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Simplified, Rapid Diagnosis of Respiratory Syncytial Virus from Clinical Specimens

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SUMMARY **DOT ELISA was compared with RT-PCR and tissue culture to detect RSV from nasopharyngeal aspirates. DOT ELISA had diagnostic sensitivity and specificity of 65.62% and 93.92%, respectively. The results indicate that DOT ELISA can be used for screening detection of RSV from clinical specimens and is suitable for small laboratories in the provincial areas of developing countries.**

Respiratory syncytial virus (RSV) is one of the most common causes of acute respiratory tract infection in infants and young children around the world.[1-3] In temperate countries, epidemics have been observed annually for a period of about three to four months.[1] In the tropics, large proportion of children become ill because of respiratory tract infections during the rainy season. Studies of Tantivanich *et al.*[2] and Vathanopas *et al.*[3] have shown that RSV was one of the leading causes of such infections. Although RSV infection could be readily treated by ribavirin, delayed and/or inappropriate treatment due to mis-diagnosis has resulted in high mortality rate among the infected infants.[4] Thus, rapid and accurate diagnosis of this infection is needed.

Tissue culture method has been used for diagnosis of RSV infection. It seems to be a specific means of diagnosis. However, the sensitivity of the technique is affected by several factors including specimen collection, sensitivity versus refractoriness of the cell line(s) used for cultivation of the virus among many other attributes. Moreover, the technique is time consuming and laborious. Most of all it requires tissue culture which is not usually available in small health settings in the developing areas. Several serological assays for RSV detection, *ie.* immunofluorescence (IFA) and enzyme-immunoassay (EIA) have been developed.[5] The IFA and EIA were found to be more rapid than the tissue culture method. However, their specificities were variable and depended upon the quality of the antibodies used.[6-9] With the advent of the DNA amplification technique using polymerase chain reaction (PCR), many infections have been accurately, sensitively and specifically diagnosed. RT-PCR has been, as well, adapted for diagnosis of RSV. It was relatively rapid, sensitive and specific provided that specific DNA primers are available. RT-PCR is now a standard method with which other developing techniques are com-pared.[10] For RSV infection, it is relatively more sensitive than the culture method. However, RT-PCR encounters several disadvantages including high cost of reagents, requirement of equipment, precautions and skilled personnel. In this study, a dot-blot ELISA using specific monoclonal antibody as a

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detection reagent was developed for the detection of RSV antigen in the nasopharyngeal swabs of patients with suspected RSV infections. Its diagnostic accuracy, specificity and sensitivity were compared with the tissue culture technique using RT-PCR as a standard.

MATERIALS AND METHODS

Specimens

Nasopharyngeal aspirates (NPAs) were collected from 180 children and infants with suspected of respiratory infections at the Children's Hospital, Bangkok, during June to July 1992. The subjects aged between 1 to 6 months old. All specimens were dipped in MEM with 2% fetal bovine serum and antibiotics, then transported to the laboratory in an ice bucket. These specimens were subjected to the RT-PCR, tissue culture, and DOT ELISA for RSV detection, respectively.

DOT ELISA

Nitrocellulose membranes with 0.45 μm pore size were purchased from Sigma Chemical CO., Missouri, USA. The sheet were cut into strips of convenient size, approximately 90 x 10 mm. RSV subgroups A and B were used as the positive control antigens. Monoclonal antibodies from clones 2A-1, 5B-122 and 4C-27 were produced in our laboratory.[11] The monoclonal antibodies 2A-1 and 5B-122 were specific to RSV subgroups A and B, respectively, and they were used to determine the subgroup specificity; the monoclonal antibodies from clone 4C-27 were specific to both subgroups A and B. Rabbit anti-mouse immunoglobulins-alkaline phosphatase conjugate was purchased from Dako Corporation, California, USA. The substrate BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium) was purchased from Sigma Chemical Co. DOT ELISA was performed by the method of Gandhi and Kumar *et al.*[12-13] with modifications. The nitrocellulose strips were marked with waterproof ink for identification. The nasopharyngeal aspirates (NPAs) and the positive control were spotted on each mark on the nitrocellulose strips by using 3 μl per spot and air dried. The strips were placed in a blocking solution (Tris pH 7.5-Tween buffer containing 5% fetal bovine serum and 1% BSA) and incubated at room temperature for 30 minutes then washed with Tris pH 7.5-Tween buffer 4 times for 3 minutes each. The strips were blot dried with Whatman filter paper and 3 μl of monoclonal antibody were added on each the original spots, then incubated for 30 minutes at room temperature. The strips were washed in Tris pH 7.5-Tween buffer 4 times for 3 minutes each, then incubated in the rabbit anti-mouse antibody conjugated with alkaline phosphatase diluted 1:1,000 for 30 minutes at room temperature. The strips were then washed 4 times as above and finally with 0.15 M Tris, pH 9.6. The substrate solution was freshly prepared by adding one tablet of the substrate in 10 ml distilled water, then the strips were transferred into the substrate solution and incubated about 10 minutes. To stop the reaction, the strips were rinsed with tap water then air dried. The color of the positive samples was purple while the negative results gave no color. The positive color can remain for many months.

Tissue culture

RSV subgroup A, RSV subgroup B, and NPAs were propagated in HEp-2 cells cultures in Eagle's minimal essential medium, 5% fetal bovine serum and antibiotics. The infected cells were incubated at 37°C in CO_2 incubator. The presence of CPE was observed daily. The positive isolations were confirmed by IFA. adenovirus, parainfluenza virus, influenza virus and uninfected HEp-2 cells were used as the controls. adenovirus, parainfluenza virus and influenza virus were propagated in HEp-2 cells and MDCK cells, respectively.

Preparation of samples for RT-PCR

Total RNA was extracted from RSV infected HEp-2 cells according to the procedure described by Chomczynski and Sacchi[14] with a slight modification. An aliquot of RSV subgroup A long strain or RSV subgroup B (HC 306/91, Thai strain) was extracted by adding 0.5 ml of lysis buffer (4M guanidium thiocyanate, 25 nM sodium citrate, pH 7.0, 0.5% of sarcosyl, 0.1 M 2-mercaptoethanol), 50 μl of 2M sodium acetate, pH 4.0, 0.5 ml of phenol and 0.1 ml of chloroform to the virus preparation. The preparation was mixed and centrifuged at 12,000 x *g* for 15 minutes at 4°C. The aqueous phase was further extracted with an equal volume of isopropanol, kept in -70°C for 10 minutes, then centrifuged at 12,000 x *g* at 4°C for 15 minutes. The pellet was suspended in 200 μl of diethylpyrocarbonate (DEPC) treated water and 15 μl of 2 M sodium acetate pH 5.2, precipitated with 2 vol-

umes of ethanol and washed with 500 µl 70% ethanol. The RNA pellet was dissolved in 4.5 µl DEPC treated water and kept for RT-PCR. Extraction of RNA from adenovirus, parainfluenza, and influenza viruses was done as for the RSV.

Extraction of RNA from clinical specimens was performed as above after adding 500 µl of lysis buffer to 250 µl of clinical specimens. The RNA pellet was dissolved in 9 µl of DEPC treated water and 4.5 µl was used for RT-PCR.

Reverse transcription and PCR

The cDNA was synthesized in 10 µl of the reaction mixture containing 50 mM Tris HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 mM each of four dNTPs (Boehringer Mannheim, Germany), 5 units of human placental ribonuclease inhibitor (BRL, USA), 1 µM each of two RSV specific primers, FIA (5′- TTAACCAGCAAAGTGTAAGA-3′) and FIB (5′-TTTGTTATAGGCATATCATTG-3′),[15] extracted RNA, and 100 units of SUPERSCRIPT reverse transcriptase (BRL, USA). The mixture was incubated at 42°C for 15 minutes. The reaction mixture was made up to 50 µl by adding 10 mM Tris HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 0.1 mM each of four dNTP's, 2 µM each of the two primers, FIA and FIB, and 2.5 units of *Taq* DNA polymerase (Promega, USA). The reaction mixture was overlaid with 50 µl mineral oil and subjected to 1 amplification cycle, consisting of sample denaturation at 94°C for 2 minutes, primer annealing at 52°C for 45 seconds and primer extension at 72°C for 45 seconds. The amplification was repeated 44 more cycles.

Analysis of the amplified products

Ten µl of the 243 bp amplified products was analysed by electrophoresis on 3% agarose containing ethidium bromide for 30 minutes. The gels were photographed under UV illumination.

Southern blot hybridization

The DNA amplified products were transferred from agarose gel to a Hybond-N nylon membrane (Amersham, UK). DNA sample was denatured on the membrane for 10 minutes in 0.4 M NaOH, and soaked in 5 x SSC for 1 minute, then fixed under UV light. The membrane bound DNA was hybridized with an oligonucleotide probe (5′-AGTGTTAATGCAGGTGTAAC-3′) at nucleotide positions 725-744 of the RSV sequence[16] and labeled for chemiluminescence according to the method of enhanced chemiluminescence (ECL) 3′-oligo labeling and detection systems (Amersham, UK). The hybridized DNA-membrane was then exposed to X-ray film.

Determination of sensitivity and specificity of the test

Sensitivity and specificity among RT-PCR, tissue culture, and DOT ELISA were compared by using RT-PCR as a standard method. The results of these methods were compared and calculated by using Cochran Q test, and comparison between each method were calculated by using Mc Nemar's χ^2 test.

RESULTS

Tissue culture, RT-PCR, and DOT ELISA were compared in the detection of RSV from 180 nasopharyngeal aspirates. Four out of 180 (2.2%) were positive for RSV by tissue culture while 10 out of 180 (5.6%) and 21 out of 180 (11.7%) were positive for RSV by RT-PCR and DOT ELISA respectively (Table 1). Comparison among the 3 methods to detect the presence of RSV from nasopharyngeal aspirates is demonstrated in Table 2. The percentage of positive RSV isolates among RT-PCR, tissue culture, and DOT ELISA were significantly different ($p < 0.001$). Comparison between RT-PCR and tissue culture, RT-PCR and DOT ELISA, tissue culture and DOT ELISA were different at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Statistical analysis by the method of Galen [17] revealed that the diagnosis specificity, diagnosis sensitivity of the DOT ELISA and tissue culture using RT-PCR as a standard method was 93.9%, 62.6% and 96.7%, 40% respectively. Tissue culture had 6 false negative results while DOT ELISA had 11 false positive results. The kappa coefficient beyond chance between RT-PCR and tissue culture and RT-PCR and DOT ELISA were 0.56 and 0.62, respectively, indicating good agreement of the RT-PCR with tissue culture method and DOT ELISA while the kappa coefficient beyond chance between tissue culture and DOT ELISA was only 0.29 indicating fair agreement.

Table 1. Comparison of tissue culture, RT-PCR, and DOT ELISA for detection of respiratory syncytial virus from 180 nasopharyngeal aspirates

	No. positive (%)	No. negative (%)
Tissue culture	4/180 (2.2%)	176/180 (97.8%)
RT-PCR	10/180 (5.6%)	170/180 (94.4%)
DOT-ELISA	21/180 (11.7%)	159/180 (88.3%)

Table 2. Comparison of tissue culture, RT-PCR, and DOT ELISA for detection of respiratory syncytial virus from 21 nasopharyngeal aspirates

Nasopharyngeal aspirate number	Tissue culture	RT-PCR	DOT ELISA
010	-	-	+
012	-	-	+
033	-	-	+
045	-	-	+
046	-	-	+
060	-	-	+
075	-	-	+
077	-	-	+
103	-	-	+
185	-	-	+
187	-	-	+
014	-	+	+
027	-	+	+
036	-	+	+
078	-	+	+
149	-	+	+
179	-	+	+
034	+	+	+
048	+	+	+
104	+	+	+
176	+	+	+

DISCUSSION

Various methods have been used for detection of RSV from clinical specimens *eg* RT-PCR, IFA, and EIA.[5-7] Among these, RT-PCR was found to have the highest sensitivity and specificity.[10,15] In this study, RT-PCR was used as the standard method to compare the sensitivity and specificity of a DOT ELISA, and a tissue culture technique for the detection of RSV or its antigen in patients' nasopharyngeal aspirates. It was found that the tissue culture method had false negative results which indicate its lower sensitivity than the RT-PCR. Many reasons may be given to explain the finding. It may depends on the viability of the virus in the specimens during specimen collection and transportation or the other unforeseen factors that prevent the virus to complete their proliferation cycle and production of CPE. RT-PCR had higher specificity than DOT ELISA since the specificity of RT-PCR depended on the highly specific primers from the F1 gene conserved region of RSV for amplification of RSV-cDNA[15,16] while the specificity of DOT ELISA depended on the binding of monoclonal antibodies to the capsid or envelop proteins of the virus. False positive by DOT ELISA can be occurred if the nasopharyngeal aspirates contained the endogenous enzyme. This enzyme can also bind to the monoclonal antibodies and gave positive results. It is not possible to destroy the foreign proteins in the clinical specimens by boiling since it will also destroy the capsid proteins of the virus.

Even though DOT ELISA has low specificity, its sensitivity is satisfactorily high. The kappa coefficient value between RT-PCR versus DOT ELISA is higher than that of RT-PCR versus tissue culture. This comparison suggests that the DOT ELISA may be a better screening method than the tissue culture for RSV. However, confirmation is needed for DOT ELISA positive specimens when accurate diagnosis is requested. The DOT ELISA is relatively simple, inex-

pensive and does not require highly trained personnel. Moreover, it can be also performed under the field conditions. These features emphasis the possibility of using the monoclonal antibody based DOT ELISA as a presumptive test for RSV infection in developing areas.

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