



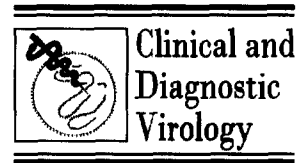
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Advances in the diagnosis of respiratory virus infections

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Abstract

Background: Advances have been made in selecting sensitive cell lines for isolation, in early detection of respiratory virus growth in cells by rapid culture assays, in production of monoclonal antibodies to improve many tests such as immunofluorescence detection of virus antigens in nasopharyngeal aspirates, in highly sensitive antigen detections by time-resolved fluoroimmunoassays (TR-FIAs) and biotin-enzyme immunoassays (BIOTH-E), and, finally, in the polymerase chain reaction (PCR) detection of respiratory virus DNA or RNA in clinical specimens. All of these advances have contributed to new or improved possibilities for the diagnosis of respiratory virus infections.

Objectives and study design: This review summarizes our experiences during the last 15 years in the development of diagnostic tests for respiratory virus infections, and in use of these tests in daily diagnostic work and in epidemiological studies.

Results: Immunofluorescence tests based on monoclonal antibodies, all-monoclonal TR-FIAs, and biotin-enzyme immunoassays (EIAs) have about the same sensitivities and specificities. They compare well with the sensitivity of virus culture. PCR followed by liquid-phase hybridization is a sensitive method for detecting adenovirus DNA and enterovirus and rhinovirus RNA in clinical specimens. IgG EIA on paired acute and convalescent phase sera is the most sensitive serological test for respiratory virus infections and is a valuable reference method when evaluating the sensitivity of new diagnostic tests. The IgG avidity test can distinguish primary infections from re-infections at least in respiratory syncytial virus (RSV) infections. IgM antibody assays, on the other hand, had low sensitivities in our studies.

Conclusions: The choice of diagnostic methods for respiratory virus infections depends on the type and location of the laboratory, the number of specimens tested, and the previous experience of the laboratory. Virus culture, whenever possible, should be the basic diagnostic method; the results, including identification of the virus, should be available no more than 24 h later than the results of rapid diagnostic tests. In small laboratories, especially in hospitals where specimen transportation is well organized, immunofluorescence may be the best choice for antigen detection with the provision that an experienced microscopist and a good UV microscope are available. If the laboratory receives a large number of specimens and has previous experience with EIAs, then biotin-EIAs or TR-FIAs may be the most practical techniques. Their advantages include the stability of the antigens in clinical samples since intact, exfoliated epithelial cells are not required, treatment of specimens is practical, testing of large numbers of specimens is possible, and reading the printed test result is less subjective than reading fluorescence microscopy. The larger role of PCR in the diagnosis of respiratory virus infections depends on future developments such as practical methods to extract DNA or RNA and to purify the extracts from nonspecific inhibitors, plus further improvements to minimize cross-contamination.

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Group-specific detection of enteroviruses and rhinoviruses is an example of the potential for PCR technology. In experienced laboratories, EIA IgG antibody tests should be available. Recombinant antigens may be a useful part of such assays.

Keywords: Respiratory viruses; Polymerase chain reaction (PCR); Antigen detection; Rapid culture assay

1. Introduction

In general, the new developments in respiratory virus diagnosis follow the same trends as in viral diagnosis. Rapid diagnostic methods are of particular importance for respiratory viruses since they have short incubation periods and epidemics spread quickly, even globally. The improvements in respiratory virus diagnostic methods include introduction of more sensitive cell lines for virus culture and an early detection of viral antigens in these cultures by immunoperoxidase staining (IPS) with monoclonal antibodies (rapid culture assay), application of monoclonal antibodies for immunofluorescence, enzyme immunoassay (EIA) and time-resolved fluoroimmunoassay (TR-FIA) detection of viral antigens directly in clinical specimens, and polymerase chain reaction (PCR) detection of viral DNA or RNA in clinical specimens followed by demonstration of specific PCR products with liquid-phase hybridization. This review focuses on our 15-year experience in the development of these diagnostic methods for respiratory viruses and particularly on the newer trends. More conventional techniques are only briefly discussed.

2. Specimen collection and shipment

An excellent specimen for virus culture, antigen detection and PCR is the nasopharyngeal aspirate (NPA) collected with a disposable mucus extractor (Waris et al., 1990). For virus culture, a cotton stick is dipped into the freshly collected aspirate specimen and then placed in viral transport medium. Specimens for virus isolation and for immunofluorescence assays (IFA) must be kept refrigerated until they are processed in the laboratory. If specimens for virus isolation cannot be processed within 48 h, storage at -70°C should be considered. For antigen assays such as TR-FIA and biotin-EIA, transportation and storage of the specimens are less critical.

Other specimens commonly used for the diagnosis of respiratory virus infections are nasal washes as well as throat and/or nasal swabs. If clinically indicated, bronchoalveolar lavage or transtracheal aspirates should be considered. For PCR, all specimens can be used because a minimal amount of virus is required (Hierholzer et al., 1993c). Details of specimen collection are provided elsewhere (Hierholzer, 1993; Johnson, 1990; Lennette et al., 1995; Schmidt and Emmons, 1989).

3. Cell cultures

When processing for viral isolation, specimens are treated with antibiotics, mixed well, clarified with low-speed centrifugation, and inoculated onto cell culture monolayers. The cultures should include a continuous human epithelial line (HEp-2, A-549, HeLa), a human embryonic lung diploid fibroblast cell strain (HLF, HELF, MRC-5, WI-38), human lung mucoepidermoid cells (NCI-H292) to replace primary rhesus monkey kidney (RMK) cells for most applications (Castells et al., 1990; Hierholzer et al., 1993b), and human rhabdomyosarcoma cells (RD) for a broad, practical coverage of viruses (Hierholzer, 1993; Hierholzer and Hatch, 1985). The inoculum is adsorbed to the monolayers at ambient temperature, and the cultures are then fed with maintenance medium and incubated at $35-36^{\circ}\text{C}$ for several weeks, with subpassaging as required. Tube cultures of MK or AGMK monkey kidney cells and their derivative lines (Vero, BSC-1, LLC-MK2, etc.), all diploid fibroblast cell cultures, and NCI-H292 cells should be rolled in roller drums or agitated on rocker platforms. Cultures of HEK, HEp-2, KB, A-549, and HeLa do not need to be rolled except for measles virus isolation in HEK and respiratory syncytial virus (RSV) isolation in HEp-2. Viral growth is detected by cytopathic effect (CPE) under light microscopy, hemadsorption, or HA.

Herpes simplex grows well in many cell types, notably primary rabbit kidney, HEK, HEp-2, HeLa, A549, HLF, NCI-H292 and mink lung cells; type 2, usually a genital isolate, grows more slowly than type 1. Cytomegalovirus (CMV) a notably labile virus, is the only herpesvirus that is shed in great amounts in the urine, and replicates slowly in roller cultures of diploid fibroblast cells, producing giant cells in 12–30 days. Herpesviruses are readily visualized by EM, with the cubic icosahedral shape of the capsid being prominent (Palmer and Martin, 1988). The viruses are commonly identified by IFA, EIA, IPS, DNA probes, and PCR.

Adenoviruses replicate readily in HEK, HEp-2, A-549, and NCI-H292 cells, with or without rolling. They are among the easier viruses to identify because they produce excess quantities of soluble antigens. Adenoviruses are differentiated from other viruses, which may grow in the same cells and with similar CPE, by EM, complement fixation (CF) latex agglutination (LA), IFA, EIA, TR-FIA, DNA probes, restriction enzymes, PCR with suitable primers, and cytological methods; they are then serotyped by hemagglutination-inhibition and serum neutralization tests.

Parainfluenza and mumps viruses replicate well in roller cultures of NCI-H292 cells under a fortified medium containing trypsin, and in MK cells without trypsin (Castells et al., 1990). The CPE induced by these viruses may develop in 4–7 days, but the cultures should be blind-passaged and held an additional week to ensure viral growth. The cells rarely become detached, and may not show obvious CPE at all, so that NCI-H292 or MK cultures for these viruses must be hemadsorbed with guinea pig, human, or monkey erythrocytes at the end of the culture period, and the viruses then typed by hemagglutination inhibition (HI), IFA, EIA, or TR-FIA tests. Measles virus causes CPE in AGMK, Vero, and HEK cells after 7–14 days of roller culture. The CPE usually develops slowly and may not be recognized and as a result, the monolayers should be hemadsorbed with vervet monkey erythrocytes; the virus is then identified by HI tests with vervet erythrocytes, IFA, EIA, and neutralization tests, or by hybridization and PCR tests. RSV produces distinct syncytia in HEp-2, HeLa, and NCI-H292 cells in 5–12 days in roller cultures.

Groups A and B strains of RSV are readily identified by IFA, EIA, and TR-FIA tests (Anderson et al., 1985; Hierholzer et al., 1990, 1994b; Lennette et al., 1995; Waris, 1991).

Influenza A, B, and C viruses are best recovered in roller cultures of MK or MDCK cells, and in embryonated eggs. Chick embryo, MDCK, and other cells require a fortified medium containing trypsin for optimal sensitivity (Frank et al., 1979; Klenk et al., 1975; Meguro et al., 1979). The viruses are detected in MK cells by hemadsorption and in MDCK cells by HA, and are then identified by IFA, IPA, EIA, HI, or neutralization tests (Grandien et al., 1985; Ziegler et al., 1995).

The enteroviruses and rhinoviruses produce CPE in NCI-H292, MK, HeLa, RD, trypsin treated MA-104, and diploid fibroblast cells, preferably in roller cultures. Some Coxsackie A viruses grow only in suckling mouse brain. Enteroviruses are distinguished from rhinoviruses by the former's acid stability. The viruses are serotyped by neutralization tests (Agbalika et al., 1984; Hierholzer and Hatch, 1985; Hyypää and Stanway, 1993; Lennette et al., 1995).

The coronaviruses are best identified directly in nasal and throat specimens by FA, EIA, and TR-FIA because the viruses are extremely labile and difficult to recover in the laboratory. The peplomers constitute the primary antigen detected in all tests, including the HI test for strain OC-43 (Hierholzer et al., 1994a).

4. Rapid culture assays

Rapid culture assays combine the high sensitivity of standard virus isolation with the speed of cell culture independent methods for the detection of viral antigens or nucleic acids in clinical specimens. Viral antigens are detected in cell cultures by immunological staining 16–48 h after inoculation, usually before a distinct, virus induced CPE is microscopically visible. Cells are grown in shell vials or in 24-well cell culture dishes. Clinical specimens are inoculated into an adequate number of vials or wells, and the cultures are then centrifuged at approximately $700 \times g$ at ambient temperature for 30–60 min. It has been documented that this centrifuged inoculation increases the sensitivity of

the rapid culture assay for a number of viruses. Thereafter, the cultures are incubated at 37°C in a 5% CO₂ atmosphere for an appropriate period. Next, the culture medium is removed, the cells are washed with phosphate-buffered saline (PBS) and then fixed with acetone or methanol. For cells grown in plastic dishes, acetone should be used at a concentration of 80% in PBS, as undiluted acetone will melt the plastic. Viral antigens in the cultured cells are detected by IFA or by IPA. For IFA, cultures are usually grown on coverslips in shell vials, and after staining, the coverslips are mounted on microscope slides. IPA staining can be performed in the 24-well plates and read at 40 × – 100 × magnification with an inverted microscope. Under optimal conditions viral plaques can grow large enough during an overnight incubation that they can be identified by the naked eye after staining (Ziegler et al., 1988, 1995). The theoretical sensitivity of rapid culture assays is one infectious virus per inoculation volume.

Rapid culture assays first found wide application for the detection of CMV induced early nuclear antigens in infected human diploid fibroblast cells (Gleaves et al., 1984; Griffiths et al., 1984). Identification and subtyping of herpes simplex viruses (HSV) is another application for rapid culture assay (Gleaves et al., 1985; Ziegler et al., 1988). The technique has been adapted for a rapid diagnosis of infections caused by respiratory viruses (Waris et al., 1990; Ziegler et al., 1995). A recently described rapid culture assay allows for the type- and subtype-specific identification of influenza viruses directly from clinical specimens after an overnight incubation in MDCK cultures (Ziegler et al., 1995).

Although rapid culture assays as such do not yield an isolate for further analysis, positive specimens can be reinoculated into standard cultures without considerable loss of infectivity once the result of the rapid culture is known. Alternatively, the culture supernatant can be stored and used for reinoculation.

5. Antigen detection in clinical specimens

5.1. Immunofluorescence (IFA)

Detection of viruses in nasopharyngeal aspirates

of patients with acute respiratory disease by IFA was developed in 1968 using rabbit antisera and antispecies conjugates. The method was a breakthrough in the rapid diagnosis of respiratory viruses, and excellent results were obtained when nasal aspirates were collected by specifically trained persons, exfoliated epithelial cells were carefully washed out from mucus and other nonspecifically staining material in specimens, and reading was done by an experienced microscopist. Later, this technology was improved by preparing the immunoreagents from monoclonal antibodies; non-specific fluorescence was reduced and the intensity of specific fluorescence increased (Kim et al., 1983). These reagents are now commercially available (Hierholzer, 1991).

The collection of nasopharyngeal specimens requires expertise, and for IFA, the transportation of the specimen requires special attention. The specimen should be kept cold at all times until the cells have been separated and fixed on microscope slides. IFA technology is particularly useful in small hospital laboratories where transportation of specimens is quick, the number of specimens tested daily is limited, and an experienced reader is always available.

5.2. Solid-phase immunoassays (RIA, EIA, TR-FIA)

Later, more sensitive immunoassay (RIA, EIA and TR-FIA) tests were developed for the detection of respiratory viruses (Arstila and Halonen, 1988; Grandien et al., 1985; Halonen et al., 1983, 1985, 1989). These assays had additional advantages: specimen transportation was not critical since intact cells are not required; treatment of the specimen is practical; bulk testing of specimens is possible; and reading the printed out test results is more objective than reading immunofluorescence. However, the final interpretation of the test results often requires expertise (McIntosh et al., 1993). When TR-FIA and biotin-EIAs based on monoclonal antibodies and the one-incubation principle were developed, sensitivity was considerably improved and at the same time the tests became more practical, with fewer incubation and washing steps (Halonen et al., 1985; Walls et al., 1986; Hierholzer et al., 1990; Scalia et al., 1995). The format of the TR-FIA is

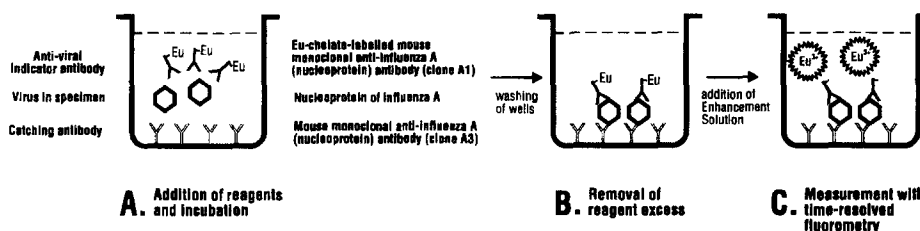


Fig. 1. Principle of the monoclonal one-step time-resolved fluoroimmunoassay for the detection of influenza A virus.

shown in Fig. 1. Treated specimens are added simultaneously with the label led (Eu-chelate or biotin) detector monoclonal antibody to the wells of polystyrene strips previously coated with the optimal concentration of the capture monoclonal antibody. After incubation for 1 h at 37°C, the wells are washed and the enhancement solution (TR-FIA) or peroxidase labelled avidin (biotin-EIA) is added. In this test format, the antigen in the specimen and the detector antibody react quickly and efficiently in liquid-phase, and later the formed antigen-antibody complex binds to the capture antibody on solid phase. The format can be used only when immunoreagents are prepared from monoclonal antibodies. The sensitivity is higher than in conventional two-step assays where the antigen is first bound to catching antibody on solid phase during the first incubation, and, after washing off the excess antigen and other nonbound material, the detector antibody is added and wells are incubated again. Sensitivities up to 0.1 ng/ml of antigen can be obtained with all monoclonal one-incubation TR-FIAs (Hierholzer et al., 1990; Arstila and Halonen, 1988).

Monoclonal TR-FIAs have been used at the Department of Virology, University of Turku in daily diagnosis for influenza A and B, parainfluenza 1, 2, and 3, RSV, and adenoviruses (group-reacting hexon test) since 1987 and 2000–4000 nasopharyngeal aspirates have been tested during each epidemiological year. During these 8 years, more than 20 000 specimens, including thousands of positives, have been tested. The tests have been done primarily for rapid diagnosis of hospitalized children with acute respiratory infection, but the results have also been used in many clinical and epidemiological studies.

In addition to the seven respiratory viruses indicated above, monoclonal TR-FIAs have been developed for parainfluenza 4 and mumpsvirus (Hierholzer et al., 1993a), the enteroviruses responsible for acute hemorrhagic conjunctivitis (Hierholzer et al., 1990), the human respiratory coronaviruses (Hierholzer et al., 1994a), and for the A- and B-grouping of RSV strains (Hierholzer et al., 1994b; Waris, 1991).

Recently, EIA kits which allow the detection of RSV or influenza virus type A in clinical specimens within approximately 20 min have become commercially available (Dominguez et al., 1993; Krilov et al., 1994; Ryan-Poirier et al., 1992). These tests are sufficiently sensitive and can be performed at bed-side or in a physician's office. Weak positive results, however, should be confirmed by a standard method.

6. PCR followed by liquid-phase hybridization in detection of respiratory virus DNA/RNA in clinical specimens

Viral diagnosis based on the detection of viral nucleic acid in clinical specimens is a new and challenging possibility which is being used for the detection of respiratory viruses. However, the technology may not yet have reached the practicality and reliability required. First, a detection system for adenoviruses was developed (Hierholzer et al., 1993c) and later expanded our experience to enteroviruses and rhinoviruses (Halonen et al., 1995). Enteroviruses and particularly rhinoviruses are interesting groups in that no reliable antigen tests and no group-reacting tests are avail-

able for diagnosis. The flexibility of PCR and hybridization make it possible to design tests for a wide range of specificities. The reagents can be selected for group, subgroup, type or even strain specificities.

In developing our new PCR formats, we wanted to have group-specific tests for all rhinoviruses and another test for all enteroviruses. In addition, we preferred to adapt technology already in use in diagnostic laboratories as much as possible. We used 12-well microtiter strips for the detection of PCR products by liquid-phase hybridization, which resulted in test formats nearly identical to those used in one-step antigen assays.

Special attention was paid to removing nonspecific inhibitors of PCR from clinical specimens, because negative results in PCR may just indicate that these inhibitors were not correctly removed. Often, PCR is so sensitive that only a limited number of viral genomes are required for a positive result. But the sensitivity may decrease several logs or the test be negative, even in the presence of large amounts of virus, if these inhibitors are not completely removed.

6.1. *Extraction and purification of sample RNA*

We used a commercial reagent, Ultraspec (Cinna-Biotecx Laboratories Inc., Houston, Texas) for the extraction of RNA in clinical specimens. It contains guanidinium isothiocyanate which denatures all proteins including enzymes in specimens; no proteinase K is required, which may be another source of PCR inhibitors. RNA was precipitated from the extract with cold isopropanol. If the precipitate is purified by washing with 70% cold ethanol, it should be repeated at least three times. It may be important that the final precipitate is not dried but dissolved in distilled water at 56° for 15 min, otherwise, it may be difficult to dissolve the precipitated RNA. We purified the isopropanol precipitate by filtration and washing steps using Millipore Ultrafree-MC 10000NMWL filter units with polysulfone type PTGC membranes. These are highly protein retentive and porous for small molecular size material, but nucleic acids can be resuspended from the top of the filter.

6.2. *Construction of primers and probes*

Our experience with liquid-phase hybridization indicates that the primers should be constructed to produce PCR products which are less than 200 bp long (longer may form loops which prevent hybridization with probes). Optimal length may be between 100 and 150 bp. The probes should be selected so they do not overlap with primer regions or with each other and they should be 18–25 mers.

6.3. *Coating of microtitration strips*

We used microtitration strips coated with streptavidin and then with biotinylated probes in our liquid-phase hybridization for the assay of PCR products. However, if streptavidin is coated directly on polystyrene, it is in only partially active form. We coated the strips first with biotinylated bovine albumin followed by streptavidin (Dahlen et al., 1991) and then biotinylated probes. These triple-coated strips can be stored refrigerated for months.

6.4. *Hybridization assay*

The format of the hybridization assay is shown in Fig. 2. Single-stranded PCR products (boiled and cooled or exonuclease-treated) are added together with a probe labelled with Eu-chelate to the biotinylated probe-coated wells and then incubated for 1 h at ambient temperature. After washing steps, the enhancement solution is added and the fluorescence measured with a fluorometer.

The sensitivity of the enterovirus PCR/hybridization was approximately 1 molecule of purified coxsackie A9 RNA; compared with infectivity using two echovirus strains, it was about the same or higher (Halonen et al., 1995). More rhinovirus-positive specimens were found by PCR/hybridization than by culture, which is similar to the finding reported by other groups. However, one culture-positive specimen was PCR negative. In enterovirus PCR, all culture positive respiratory specimens were positive and all culture-negative specimens were also negative by PCR. In adenovirus PCR, slightly more respiratory speci-

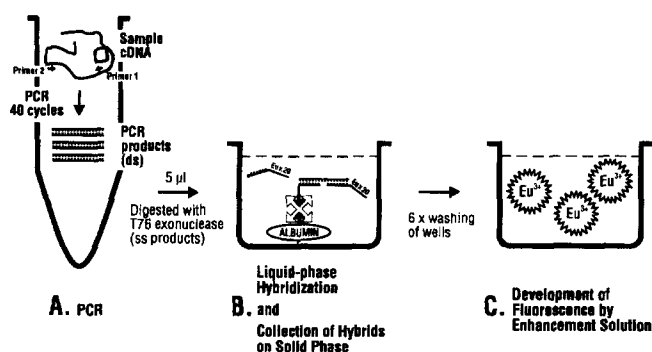


Fig. 2. Principle of liquid-phase hybridization by time-resolved fluorometry in detection of PCR products. Eu-labelled probe ($\text{Eu} \times 20$); biotinylated probe (\downarrow); Streptavidin (4-point arrows); biotinylated albumin (ALBUMIN).

mens were positive with PCR than with culture. A few culture positive but PCR negative specimens may indicate that removal of PCR inhibitors from the clinical specimens was incomplete.

7. Serological diagnosis

EIA IgG antibody assay in paired serum specimens is the most efficient serodiagnostic method for respiratory viruses and is vastly superior to the complement fixation test, whereas IgM assays have been less sensitive in our comparisons (Koskinen et al., 1987; Vuorinen and Meurman, 1989; Nohynek et al., 1991). The IgG antibody avidity test can be used for distinguishing primary infections from reinfections, at least with RSV (Meurman et al., 1992). IgG antibody assays often complement rapid viral diagnostic methods, because acute phase specimens for virus isolation, antigen detection or PCR are not always available. In addition, subclinical infections can be diagnosed only by serology; they remain useful for the evaluation of new diagnostic methods as well. Recombinant antigens may prove valuable in the future for respiratory viruses (Harmon et al., 1989).

8. Conclusion

In the fully equipped diagnostic laboratory,

virus culture and/or rapid culture assays should be the front line diagnostic methods for acute respiratory infections. Simultaneously an antigen detection should be carried out whenever rapid diagnosis is required. For many viruses, results from rapid culture assays are available as quickly as the results from antigen detection assays or PCR. If the number of respiratory specimens sent to the laboratory is large, cultures may be practical only for selected specimens. If the laboratory has no cell cultures available, the results of highly sensitive antigen assays are almost identical with cultures (Waris et al., 1990). Why then standard isolation or rapid culture assays? The advantage is that the cultured virus is available for further analysis if required, e.g. for drug-susceptibility testing or for sequence analysis. Although the total diagnostic positivity-rate is almost identical with both methods, there may be specimens which are positive with only one of these.

Another question is, which method is used for the antigen detection. As discussed earlier, it depends on laboratory, its location, number of specimens tested, previous experience of the laboratory, and expertise available. Also, the availability of well-characterized immunoreagents is important. Again, the overall results with IFA using monoclonal antibodies as immunoreagents and an experienced reader with a modern UV

microscope are almost identical with highly sensitive solid-phase immunoassays.

The future role of PCR depends on new developments in this promising technology. We need a more efficient and simple method for the extraction and purification of DNA and RNA, a simple test for specific identification of PCR products and preferably a more practical DNA amplification technique, such as isothermal amplification (Walker et al., 1992). Before a new extraction and purification method is developed, we need internal controls for checking the removal of inhibitors from each individual specimen. Otherwise, negative results may not be correct. We also need improved methods to control for cross-contaminations. This requires, in addition, that we minimize the possibilities of contaminating specimens at the time of specimen collection and handling, i.e. before they even reach the laboratory. Such contamination problems are difficult to avoid, if the assay sensitivity is at the level of one DNA or RNA molecule. Some of these problems might be overcome by lowering the sensitivity of PCR. It is likely that a less sensitive PCR system still would pick up most, if not all, isolation-positive specimens. The most natural targets for PCR diagnosis of respiratory infections are those with difficulties in culture or identification, such as coronaviruses. Broad tests such as group reacting rhinovirus and enterovirus tests are also examples of future respiratory virus PCRs.

Economic aspects of respiratory virus diagnosis have to be kept in mind. One of the ultimate goals of the laboratory diagnosis in uncomplicated upper respiratory infections is to reduce the number of unnecessary prescriptions for antibiotics. That means, the laboratory diagnosis of such an infection should not be more expensive than a course of antibiotic treatment. To date, unfortunately, few diagnostic techniques meet this criteria, but relatively sensitive methods such as latex agglutination tests and self-contained EIA kits are becoming available and may, in the future, offer a more wide-spread, cost-effective use of virus diagnostic services in hospitals and physician's offices.

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