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Sensitivity of Respiratory Virus Culture When Screening with R-Mix Fresh Cells

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Use of R-Mix Fresh Cells has been shown to be a rapid and sensitive method for the detection and identification of respiratory viruses. We prospectively evaluated the impact of incorporation of R-Mix shell vials on the sensitivity and time to detection of seven respiratory viruses recovered in a comprehensive culture during the course of an entire respiratory season in a high-volume clinical laboratory. In this study, R-Mix shell vials were used as part of the culture of 3,803 respiratory specimens. A total of 428 respiratory viruses were recovered. Staining of R-Mix vials after overnight incubation allowed initial detection of 274 of 279 influenza viruses, 33 of 38 parainfluenza viruses, 35 of 51 adenoviruses, and 52 of 60 respiratory syncytial viruses (RSVs). The time to reporting of all positive cultures after in-lab specimen receipt was 2.9 days on average and those initially detected in R-Mix cells were reported in 2.3 days on average. A combination of direct fluorescent-antibody (DFA) staining and virus culture was performed on a subset of 711 respiratory specimens. Of 152 viruses identified, 57 were observed only with DFA testing (55 RSV and 2 influenza A viruses) and 31 were recovered only in cell culture. After overnight incubation, R-Mix cells detected 87.1% of respiratory viruses not observed by DFA testing and 96.9% of viruses positive by both methods. The sensitivities of DFA testing and R-Mix cells for identification of influenza viruses were 70.5% and 96.7%, respectively. The R-Mix method detected influenza virus in 18 samples that were negative by DFA testing.

The availability of an expanded selection of antiviral agents makes rapid detection and identification of respiratory viruses an important tool for patient management. Rapid diagnosis of respiratory virus infections has also been shown to reduce antibiotic usage, duration of hospitalization, and unnecessary laboratory testing (1, 3, 13). Clinical laboratories now have a choice among many commercially available products that identify respiratory viruses (e.g., influenza virus and respiratory syncytial virus [RSV]) with antigen detection-based assays that require only 15 min to a few hours to complete (8, 10, 12). Unfortunately, many of these rapid methods are typically insensitive compared to conventional cell culture techniques (4, 6, 9). The major drawback for cell culture, however, is the length of time necessary to identify viruses in culture.

Several studies have demonstrated the efficacy of R-Mix cells for the detection of several respiratory viruses from clinical specimens (2, 5, 7, 11). This mixed cell line combines the greater sensitivity afforded by culture with a relatively rapid turnaround time for reporting of results compared to traditional tube or shell vial culture. Optimally, the combination of A549 and Mv1Lu cells in a single monolayer will also allow the recovery of a broader spectrum of respiratory viruses at one time.

The analytical and clinical sensitivity of R-Mix cells for the detection of influenza viruses is well documented. Screening of R-Mix cells after 24 h detected 100-fold fewer influenza A virions than cell culture examined for up to 14 days with multiple hemadsorptions (11). With serial dilutions of influenza A virus, R-Mix cells detected 4 to 5 log-fold fewer infectious particles than two commercially available immunoassays (11). In a prospective evaluation of culture for 396 respiratory specimens, 15 and 13 (both of 19) influenza A viruses were identified in R-Mix vials and cell culture, respectively (2). Other clinical studies have shown sensitivities (R-Mix versus cell culture) of 100% versus 67% and 100% versus 90% for detection of influenza viruses (5, 11). Direct fluorescent-antibody (DFA) testing for influenza viruses identified 67 to 80% of positive specimens compared to the 95 to 97% seen with R-Mix cells (5, 11).

While the data for use of the R-Mix method in detecting influenza viruses is notable, little is known about its routine clinical utility for detecting significant numbers of other respiratory viruses. In a retrospective study with fluorescent-antibody-positive specimens, R-Mix cells were of comparable sensitivity to individual cell lines for the detection of parainfluenza viruses, adenovirus, and RSV (7). We prospectively evaluated the sensitivity of screening R-Mix cells after overnight incubation in the course of an entire respiratory season that included the culture of 3,803 clinical specimens. In addition, we compared the utility of the R-Mix method to direct antigen detection on a subset of 711 clinical samples evaluated by DFA, R-Mix shell vials, and supplemental cell lines. In contrast to previous studies, we attempted to detect all influenza virus, parainfluenza virus, adenovirus, and RSV pathogens.

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MATERIALS AND METHODS

Clinical specimens. Specimens submitted for respiratory virus DFA and/or culture between 1 November 2001 and 30 April 2002 were included in the data analysis. Physicians had several individual test options to identify respiratory viruses. These included DFA testing for all seven common respiratory viruses

(influenza A, influenza B, parainfluenza viruses types 1, 2, and 3, RSV, and adenovirus), only influenza A and B viruses, or only RSV. Cell culture could be ordered with or without DFA testing. Viral cultures were performed for 3,803 respiratory specimens. A total of 711 samples were tested by both DFA and cell culture.

Cell lines. R-Mix shell vials (containing both human lung carcinoma [A549] and mink lung [Mv1Lu] cells) were purchased from Diagnostic Hybrids, Inc. (Athens, Ohio), and PRMK cells were purchased from ViroMed Laboratories (Minneapolis, Minn.). Buffalo green monkey kidney (BGM), A549, and MRC-5 shell vials were obtained from the ARUP Cell Culture Laboratory (Salt Lake City, Utah). R-Mix shell vials were maintained according to the manufacturer's instructions, and the other cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum at 37°C in a CO_2 incubator.

Viral culture. Approximately 0.2 ml of prepared clinical samples was inoculated into each of two R-Mix, one BGM, one A549, one MRC-5, and one PRMK shell vial according to standard laboratory protocol (4). R-Mix shell vials were readied according to the manufacturer's instructions. Vials were centrifuged at $3,000 \times g$ for 15 min at 20°C and incubated at 35°C in 5% CO_2. One R-Mix shell vial was stained at 18 to 24 h postinoculation (see below), and the remaining vials were observed for cytopathic effect up to 10 days, at which time hemadsorption was performed on the PRMK shell vial.

Immunofluorescent stains. Samples for DFA testing were brought to a total volume of 4 to 5 ml with phosphate-buffered saline, centrifuged, and the pellets resuspended in the residual fluid. Following a second wash with phosphate-buffered saline, pellets were spotted to eight-well slides. Dried and fixed slides were then stained according to the manufacturer's instructions (Bartels, Inc., Division of Trinity Biotech, Wicklow, Ireland). A minimum of 20 respiratory epithelial cells per spot was considered an adequate sample, and the presence of one or more characteristically staining respiratory tract cells was considered a positive result.

Screening of the R-Mix shell vial at 18 to 24 h postinoculation was accomplished with the first R-Mix shell vial. Coverslips were fixed with acetone and stained in the vial with a respiratory virus fluorescent antibody pool (Bartels, Inc.) according to the manufacturer's instructions. Positive screens were further identified with the second R-Mix shell vial. Coverslips were scraped and spotted to eight-well slides. Dried and fixed slides were then stained with virus-specific monoclonal antibodies (Bartels, Inc.). If the initial R-Mix screen was negative, the second vial was discarded. Viruses recovered in cell lines other than R-Mix were identified by scraping cells, spotting them to eight-well slides, and staining with virus-specific monoclonal antibodies (Bartels, Inc.).

Data analysis. Turnaround times were calculated based on when specimens were received and entered into the laboratory information system and the time of result entry for the specified virus. All positive culture results were manually verified to determine the initial R-Mix shell vial status.

RESULTS

Influenza A virus was the predominant virus identified in cell culture, accounting for over half of all positive cultures. After 18 to 24 h of incubation, nearly all influenza A (238 of 241) and influenza B (36 of 38) viruses were identified in R-Mix vials (Table 1). Parainfluenza viruses represented a smaller proportion of positive cultures but were identified after overnight incubation in R-Mix vials approximately 87% of the time. Adenoviruses were observed in 35 of 51 cultures upon initial screening of the R-Mix shell vial. Use of R-Mix cells permitted identification of 52 of 60 (86.7%) RSV isolates after overnight incubation (Table 1).

All positive viral culture results were reported in 2.9 days on average for the seven respiratory viruses (Table 1). For those cultures positive by the R-Mix method, the average turnaround time from in-lab receipt was 2.3 days. Turnaround times of ≤2 days for all positive culture reports were observed for influenza A and parainfluenza type 2 viruses, the viruses most readily identified with the R-Mix vial. All adenoviruses were reported in 5.5 days on average and were the least likely viruses to be identified in R-Mix cells after overnight incubation (≈69% of the time). For the remaining viruses, average reporting times for all positives varied from 2.9 to 5.3 days and did not appear to correlate with the R-Mix positivity rate (Table 1). However, shorter turnaround times were observed for each virus type when R-Mix cells were employed.

TABLE 1. Sensitivity of screening R-Mix cell cultures after overnight incubation compared to supplemental cell culture testing and average turnaround times for respiratory virus reporting (n = 3,803 specimens)

Virus	Total no. of positive cultures	No. (%) positive by R-Mix	Avg time to positivity (days)	
			R-Mix	All cultures
Influenza A	241	238 (98.8)	1.9	2.0
Influenza B	38	36 (94.7)	3.1	3.5
Parainfluenza 1	30	26 (86.7)	3.8	5.3
Parainfluenza 2	2	2 (100)	1.7	1.7
Parainfluenza 3	6	5 (83.3)	3.7	4.8
RSV	60	52 (86.7)	2.1	2.9
Adenovirus	51	35 (68.6)	3.5	5.5
Total	428	394 (92.1)	2.3	2.9

Respiratory viruses were identified in 152 of 711 specimens that were analyzed by both DFA and cell culture (Table 2). RSV accounted for the vast majority of viruses identified only by DFA. Along with two samples containing influenza A virus, specimens positive by DFA alone amounted to 37.5% of all virus-containing samples. Samples in which viruses were identified only by the culture portion of testing accounted for 20.4% of all positives, and the R-Mix method detected 87.1% of these after less than 24 h of incubation. For those samples positive by both DFA and culture methods, 62 of 64 (96.9%) were identified in R-Mix shell vials (Table 2).

DISCUSSION

Routine incorporation and screening of R-Mix cells as part of the viral culture testing performed for 3,803 clinical specimens allowed for prompt detection of a cumulative 92.1% of all respiratory viruses. Influenza viruses were identified 98.2% of the time by the R-Mix method compared to cell culture (Table 1). Compared to DFA, R-Mix cells were approximately 97% sensitive for influenza viruses and identified 18 more virus-containing specimens (DFA sensitivity = 70.5%) (Table 2). Therefore, clinical laboratories performing culture only for

TABLE 2. Sensitivity of screening R-Mix cells compared to DFA and supplemental cell culture testing (n = 711 specimens)

Virus	No. of specimens			Total no. (%) positive by R-Mix
	DFA positive, culture negative	DFA negative, culture positive (by R-Mix)	DFA positive, culture positive (by R-Mix)	
Influenza A	2	14 (14)	34 (34)	48 (96.0)
Influenza B	0	4 (4)	7 (7)	11 (100)
Parainfluenza 1	0	2 (2)	2 (2)	4 (100)
RSV	55	5 (5)	18 (16)	21 (26.9)
Adenovirus	0	6 (2)	3 (3)	5 (55.6)
Total	57	31	64	89 (58.6)

influenza viruses could expect excellent results with just R-Mix cells. Theoretically, a single vial stained after overnight incubation with a dual-fluorescent anti-influenza virus A/B reagent (8) would identify nearly all influenza virus-containing specimens.

Parainfluenza viruses were less likely to be recovered by the R-Mix stain after overnight incubation than influenza viruses. It has been shown that R-Mix cells support the recovery and identification of all three types of parainfluenza viruses with 100% sensitivity in known positive specimens (7). In the course of our prospective evaluation, we found that only about 87% of all parainfluenza viruses were identified by the R-Mix method compared to supplemental cell culture (Table 1). However, with only two parainfluenza type 2 and six parainfluenza type 3 isolates recovered during this particular respiratory season, we cannot draw any significant conclusions about the efficacy of R-Mix cells for these viruses. On the other hand, for culture of parainfluenza type 1 viruses, 26 of 30 were identified in R-Mix cells and, compared to DFA, two additional isolates were recovered in the R-Mix shell vial. However, more work needs to be done to demonstrate the utility of R-Mix cells for these types of viruses compared to DFA and cell culture.

While the traditional cell lines used for recovery of RSV in culture (e.g., HEp-2) were not routinely used in this study, it is interesting that a significant proportion of RSV isolates were identified by the R-Mix method. We suspected that at the time of the first stain of the R-Mix cells, these isolates were probably not actively growing in culture. More likely what was observed was positive staining of patient respiratory tract cells harboring viruses that were centrifuged onto the R-Mix monolayer. Compared to observation of individual cell lines for up to 10 days, the first R-Mix stain identified 52 of 60 RSV isolates (Table 1). Similarly, a previous study found that R-Mix cells stained at 24 and/or 48 h detected five of seven RSV strains compared to cell culture that included HEp-2 cells (2).

In our prospective evaluation of the R-Mix method compared to direct antigen testing for RSV, we found DFA to be much more sensitive overall. Of 78 total specimens positive for RSV, approximately 94% were identified by DFA (Table 2). The R-Mix method detected only 27% of these but also recovered an additional five RSV isolates not observed by DFA. Data from Fong et al. (5) also demonstrated that R-Mix cells were much less sensitive than DFA. In that study, of the seven DFA-positive samples evaluated, R-Mix and cell culture recovered three and one RSV strain, respectively. It is apparent that R-Mix cells will not entirely replace conventional antigen detection techniques for the identification of RSV but rather serve as a supplemental method for specimens deemed negative by rapid methods.

Heretofore, no study has evaluated the prospective utility of the R-Mix method for a significant number of specimens harboring respiratory adenoviruses. These viruses will grow in a variety of commonly used, continuous cell lines such as A549, HeLa, HEp-2, and MRC-5. One would suspect that the incorporation of A549 cells in the mixed cell population of R-Mix monolayers would provide sufficient sensitivity to allow detection of the adenoviruses present in most samples. However, in our protocol, staining of R-Mix vials after overnight incubation yielded only ≈69% of adenoviruses (Table 1). In fact, most of the isolates later identified in cell culture were recovered from A549 shell vials (data not shown).

Since our procedure was to stain after 18 to 24 h of incubation, there may have been insufficient time for detectable concentrations of adenovirus to accumulate in the A549 cells present in R-Mix vials. While the manufacturer recommends screening for respiratory viruses other than influenza virus after 48 to 72 h of incubation, we and others have modified this protocol to limit the number of necessary vials, shorten the time to detection, and maximize the recovery rate for influenza viruses (4, 11). Unfortunately, a comparison to other studies cannot be made because, in those reports, no adenoviruses were identified in clinical specimens where staining at both 20 to 24 h and 40 to 48 h was employed (2, 5). Regardless, we are currently investigating the need for further screening to enhance the rapid recovery and identification of adenoviruses.

The average time to reporting of the 428 positive cultures during this particular respiratory season was 2.9 days (Table 1). This time was shortened to 2.3 days when only the R-Mix positive samples were evaluated. These data reflect the average amount of time from in-lab receipt to final reporting of the specified virus. Other studies have reported that the identification of respiratory viruses (primarily influenza viruses) in R-Mix cells can be accomplished in 1 to 1.4 days (2, 5, 11). Taking into account only the influenza viruses in our study, the average turnaround time was 2.0 days. These differences may be explained by the fact that the two studies reporting a 1-day turnaround time used monoclonal antibodies for influenza A and/or B viruses for the initial R-Mix stain compared to the pooled antibody reagent used in the present study (5, 11).

In several instances, we also found that if the first R-Mix vial was positive with the pooled stain for respiratory viruses, the scraping, spotting, and staining of the second R-Mix vial did not always yield an identifiable virus. It was, therefore, necessary to prolong the duration of the culture (usually 1 to 2 days) until another cell type could be used for identification purposes. This type of occurrence was noted by Barenfanger et al. who documented forty-six samples positive by the initial 24-h R-Mix screen from which a specific virus could not be identified by staining of the cells from the second R-Mix vial (2). They suspected that the virus was in such low titer that it went undetected in the cells from the second vial. Alternatively, it was possible that the screening antisera may have been positive due to cross-reaction with other antigens. However, they were able to identify respiratory viruses in 24 of these occurrences by alternative methods such as conventional cell culture, DFA testing, or immunoassays for influenza A virus and/or RSV. These specimens and the length of time required to perform supplemental tests were not included in their calculation of turnaround time (2). We also confirmed the presence of those respiratory viruses that were detected by the initial R-Mix stain but unidentified in the second vial with the supplemental cell lines incorporated into the protocol. For these occurrences, the additional time required to identify the specified virus was incorporated into the total turnaround time.

In summary, during the course of the respiratory season for this study, screening of R-Mix cells after overnight incubation was found to be a very sensitive method for detection of influenza viruses and was more reliable than direct antigen testing. Parainfluenza viruses and adenoviruses were generally less

likely to be identified with the R-Mix method, and recovery of additional isolates in this mixed cell line may require extended incubation. DFA remained the most sensitive method for detection of RSV, but R-Mix cells were useful for identifying the few viruses not seen on the direct stain. Finally, with R-Mix cells used to screen for these seven respiratory viruses, we found that a high-volume clinical laboratory could realistically expect most confirmed positive reports to be generated within 2 to 3 days from in-lab receipt of specimens.

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