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Comparative Study of Respiratory Syncytial Virus in Nasopharyngeal Aspirates Using Conventional Cell Culture, Shell Viral Centrifugation Culture, Immunofluorescence and Biotin-Avidin Enzyme Linked Immunosorbent Assays

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SUMMARY **133 nasopharyngeal aspirates (NPA) were simultaneously tested for the presence of respiratory syncytial virus (RSV) by conventional cell culture (CCC), shell vial centrifugation culture (SVC), immunofluorescence assay (IFA) and biotin-avidin enzyme linked immunosorbent assay (B-A ELISA). These yielded positive results in 32(24%), 45(33.8%), 36(27%) and 40(30%) of specimens, respectively. Specimens positive by IFA and B-A ELISA were all also positive by SVC. The sensitivity of CCC, IFA, and B-A ELISA comparing to SVC was 71%, 80%, and 88.9%, respectively. For rapid detection of RSV, we recommend the SVC method where a cell culture laboratory is available and the B-A ELISA method where a cell culture laboratory is not available.**

Respiratory syncytial virus (RSV) is a leading cause of lower respiratory tract infection in young children.[1,2] Pneumonia and bronchiolitis are the two common manifestations of RSV infection[3,4] and usually require hospitalization. In addition, RSV has been known to cause nosocomial infection in pediatric wards.[5,6]

The availability of specific antiviral therapy, ribavirin, for the treatment of RSV infections[7,8] has emphasized the importance of rapid diagnosis of this pathogen. For many years, the immunofluorescence test (IFA)[8-11] performed directly on infected epithelial cells in nasopharyngeal secretions has been the mainstay for such diagnosis. Enzyme linked immunosorbent assay (ELISA) has become an alternative method for viral antigen detection.[12,13] A number of ELISA kits for RSV detection are commercially available.[14-16]

Isolation of viruses in cell culture remains the gold standard to which other methods are compared. The conventional cell culture (CCC) method for viral isolation can take up to 14 days or longer. Recently, a variation of traditional cell culture has been described which combines cell culture in shell vials with centrifugation.[17,18] The shell vial centrifugation cell culture (SVC) technique has been introduced for detection of many viruses with accuracy and speed.[19-21] It has reduced the waiting period to 2 days.

A biotin-avidin enzyme linked immunosorbent assay (B-A ELISA) for detection of RSV antigen has recently been developed in our laboratory, using commercial antisera : rabbit anti-RSV immunoglobulin as capture antibody and biotinylated rabbit anti-RSV immunoglobulin with peroxidase conjugated avidin as detector system. The optimal dilutions of the reagents were obtained by performing checkerboard titration.

The objective of this study was to comparatively evaluate the sensitivity and specificity of the 4 different methods (CCC, SVC, IFA and B-A ELISA) used for the detection of RSV in the nasopharyngeal aspirates (NPA).

MATERIALS AND METHODS

Specimens

Clinical nasopharyngeal aspirates (NPA) for RSV testing were obtained from 133 pediatric patients who were admitted with a provisional diagnosis of pneumonia and bronchiolitis. The nasopharynx secretion was collected by pernasal aspiration

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and trapped in a sterile mucous extractor. The specimens were transported to the laboratory on wet ice, and processed immediately.

Preparation of specimen

The NPA was transferred to a 15 ml conical centrifuge tube; phosphate-buffered saline (PBS) containing antibiotics was added up to a volume of approximately 5 ml. The specimen was broken up into a fine cell suspension by gently aspiration and expulsion 6-7 times through the tip of a pasteur pipette. The suspension was then centrifuged at 1,500 rpm, 4°C for 10 minutes. The supernatant fluid was collected for CCC, SVC and B-A ELISA which were processed within 2-3 hours of arrival in the laboratory. The cell pellet was kept for IFA, it was washed once in PBS to remove residual mucus and then resuspended in sufficient amount of PBS to yield a visibly turbid suspension. The cell suspension was spotted onto a 2-well glass slide, air dried, fixed in cold acetone, kept at -20°C. The slide was stained for RSV infected cells within 1-3 days.

Conventional cell culture (CCC)

HEp-2 cells were prepared in-house by conventional methods, cells were fed with MEM Earle's salts (Gibco, Grand Island, U.S.A.) supplemented with fetal bovine serum (Gibco, Grand Island, U.S.A.) and antibiotics. Duplicated HEp-2 tubes were inoculated with 0.2 ml of supernatant fluid and incubated at 33°C. The cells were observed daily for 10 days for evidence of typical RSV cytopathic effects (CPE) of syncytium formation. Syncytium positive tubes were confirmed as RSV-positive by IFA using rabbit anti-RSV immunoglobulin (Dakopatts, Denmark) and fluorescein-isothiocyanate conjugated swine anti-rabbit immunoglobulin (Dakopatts, Denmark). If CPE was absent, blind passage was performed and then observed for 10 days. The specimen was considered negative when blind passage was done twice. Control cells and control RSV positives were included in each run.

Shell Vial Centrifugation Culture (SVC)

One ml of HEp-2 cell at a concentration of 2×10^5 cells/ml was seeded into shell vial; each vial contained a 12 mm round cover slip. After incubation at 37°C for 24 hours, the cells became confluent over the cover slip. Duplicated vials were inoculated with 0.2 ml of NPA supernate, then centrifuged at 2,600 rpm, 25°C for 1 hour. The cells were washed with maintenance media twice, then fed with 0.5 ml of maintenance media and kept at 33°C. After 18 hours, the cover slip was taken out and the RSV infected cell was identified by indirect immunofluorescent test. The presence of one or more cells with cytoplasmic fluorescing granules were read positive. Positive and negative controls were done in parallel, using stock RSV and maintenance media respectively as inocula.

Immunofluorescence assay (IFA)

The epithelial cells from the NPA pellet that were fixed on 2-well slide were tested for RSV antigen. Rabbit anti-RSV immunoglobulin at dilution 1:20 was dropped into one well and non-immune rabbit immunoglobulin (Dakopatts, Denmark) at the same concentration was dropped into another well as a non-specific fluorescence control. The slide was incubated at 37°C in a humidified chamber for 30 minutes, rinsed with PBS (pH 7.2) and then washed three times in PBS, 10 minutes each. Both wells were then overlaid with fluorescein isothiocyanate conjugated swine anti-rabbit IgG at a dilution of 1:40. The steps of incubation and washing were repeated. The slide was then immersed in a 1:30,000 dilution of Evan Blue for 5 minutes and rinsed with distilled water for 1 minute, dried and mounted in PBS buffered glycerol. The slides were read blindly under a fluorescence microscope (BH, Olympus, Japan) at × 400 magnification. The specimen was considered adequate when more than 100 nasopharyngeal epithelial cells were present per well. The presence of RSV antigen was indicated by the appearance of intracellular cytoplasmic inclusion-like bodies or particles fluorescing an apple-green color, in one or more respiratory epithelial cells.

Biotin-avidin enzyme linked immunosorbent assay (B-A ELISA)

The double-antibody sandwich ELISA technique was performed on microwell strip, flat bottom (Nunc Immuno Module) with 100 μl of each reagent. Each specimen was tested in duplicate. Rabbit anti-RSV immunoglobulin and non-immune rabbit immunoglobulin, each at a protein concentration of 10 μg/ml, were coated to each of adjacent wells. The nonimmune rabbit immunoglobulin coated well served as a nonspecific back ground control for each individual sample. The microwell strip was incubated at 37°C for 1 hour, then washed 5 times with PBS containing 0.1% Tween (PBS-Tween). NPA supernate was applied to both wells, incubated at room temperature for 2 hours, washed and biotinylated rabbit anti-RSV immunoglobulin (Dakopatts, Denmark) at a dilution 1:3,000 was added, incubated at room temperature for another hour, then washed and peroxidase conjugated avidin (Egg White; Dakopatts, Denmark) at a dilution of 1:7,000 was added. This was incubated at room temperature for 1 hour and washed again. The reaction was detected by adding O-phenylenediamine dihydrochloride (OPD) with H_2O_2 as a substrate and incubated in a dark room for 15 minutes. Finally, 4N H_2SO_4 was used to stop the reaction. The solution was read by spectrophotometer at a wave length of 492 nm. The absorbance value of the

result (△ OD) was calculated as the difference between the absorbance value of the test well and that of the control well. NPA with △ OD value equal to or greater than 0.20 was considered positive; less than 0.20 was defined as negative.

RESULTS

Specimens sent under improper conditions were excluded; 133 specimens were used in this study. A total of 45 specimens were positive by the SVC technique. Of these 45 positive specimens, 32, 36 and 40 were positive by CCC, IFA and B-A ELISA, respectively. The sensitivity and specificity of each test were calculated by comparison with SVC (Table 1). All methods possessed 100% specificity. The highest sensitivity (89%) was the B-A ELISA while the lowest (71%) was the CCC method.

Table 1. Comparison of results of RSV detection by CCC, IFA, and B–A ELISA to SVC in 133 NPA specimens.

	No. of SVC			
	RSV positive	RSV negative	Sensitivity %	Specificity %
CCC positive	32	0		
CCC negative	13	88		
Total no. test	45	88	71	100
IFA positive	36	0		
IFA negative	9	88		
Total no. tested	45	88	80	100
B–A ELISA positive	40	0		
B–A ELISA negative	5	88		
Total no. tested	45	88	89	100

RSV = respiratory syncytial virus, CCC = conventional cell culture
IFA = immunofluorescence assay, B–A ELISA = biotin–avidin ELISA
SVC = shell vial centrifugation culture, NPA = nasopharyngeal aspirate

DISCUSSION

We have evaluated the efficacy of the 4 methods in the detection of RSV infection; CCC, SVC, IFA, and B-A ELISA. Samples were NPA which were known to contain more cells than other types of clinical specimens and provided a faster rate and greater percentage of RSV isolation.[22] The HEp-2 cell line was used for viral culture because it had been demonstrated to be sensitive for isolating RSV.[22,23] The HEp-2 cells were prepared in-house and used within 24 hours of passage.

SVC appeared to be the most sensitive method. It could detect 45(33.8%) RSV positive samples from a total of 133 samples. Only 32(24%), 36(27%), and 40(30%) samples were positive by CCC, IFA, and B-A ELISA, respectively. All positive samples of B-A ELISA and IFA were confirmed by SVC.

The average period for CPE appearance in CCC was about 5-6 days (range 3-28 days) and 84.4% (27/32) of the isolates were detected in the first passage. By the SVC, the results were obtained on the next day. The number of visualized fluorescent RSV infected cells from CCC was more than from SVC (20-40% of cells per field from the CCC and 2-3 cells to 10 cells per field from the SVC method).

Our study showed that SVC was superior to CCC in detecting RSV by cell culture technique. This observation corresponded to that of previous authors.[19-21] Thirteen isolates were detected by SVC but could not be detected by CCC. The mechanism by which centrifugal inoculation increases the sensitivity of cells to viral infection is unclear. However, we found that centrifugation decreased the infective dose of RSV into HEp-2 cells by 100 times (data not shown). This finding is in agreement with previous reports.[24,25]

Detection of RSV antigens was more sensitive with the B-A ELSA than with IFA. The IFA method has several disadvantages, i.e. it is time consuming due to the process of washing infected epithelial cells in NPA to eliminate mucus and debris which may cause nonspecific staining. In addition, many epithelial cells may be lost during the washing procedure and cause false negative results. The efficiency of the in-house prepared B-A ELISA used in this study is comparable to that of commercial ELISA kits which had a sensitivity from 82-90%.[14-16] The optimal dilution of anti-sera and reaction times in our-B-A ELISA procedure were determined by performing a series of checkerboard titrations. When a new lot of reagent was used, it was again titrated by same procedure. B-A ELISA had several advantages, it is rapid and inexpensive, as the purchased anti-RSV sera can be used in very high dilution, handling of specimens is simpler, processing can be delayed by keeping specimens at -20°C until assay. The interpretation of results is objective. Positive and negative results are clearly seen in differentiated colors.

The NPA contains many substances such as mucus, remnants of cell which may cause heavy nonspecific background. The absorbance value of the nonimmune rabbit immunoglobulin coated well served as a nonspecific control for each specimen. In our experience on performing the test, we found that the nonimmune

rabbit immunoglobulin coated wells gave a background absorbance value higher than the reagent control wells. Determination of the result of the test by using the absorbance value of test well minus the absorbance value of the nonimmune rabbit serum coated well ($\triangle$ OD) should yield high specificity as supported by this study. The cut-off point at 0.20 was derived experimentally on simulated RSV positive and negative specimens, and on clinical samples; it gave satisfactory discrimination between positive and negative specimens. In this study, no NPA had Δ OD value that was equivocal or close to the cut-off value. The mean Δ OD of positive samples was 0.910 (range 0.220 - $>$ 2.0) and that of negative samples was 0.022 (range 0.002 - 0.172).

In conclusion, we suggest that SVC is the method of choice for detection of RSV as it is the most sensitive and rapid. For a laboratory where cell culture is not available, the in-house prepared B-A ELISA is recommended, it has many advantages including a high sensitivity (89%), specificity (100%), speed and low cost.

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