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Simultaneous Rapid Culture for Four Respiratory Viruses in the Same Cell Monolayer Using a Differential Multicolored Fluorescent Confirmatory Stain

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A simultaneous rapid culture for influenza virus types A and B, parainfluenza virus, and respiratory syncytial virus was developed in a 96-well plate format with a culture-confirmatory stain using multiple fluorescent tags. Performance characteristics were comparable to those of standard and/or single rapid-culture methods as shown by parallel testing of 590 fresh clinical specimens and retrospective testing of 190 previously positive frozen specimens. The quadruple culture required less specimen volume than separate cultures, was significantly quicker than standard tube culture, was less labor intensive than separate cultures, and was less expensive than the other methods.

Acute respiratory disease occurs annually during the winter months in temperate climates. Influenza virus (types A and B), parainfluenza virus (types 1 to 3), and respiratory syncytial virus (RSV) are the most common viral pathogens encountered during these seasonal outbreaks. Disease syndromes are not always distinct, but treatment protocols are virus specific; therefore, differentiation among the etiologic agents is of paramount importance.

Culture is often used for identification of the etiologic agent. A single cell type can be used to isolate all four viruses, which makes culture a cost-effective diagnostic method for virus differentiation. Conventional virus culture, however, can require up to 2 weeks for a final result; this is too slow to be useful in patient treatment and management. Many laboratories currently employ rapid-culture methods, with sensitivities approaching or even exceeding those of conventional culture for isolation of respiratory viruses (1, 7). Results are usually available within 48 h, but separate tests must be run for each suspected virus. This makes rapid culture less practical and cost-effective when multiple etiologic agents are suspected.

Simultaneous rapid culture for adenovirus, cytomegalovirus, and herpes simplex virus in the same shell vial with a multicolored differential fluorescent confirmatory stain has been reported (2). We used the same stain (three fluorochromes) to differentiate respiratory viruses isolated in a 96-well plate format that was previously described for influenza virus culture (1). We expanded the culture-confirmatory stain by adding a fourth fluorescent marker and used it to detect and differentiate among four viruses.

We evaluated the performance of the quadruple rapid respiratory culture by using 590 fresh specimens and two sets of frozen specimens: one set of 72 specimens previously culture positive for any of the four respiratory viruses and a second set of 118 specimens previously positive for RSV by direct immunofluorescence (DIF). We also compared the performance of four different cell types in the quadruple rapid system.

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MATERIALS AND METHODS

Specimens. Fresh respiratory specimens (nasopharyngeal swabs and washes, nasal turbinate swabs, throat swabs and washings, sputum, and bronchial washings and lavages) were submitted to our laboratory from January through May of 1995. Swabs were collected and transported in viral transport medium (various formulations). Washes were collected and transported in saline. Specimens were refrigerated and transported to our laboratory within 24 h and processed within 12 h of receipt. The first set of 72 frozen specimens consisted of parainfluenza virus-positive specimens (submitted in 1993 and 1994) and RSV- or influenza virus-positive specimens (submitted in November and December of 1994). Frozen specimens were randomized throughout the study with the fresh specimens. Quadruple rapid culture readers were unaware of other test results and which specimens were fresh or previously frozen. The second set of 118 frozen specimens was positive for RSV during the 1994 to 1995 season by DIF and was not initially cultured. These specimens were maintained frozen at −70°C and were cultured retrospectively in parallel by using the quadruple method (these specimens were stained for RSV only) and a reference method (7). The two culture sets were both read by two different individuals. Inoculation volumes were kept consistent among the quadruple and reference culture methods (0.1 ml per tube, shell vial, or well).

Reference methods. The reference methods used for the four viruses are listed in Table 1. The rapid culture for influenza virus types A and B was performed in 96-well plates as described previously (1). Specimens with discrepant results were cultured by conventional tube culture with hemadsorption (3). Specimens which were negative for RSV by DIF or inadequate (containing fewer than 20 ciliated respiratory cells) were cultured (6). Parainfluenza virus cultures were performed with primary rhesus monkey cells with periodic hemadsorption challenges and a blind stain at the end of 2 weeks of incubation with a parainfluenza virus group-specific monoclonal antibody (3).

Plate seeding. Sterile 96-well plates (Laboratory Disposable Products, Inc.) were seeded with four different cell types in sets of four adjacent wells. Each set contained one well each of RMK cells (ViroMed Laboratories, Inc.) at a concentration of 1.6×10^4 to 2.4×10^4 per well, MDCK cells (ATCC CCL34) at a concentration of 2.0×10^4 to 6.0×10^4 per well, A549 cells (ATCC CCL185) at a concentration of 2.0×10^3 to 4.0×10^3 per well, and LLC-MK_2 cells (ATCC CCL7) at a concentration of 1.2×10^4 to 1.6×10^4 per well. RMK cells were subcultured from flasks of primary cells, MDCK cells were used at passages 58 to 75, A549 cells were used at passages 87 to 100, and LLC-MK_2 cells were used at passages 294 to 304. Wells between sets were left empty to minimize the risk of cross contamination.

Quadruple culture. Specimens were processed, and plates were inoculated, incubated for 40 h to 4 days, and fixed as previously described for influenza virus culture (1). Positive controls (a mixture of influenza virus types A and B, parainfluenza virus type 3, and RSV, each at a dilution to infect 10 to 50% of the cell monolayer at 2 days of incubation) and a negative control (cell maintenance medium) were included in each run. The positive control mixture was inoculated into a single set of wells, and the negative control was inoculated into a second set. After fixation, plates were washed once with phosphate-buffered saline (PBS; pH 7.6 ± 0.1). Cell monolayers were then stained directly in the plates by a four-step indirect immunofluorescence-alkaline phosphatase stain using pooled reagents. Influenza virus types A and B, were differentiated by using monoclonal antibodies of different isotypes (8). Parainfluenza virus and RSV were identified by using polyclonal antibodies. Antibodies and fluorescent conjugates specific for

TABLE 1. Quadruple culture: antibodies, fluorophores, and reference methods

Test condition	Variant used to detect:			
	Influenza virus type A	Influenza virus type B	RSV	Parainfluenza virus
Primary antibody	Mouse monoclonal IgG2a	Mouse monoclonal IgG1	Rabbit polyclonal	Biotinylated goat polyclonal
Secondary antibody	Goat anti-mouse IgG2a	Goat anti-mouse IgG1	Donkey anti-rabbit	Streptavidin
Fluorescent tag	FITC	Alkaline phosphatase-ELF[a] stain	Cy3[b]	AMCA[c]
Filter set	FITC	UV	Dual FITC-rhodamine	UV
Excitation wavelength(s) (nm)	450–490	330–390	475–495, 540 (spike)	330–390
Barrier wavelength (nm)	520	420	515–530, 580–630	420
Fluorescence	Apple green	Yellow-green	Orange[d]	Blue
Counterstain	Red	Pink	Red	Pink
Reference method	Rapid-tube culture	Rapid-tube culture	DIF-rapid culture	Tube culture

[a] ELF, enzyme-labeled fluorescence.
[b] Cy3, indocarbocyanine.
[c] AMCA, aminomethylcoumarine acetate.
[d] Gold when viewed with the FITC filter set.

each virus are outlined in Table 1. Reagent 1 was a cocktail of biotinylated goat anti-parainfluenza virus types 1, 2, and 3 antibodies (ViroStat, Portland, Maine) and rabbit anti-RSV antibodies (Dako Corporation, Carpinteria, Calif.) prepared in PBS with 1% bovine serum albumin and 0.1% Tween 20. After incubation for 30 min in a 37°C incubator with reagent 1, cell monolayers were washed twice with PBS and reagent 2 was applied. Reagent 2 was a cocktail of influenza virus type A immunoglobulin G2a (IgG2a) and influenza virus type B IgG1 mouse monoclonal antibodies (Sera-Lab, Sussex, England) and indocarbocyanide-conjugated donkey anti-rabbit polyclonal antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) prepared in the same way as reagent 1. After incubation with reagent 2 for 45 min in a 37°C incubator, monolayers were washed twice with PBS and reagent 3 was applied. Reagent 3 was a cocktail of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates, Inc., Birmingham, Ala.), alkaline phosphatase-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates), and aminomethylcoumarin acetate-conjugated streptavidin (Jackson Immunoresearch Laboratories) prepared in PBS with 1% bovine serum albumin, 0.1% Tween 20, and 0.006% Evans blue. After incubation with reagent 3 for 45 min in a 37°C incubator, plates were washed twice with PBS and reagent 4 was applied. Reagent 4 was a 1:20 dilution of enzyme-labeled fluorescence substrate (4, 5) with additives 1 and 2 prepared in enzyme-labeled fluorescence developing buffer (E-6602; Molecular Probes, Inc., Eugene, Oreg.) and mixed with an equal volume of 3 mg of levamisole per ml in PBS. Plates were incubated at room temperature (20 to 30°C) for 10 min and washed once with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 6.0)–10 mM EDTA and once with PBS. Glycergel (Dako Corporation), a buffered glycerol mounting medium which is liquid at 50°C and semisolid at room temperature, was then added (excess mounting medium was aspirated). Optimal dilutions of primary antibodies and conjugates were determined by checkerboard titration. Each of the antibodies and conjugates was tested for cross reactivity with the other target viruses and with an array of other viruses (herpes simplex virus types 1 and 2, adenovirus, cytomegalovirus, measles virus, mumps virus, and rubella virus). After staining, plates were inverted and read with three different filter combinations in a Zeiss Axioscope fluorescence microscope equipped with a clear plastic plate holder. A monolayer was scored positive if it contained one or more cells demonstrating typical fluorescence with the appropriate filters. Negative cells stained red or pink because of the Evans blue counterstain (Table 1).

RESULTS

In total, 780 specimens were tested by quadruple rapid respiratory culture: 590 fresh specimens and 190 frozen, previously positive specimens. Fresh specimens were tested simultaneously by quadruple culture and the reference methods. Of the 590 fresh specimens tested, 107 (18.1%) were positive for one or more respiratory viruses: 42 for influenza virus type A, 31 for RSV, 8 for parainfluenza virus, 25 for influenza virus type B, and 1 for both RSV and parainfluenza virus (Table 2). The sensitivity and specificity of the rapid quadruple culture were 100 and 99.1% for influenza virus type A, 56.7 and 99.6% for RSV, 100 and 100% for parainfluenza virus, and 91.3 and 99.6% for influenza virus type B, respectively. One specimen which was negative for influenza virus type B by rapid culture and positive by quadruple culture was shown to be a true positive when cultured in tubes. Performance characteristics were subsequently adjusted to 91.7% sensitivity and 99.8% specificity for influenza virus type B (Table 2).

From the first set of 72 frozen specimens randomized throughout the fresh specimens, rapid quadruple culture recovered 95.8% of the influenza virus type A-positive specimens, 21.4% of the RSV-positive specimens, 83.3% of the parainfluenza virus-positive specimens, and 100% of the influenza virus type B-positive specimens (Table 1). These specimens were not retested by the reference methods. Because no negative specimens were tested, quadruple method specificity values were not calculated for frozen specimens. The overall sensitivities and specificities for fresh and frozen (set one) specimens combined were 98.4 and 99.2% for influenza virus type A, 92.3 and 99.8% for influenza virus type B, 39.7 and 99.7% for RSV, and 88.9 and 100% for parainfluenza virus, respectively.

Because the DIF-culture combination used as the RSV reference test is more sensitive for diagnosis of RSV than culture alone (6), an additional set of frozen specimens RSV positive by DIF was cultured in parallel by quadruple culture and a reference culture method (7) to better evaluate the performance of the quadruple culture method with RSV (these specimens were stained for RSV only). Sixty-six of the 118 specimens (55.9%) were positive in culture. Quadruple culture recovered 61 (92.4%) of the 66, and the reference culture recovered 54 (81.8%).

Recovery rates of the four cell lines for fresh and frozen specimens are listed in Table 3. (The 118 specimens RSV positive by DIF were cultured in RMK cells only in the quadruple system.)

DISCUSSION

The performance of the quadruple rapid respiratory culture was comparable to that of the reference cultures for all four viruses. For influenza virus type A, quadruple culture recovered all of the isolates from fresh specimens recovered by the reference method plus an additional five that were not isolated in the single rapid culture. These five specimens were negative when subsequently cultured in tubes; therefore, they were scored as false positives. Since we have found rapid culture to be more sensitive than standard culture for isolation of influenza virus type A in our laboratory (1), these could be true positives which were missed by the reference culture. All five specimens contained low viable virus numbers, each showing

TABLE 2. Performance of rapid quadruple culture

Specimens	Result of:		No. of specimens				
	Quadruple culture	Reference method(s)	Influenza virus type A	Influenza virus type B	Influenza virus type B, resolved	RSV	Parainfluenza virus
Fresh	+	+	37	21	22	17	9
	+	−	5	2	1	2	0
	−	+	0	2	2	13	0
	−	−	548	565	565	558	581
Frozen	+	+	23	2		6	15
	−	+	1	0		22	3
	−	−	48	70		44	54
Combined	+	+	60	24		23	24
	+	−	5	1		2	0
	−	+	1	2		35	3
	−	−	596	635		602	635

only one to three infected cells in one or two of the four inoculated wells. Quadruple culture failed to recover one influenza virus type A from a frozen specimen. This specimen was not recultured by the reference method upon thawing. Thus, it is uncertain whether viable virus remained.

Quadruple culture recovered all of the parainfluenza virus positives from fresh specimens. Parainfluenza virus is less frequently isolated in our laboratory than the other three viruses (it was isolated from only nine fresh specimens during the study period). For this reason, we included 18 frozen specimens previously positive for parainfluenza virus and scattered them throughout the fresh specimens. Quadruple culture was positive for 15 of the 18 specimens. There was insufficient specimen volume to repeat the standard cultures upon thawing to ascertain virus viability after prolonged storage. It is therefore unknown if the three previous positives not recovered were actually still positive when thawed. The quadruple culture was designed to recognize all three types of parainfluenza virus but not to differentiate among them. Parainfluenza virus isolates recovered by quadruple culture were retrospectively typed; 4 of the 24 recovered were type 1, and the remaining 20 were type 3.

Recovery of influenza virus type B was comparable in the quadruple and reference cultures. One of the two specimens which were positive in the quadruple culture and negative in the reference cultures was positive by standard tube culture. This specimen was subsequently determined to be a true positive. The other positive was not confirmed by standard culture and was therefore counted as a false positive. That sample did, however, contain a low viable virus number (showing only a few infected cells in one of the four inoculated wells) and may have actually produced a reference culture false-negative result.

In our study, culture used alone was less than optimal for recovery of RSV (6). Our standard testing protocol for RSV diagnosis is DIF screening of all appropriate specimens (nasopharyngeal or nasal) with culture follow-up of negative and inadequate specimens (fewer than 20 ciliated respiratory cells). Specimens which do not contain the appropriate number of the correct cells and those which are negative by DIF are then cultured. Since DIF with culture detects more positives than DIF used alone, we felt it important to include RSV in the quadruple culture format. Although the possibility of false-positive DIF results does exist (positive results were not confirmed by a second method), RSV recovery by quadruple culture compared with the combined DIF-culture method was poor (56.7% recovery of positives from fresh specimens and 21.4% recovery of positives from frozen specimens). Its performance compared to that of the reference culture alone, however, was excellent (92.4 and 81.8% recovery for quadruple and reference cultures, respectively). We conclude that quadruple culture can be used to replace other culture methods, as long as specimens continue to be screened for RSV by DIF.

Differentiation among the four viruses in the quadruple culture was straightforward. Both influenza virus type A and RSV could be seen by using an FITC filter combination. The green (influenza virus type A) and gold (RSV) colors were easily differentiated. Both could also be observed with a dual FITC-rhodamine filter set; however, the green fluorescence was dull. The indocarbocyanide-labeled RSV cells were a brilliant orange against the red background. We chose to read FITC by using the single-filter set and to read indocarbocyanide with the dual filters; however, the single FITC filter set could be used to read both simultaneously. The aminomethylcoumarin acetate and enzyme-labeled fluorescence labels could not be seen with either of these filter sets, but both could be viewed

TABLE 3. Performance of four cell types for quadruple rapid respiratory virus culture

Organism	No. (%) positive with:				Total no. positive
	A549 cells	LLC-MK_2 cells	MDCK cells	RMK cells	
Influenza virus type A	46 (77)	44 (73)	60 (100)	51 (85)	60[a]
Influenza virus type B	7 (29)	6 (25)	19 (79)	18 (75)	24[a]
Parainfluenza virus	18 (90)[b]	19 (95)[b]	19 (79)	24 (100)	24
RSV	18 (78)	18 (78)	7 (30)	20 (87)	23[a]

[a] Excludes data from positive specimens which were not confirmed by at least one reference method.
[b] A549 and LLC-MK_2 cells were not tested for four specimens.

with a UV filter set. The blue (parainfluenza virus) and green (influenza virus type B) colors were distinct. Influenza virus type A- and RSV-positive cells could not be seen with this filter set. Resolution through the bottom of the 96-well plate was sharp, and even single infected cells were conspicuous.

We found that the use of all four cell lines was unnecessary; however, no single cell line was the most sensitive for all four viruses (Table 3). RMK cells performed the best for recovery of RSV and parainfluenza virus. MDCK cells were the most sensitive cell line for detection of influenza virus type A, and the MDCK-RMK cell combination was the most sensitive for influenza virus type B. (The influenza virus type B recovery rate for the combination was 96%.) We did not find the addition of A549 or LLC-MK_2 advantageous and subsequently removed these lines from the quadruple culture protocol.

Quadruple rapid culture has three advantages over the standard tube or single rapid culture method. First, it requires significantly less specimen volume (0.1 ml of specimen per well) for recovery of all four agents. Second, since all four agents are recovered simultaneously, labor is markedly reduced. Substitution of the quadruple rapid method for standard tube culture eliminates repeated culture readings, cumbersome hemadsorption challenges, culture medium changes, blind passages, blind stains, and confirmation testing of isolates recovered by cytopathic effect and/or hemadsorption. Substitution of a quadruple system for individual shell vial cultures eliminates three-fourths of the culture manipulations and half of the monolayer readings. The 96-well plate format is also much less labor intensive than shell vials, since there are no vials to cap and uncap and there is no coverslip manipulation. The third advantage is a reduction in reagent costs resulting from elimination of the extra tubes and/or vials needed for individual cultures. Although the quadruple culture uses a number of different antibodies and conjugates, the total cost of all of the staining reagents was $0.84 per well since the reagents are supplied in concentrated form. This is less than the cost of a single tube or shell vial. The only disadvantage of the combined culture system is the requirement for an additional filter set for the fluorescence microscope. This initial investment, however, is quickly offset by enhanced specimen utilization, less expensive reagents, reduced labor, and improved turnaround time for respiratory virus culture.

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REFERENCES

1. **Brumback, B. G., D. M. Cunningham, M. V. Morris, and J. L. Villavicencio.** 1995. Rapid culture for influenza types A and B in 96-well plates. Clin. Diagn. Virol. **4:**251–256.
2. **Brumback, B. G., and C. D. Wade.** 1994. Simultaneous culture for adenovirus, cytomegalovirus, and herpes simplex virus in same shell vial by using three-color fluorescence. J. Clin. Microbiol. **32:**2289–2290.
3. **Hsiung, G. D.** 1994. Orthomyxoviridae and paramyxoviridae, p. 141–157. *In* G. D. Hsiung, C. K. Y. Fong, and M. L. Landry (ed.), Hsiung's diagnostic virology, 4th ed. Yale University Press, New Haven, Conn.
4. **Larison, K. D., R. BreMiller, K. S. Wells, I. Clements, and R. P. Haugland.** 1995. Use of a new fluorogenic phosphatase substrate in immunohistochemical applications. J. Histochem. Cytochem. **43:**77–83.
5. **Molecular Probes, Inc.** 1994. ELF™ cytological labelling kits, p. 1–5. Molecular Probes, Inc., Eugene, Oreg.
6. **Morris, M., C. Belaski, B. Brumback, and J. Bailey.** 1991. Comparison of rapid culture and direct immunofluorescence for diagnosis of respiratory syncytial virus, abstr. C-53, p. 351. *In* Abstracts of the 91st General Meeting of the American Society for Microbiology 1991. American Society for Microbiology, Washington, D.C.
7. **Morris, M., B. Brumback, C. Bidwell, and J. Bailey.** 1990. Rapid culture of respiratory syncytial virus in 48-well flat bottom plates, abstr. C-84, p. 358. *In* Abstracts of the 90th Annual Meeting of the American Society for Microbiology 1990. American Society for Microbiology, Washington, D.C.
8. **Murphy, P., L. V. Wass, and J. L. Waner.** 1994. The use of isotype differences between monoclonal antibodies to diagnose influenza A and influenza B by direct immunofluorescence in the same viewing field, abstr. M18, p. 89. *In* Program and abstracts of the Tenth Annual Clinical Virology Symposium and Annual Meeting of the Pan American Group for Rapid Viral Diagnosis. University of South Florida, College of Medicine, Tampa, Fla.