L. Hoekstra.** 1990. Influenza infection and diabetes mellitus. Case for annual vaccination. Diabetes Care **13**:876–881.
8. **Döller, G., W. Schuy, K. Y. Tjhen, B. Stekeler, and H.-J. Gerth.** 1992. Direct detection of influenza virus antigen in nasopharyngeal specimens by direct enzyme immunoassay in comparison

with quantitating virus shedding. J. Clin. Microbiol. **30**:866–869.
9. **Havlickova, M., A. Z. Pljusnin, and B. Tumova.** 1990. Influenza virus detection in clinical specimens. Acta Virol. **34**:446–456.
10. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339**:237–238.
11. **Masurel, N.** 1969. Relation between Hong Kong virus and former A2 isolates and the A/Equi2 virus in human sera collected before 1957. Lancet **i**:907–910.
12. **Masurel, N.** 1992. Influenza in the 1991/'92 season; vaccine composition for the 1992/'93 season. Ned. Tijdschr. Geneeskd. **136**:1780–1783.
13. **Paton, A. W., J. C. Paton, A. J. Lawrence, P. N. Goldwater, and R. J. Harris.** 1992. Rapid detection of respiratory syncytial virus in nasopharyngeal aspirates by reverse transcription and polymerase chain reaction amplification. J. Clin. Microbiol. **30**:901–904.
14. **Pisareva, M., T. Bechtereva, A. Plyusnin, A. Dobretsova, and O. Kisselev.** 1992. PCR amplification of influenza A virus specific sequences. Arch. Virol. **125**:313–318.
15. **Rothbarth, P. H., J. J. Habova, and N. Masurel.** 1988. Rapid diagnosis of infections caused by respiratory syncytial virus. Infection **16**:252.
16. **Ryan-Poirier, K. A., J. M. Katz, R. G. Webster, and Y. Kawaoka.** 1992. Application of directigen FLU-A for the detection of influenza A virus in human and nonhuman specimens. J. Clin. Microbiol. **30**:1072–1075.
17. **Spada, B., K. Biehler, P. Chegas, J. Kaye, and M. Riepenhoff-Talty.** 1991. Comparison of rapid immunofluorescence assay to cell culture isolation for the detection of influenza A and B viruses in nasopharyngeal secretions from infants and children. J. Virol. Methods **33**:305–310.
18. **Sprenger, M. J. W., P. G. Mulder, W. E. P. Beyer, R. Van Strik, and N. Masurel.** 1993. Impact of influenza on mortality in relation to age and underlying disease in 1967–1989. Int. J. Epidemiol. **22**:334–340.
19. **Sutherland, J. E., V. W. Persky, and J. A. Brody.** 1990. Proportionate mortality trends: 1950 through 1986. JAMA **264**: 3178–3184.
20. **Tillett, H. E., J. W. Smith, and C. D. Gooch.** 1983. Excess deaths attributable to influenza in England and Wales: age at death and certified cause. Int. J. Epidemiol. **12**:344–352.
21. **Waner, J. L., S. J. Todd, H. Shalaby, P. Murphy, and L. V. Wall.** 1991. Comparison of directigen FLU-A with viral isolation and direct immunofluorescence for the rapid detection and identification of influenza A virus. J. Clin. Microbiol. **29**:479–482.
22. **Wright, P. A., and D. Wynford-Thomas.** 1990. The polymerase chain reaction: miracle or mirage? A critical review of its uses and limitations in diagnosis and research. J. Pathol. **162**:99–117.
23. **Yamada, A., J. Imanishi, E. Nakajima, K. Nakajima, and S. Nakajima.** 1991. Detection of influenza viruses in throat swab by using polymerase chain reaction. Microbiol. Immunol. **35**:259–265.