



Serological Response to Respiratory Syncytial Virus Infection in Pediatric Patients with a Comparison to Immunofluorescence and Virus Isolation

Pilaipan Puthavathana,¹ Supreeda Habanananda,² Rachaneeporn Toncharoensook,¹ Uraiwan Kositanont¹ and Chantapong Wasi¹

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infection (LRI) in infants and young children worldwide.^{1,2} Laboratory diagnosis of the infection can be made by several means, including rapid detection of viral antigen in nasopharyngeal secretions, virus isolation in cell culture, and detection of specific antibody in paired sera.³⁻⁶ A positive result obtained by any of the three methods provides a conclusive diagnosis. In general, however, all three methods should be employed together in order to give the highest detection rate because a case missed by one method can be discovered by another.

Previous investigations revealed that serological methods failed to diagnose some cases of RSV infection, which can be explained by the insensitivity of complement fixation (CF) and neutralization tests performed in those laboratories.⁷⁻⁹ Another explanation is the poor antibody response to viral antigens in infancy. Antibodies are not developed to all structural proteins in young infants after RSV infection.¹⁰ Young infants and adults are different in their capability to respond to certain viral antigens, and

SUMMARY : The serological response to respiratory syncytial virus (RSV) in 125 pediatric patients hospitalized with acute lower respiratory infection was investigated by enzyme linked immunosorbent assay (ELISA) for specific immunoglobulin (Ig) A, IgG, and IgM and complement fixation (CF) test. By ELISA, a 4-fold rise in IgG titre in paired sera was most commonly found, followed by a rise in IgA and IgM titres. Investigation by ELISA and CF leads to the suggestion that major CF activity against RSV antigens resides in the IgG and not the IgA and IgM classes. No case with CF activity failed to be diagnosed by ELISA. The youngest infant who could develop seroconversion was one month old, nevertheless two children older than two years could not. When the three diagnostic methods were compared, ELISA serology was the most sensitive followed by indirect immunofluorescence (IIF) for antigen detection and virus isolation, respectively, ELISA could diagnose RSV infection in 45% of the study cases, whereas IIF and virus isolation only diagnosed 26% and 14%, respectively. Half of the cases was diagnosed by all of the three methods together.

also in terms of immunoglobulin class and subclass response to specific antigens.¹⁰⁻¹³ Later reports on ELISA serology showed that young infants did develop specific IgA, IgG, and IgM antibodies in response to RSV infection. However, the criteria for determining positivity in those reports were different.^{10,12,14-17}

The present study reports on the diagnosis of RSV infection by using ELISA serology to demonstrate a 4-fold rise in specific IgA, IgG and IgM antibody titres in paired sera of children hospitalized with LRI. In

addition, correlation between the ELISA and other diagnostic methods (CF, indirect immunofluorescence [IF] and isolation) was examined, together with the relationship between antibody response and age.

From the Departments of ¹Microbiology and ²Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

Correspondence : Pilaipan Puthavathana, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

MATERIALS AND METHODS

Subjects

A total of 125 pediatric patients under five years of age who were hospitalized in the Department of Pediatrics, Siriraj Hospital, for acute LRI during June 1990 to December 1991 were included in the study. These cases were completely investigated for viral etiology by the diagnostic methods of IIF, viral isolation, CF and ELISA serologies, or else they were excluded from the data analysis.

Specimen collection and processing

Clinical specimens used for investigation were nasopharyngeal secretion (NPS) and paired sera. NPS was collected in a mucus trap (Nunc, Roskilde, Denmark) in 2-3 ml of viral transport medium and transported on wet ice to the virology laboratory. The sample was thoroughly mixed and centrifuged at 300 x g for 10 minutes at 4°C. The supernatant was kept at 4°C and used for virus isolation which was done within two days, and the pellet of exfoliated cells was further washed three times to remove excess mucus. The cells were then spotted on microscopic slides, air-dried, fixed in pre-cooled acetone for 10 minutes and kept at -70°C until stained. Acute sera were collected within seven days after the onset of illness and convalescent sera were collected two to three weeks later. All sera were separated from clotted blood and kept at -20°C until tested. Acute and convalescent sera were tested simultaneously in each experiment.

Indirect immunofluorescence

Slides of cell deposits were covered with bovine antiserum against RSV (Wellcome Diagnostics, Dartford, England) diluted to 1:10; they were then incubated for 30 minutes at 37°C in a moist chamber and washed in PBS and distilled water. The wet slides were drained of water, and anti-bovine immunoglobulin conju-

gated with fluorescein isothiocyanate (Wellcome Diagnostics) at the dilution of 1:20 was applied to the slide for another 30 minutes at 37°C. The washing step was repeated as above and was followed by counterstaining for 1 minute with Evan's blue (Sigma Chemical Co, St Louis, MO) diluted to 1:30,000.

The stained slides were examined under a fluorescence microscope (Optiphot, Nikon, Tokyo). Cell deposits containing less than 200-300 cells were discarded from the analysis. A test slide was also stained with antisera specific to respiratory viruses and represented the negative control; slides of RSV infected HEP-2 cells were used as positive controls. The immunofluorescence pattern characteristic of RSV antigen was cytoplasmic staining with rough granules.

Virus isolation

RSV was isolated in HEP-2 cell culture aged 1-3 days (American Type Culture Collection, Rockville, MD). The cells were cultured in minimum essential medium (MEM; Gibco, NY) supplemented with 10% fetal bovine serum (FBS; Gibco, NY) and maintained in MEM supplemented with 2% FBS. Penicillin (200 U/ml), streptomycin (200 µg/ml), gentamicin (10 µg/ml), and amphotericin B (1 µg/ml) were added to both growth and maintenance media.

Each duplicate tube of HEP-2 cell monolayer was inoculated with 200 µl of supernatant from a NPS sample. The inoculated cultures were incubated at 37°C and observed daily for cytopathic effects (CPE) for two weeks. The culture with appearance of viral infection in 50-75% of the cell population were harvested and stained by indirect immunofluorescence for identification of RSV.

Complement fixation test

The CF protocol was modified after Schmidt and Emmons.⁵ The

RSV test antigen and uninfected cell control antigen were purchased from the Behring Institute (Marburg, Germany). Guinea pig sera as the source of complement were used at a working concentration of 1.5-2 hemolytic units. The test was performed in 96 well microtiter plates as follow : the test sera were inactivated at 56°C for 30 minutes, and two-fold diluted serially in veronal buffer saline pH 7.2 starting from the dilution of 1:8 to 1:128 in a 50 µl volume. Then, 25 µl of test or control antigens together with 25 µl of complement were mixed and incubated overnight at 4°C. On the next day, 50 µl of hemolysin sensitized sheep red blood cells (RBC) were added to each reaction well and incubated at 37°C for 45 minutes before the degree of hemolysis was examined.

The CF titre was read when the highest serum dilution showed less than 50% hemolysis of the sensitized RBC; and paired sera which showed a four fold rise in antibody titre were considered to be CF positive.

Indirect enzyme linked immunosorbent assay

The procedure was modified from that described by Parrot *et al.*⁴ The antigen for ELISA was prepared from RSV subgroup A strain A2 and subgroup B strain 18537 which were kindly provided by Dr Larry J Anderson, Respiratory and Enterovirus Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Controls, Atlanta, GA, USA. Briefly, HEP-2 cell cultures were infected with either one of the reference strains and incubated at 37°C until 75-100% of the cell population showed morphological changes. The culture was harvested and freeze-thawed three times followed by sonication and centrifugation at 15,000 x g for 30 minutes. The supernatant was examined for protein concentration, aliquoted and kept at -70°C until used. Uninfected HEP 2 cells processed under the same

conditions were used as negative control antigen.

Equal concentrations of RSV subgroups A and B antigens were mixed and adjusted to a working concentration of 100 µg/ml, as was the control antigen. Each 100 µl of the test or the control antigen was coated on the bottom of an ELISA plate (Nunc-Immuno-Plates, Polysorp, Roskilde, Denmark) and 3% bovine serum albumin in PBS pH 7.2 was used as the blocking reagent. Test serum was two-fold diluted serially by starting from a dilution of 1:20 to 1:1,280 for the IgA and IgM assays, or from dilution 1:80 to 1:5,120 for IgG assay. All serum dilutions were allowed to react with the coated antigens at 37°C for two hours in a moist chamber. Goat antihuman IgG, IgA or IgM conjugated with horseradish peroxidase (DAKO A/S, Copenhagen, Denmark) was used as the second antibody and incubated at 37°C for 1 hour. Orthophenylene diamine dihydrochloride (DAKO A/S) was used as the chromogenic substrate. The reaction plate was read for optical density using a Titertek Multiskan spectrophotometer (Flow Laboratories, VI, USA) at the wave length of 492 nm.

Interpretation of the ELISA result

The corrected absorbance value for each serum was calculated by subtracting the OD value obtained with the control antigen from that obtained with the test antigen. Presence of specific immunoglobulin in the test serum was indicated when the corrected absorbance value of ≥ 0.2. The antibody titre was read when the highest serum dilution showed the corrected absorbance value of ≥ 0.2, and the sera test for specific IgM contained no rheumatoid factor as examined by agglutination test (Rapi Tex^R-RF, Behringwerke AG, Germany). RSV infection was diagnosed when a 4-fold rise in titre of any specific immunoglobulin class in paired sera

was detected.

RESULTS

Immunoglobulin class response to RSV infection

Paired sera from a total of 125 pediatric patients were studied, and a 4-fold rise in immunoglobulin titre was found in 56 (45%) cases by ELISA and 11 (9%) by CF (Table 1). Thirty-one (25%), 23 (18%) and 2

(1.6%) cases developed a rise in titre with one, two and all three immunoglobulin class responses, respectively. Serological response with the IgG class was the most common, followed by IgA and IgM responses, ie, 34, 21 and 12%, respectively. All cases with seroconversion by the CF test also seroconverted by ELISA. Noticeably, all of these CF-positive cases gave IgG seroconversion, while CF antibody

Table 1. Serological response to RSV infection as determined by ELISA and CF test (N = 125)

Ig class response	Number of cases (%) diagnosed by	
	ELISA	CF
IgA only	4	0
IgG only	18	3
IgM only	9	0
IgA and IgG	19	5
IgA and IgM	1	0
IgG and IgM	3	1
IgA and IgG and IgM	2	2
Total positive cases	56 (44.8)	11 (8.8)
Total IgA response	26 (20.8)	
Total IgG response	42 (33.6)	
Total IgM response	15 (12.0)	
Total cases responded with one Ig class	31 (24.8)	
Total cases responded with two Ig classes	23 (18.4)	
Total cases responded with three Ig classes	2 (1.6)	

Table 2. Diagnosis of RSV infection by IIF, isolation and ELISA techniques (N = 125)

Diagnostic methods	Numbers of RSV cases (%) diagnosed
IIF only	4
Isolation only	1
ELISA only	26
IIF and isolation	2
IIF and ELISA	16
Isolation and ELISA	4
IIF and isolation and ELISA	10
Total cases diagnosed by all test	63 (50.4)
Total cases diagnosed by IIF	32 (25.6)
Total cases diagnosed by isolation	17 (13.6)
Total cases diagnosed by ELISA	56 (44.8)

Table 3. Relationship between serological response and age to RSV infection

Age-group (month)	Number of RSV infected cases	Number of cases with ELISA	
		Positive (%)	Negative(%)
0 - 5	5	3 (60)	2 (40)
6 - 11	31	29 (93.5)	2 (6.5)
12 - 17	14	13 (92.9)	1 (7.1)
18 - 23	7	7 (100)	0 (0)
24 - 35	5	3 (60.0)	2 (40.0)
36 - 47	0	0 (0)	0 (0)
48 - 59	1	1 (100)	0 (0)
Total	63	59 (88.9)	7 (11.1)

was not found in cases which exhibited only a rise in IgA or IgM titre. The results indicate that major CF activity in RSV infection resided in the IgG fraction.

Diagnosis of RSV infection by IIF, viral isolation and ELISA

The combination of IIF, viral isolation and ELISA techniques was able to diagnose RSV infection in 63 (50%) of the 125 study cases (Table 2), ELISA diagnosed 89% (56 of 63) of the RSV infected cases and, among the three diagnostic methods, was the most sensitive, followed by IIF and viral isolation, respectively.

Relationship between age and serological response

There were 63 RSV infected cases diagnosed by the combination of IIF, viral isolation and ELISA serology. ELISA alone failed to diagnose 7 of them. Although very young infants can develop specific antibody responses, their capability to do so seems to increase after six months of age (Table. 3). On the other hand, two cases who were unable to develop antibody responses were in the age-group of 2-3 years.

DISCUSSION

It has been previously shown that RSV infection in young children can stimulate the production of specific

IgA, IgG and IgM.^{10,14-16} These immunoglobulins are detectable within a few weeks, and remain so for at least a few months to one year after a single RSV infection.^{10, 16} However, RSV reinfection can occur within two months after the primary infection.¹⁸ This finding implies that the presence of specific IgA, IgG and IgM in a single serum cannot be used as a marker for diagnosis of current RSV infection. Our study thus diagnosed the infection by relying on a 4-fold rise in RSV antibody titer in paired sera.

The present study has revealed that among 56 RSV infected children who developed seroconversion, 31 (55%) produced one class of specific immunoglobulin, 23 (41%) cases produced two classes, and only 2 (3.6%) cases responded with three classes of immunoglobulin (Table 1). IgG was the most common immunoglobulin detected followed by IgA and IgM. The lack of IgM antibody response in young infants has been noted.¹⁹ Our findings on classes of immunoglobulin response to RSV infection are also similar to those reported by Muerman.¹⁶

We could not exclude a maternal origin of part of specific IgG present in the test serum. However, its presence would not affect our interpretation which is based on seroconversion of specific immunoglobulin

classes in paired sera. It seems that the immunoglobulin class which plays the major role in CF activity is IgG, since it was found in all cases with positive CF test, whereas the cases with IgA or IgM only did not possess CF activity. It is not surprising to see that IgA cannot fix the complement but IgM, which has been known to have the best such activity, could not fix complement in our system.²¹ However, it is also shown that the CF test detects only specific IgG for most virus antigens, but for other microbial antigens it may detect both specific IgM and IgG.²²

Among the three diagnostic methods: IIF, viral isolation and ELISA serology, the latter was the most sensitive for RSV diagnosis. ELISA alone diagnosed about 45% of the study population, whereas the three methods combined diagnosed 50%. We previously investigated viral etiology in acute upper and lower respiratory tract infections in other hospitals and found that the CF test is the least sensitive test when compared with IIF and viral isolation; CF diagnosed RSV infection in 9% of the study population, while the three methods combined diagnosed 19%.⁹

Using the sensitive ELISA test, we demonstrated that most young infants can develop antibody responses after RSV infection. The youngest case showing seroconversion in our study was one month old. This case was diagnosed positive by IIF and viral isolation and by seroconversion of specific IgA only. Immunological potential in this particular child may be stronger than the others; or the rapid waning of the anti-RSV maternal antibody will diminish the suppressor effect on the child's immunological response, and thus lead to seroconversion. These explanations also seem to be hold true for older children in general, since more than 90% of the infected cases aged 6-17 months underwent seroconversion. However, failure to develop seroconversion was

found in two cases of older than two years of age.

The capability of very young infants to develop serological response against RSV infection in our study suggested that serodiagnosis is more sensitive than other diagnostic methods if an appropriate serological test system is used which is able to detect all specific immunoglobulin classes. However, its limitation is the requirement of paired blood samples which will delay diagnosis.

ACKNOWLEDGEMENTS

This work was supported by Siriraj-China Medical Board Research Grant No. 75-348-178.

REFERENCES

1. McIntosh K, Chanock RM. Respiratory syncytial virus. In: Fields BN, Knipe DM, *et al.* eds, *Virology*, 2nd ed. New York: Raven Press, 1990; 1045-72.
2. Report of a WHO Scientific Group. *Viral respiratory diseases*. WHO Tech Rep Ser 642, 1980.
3. *Manual for rapid laboratory viral diagnosis*. WHO Offset Publication No. 47, 1979.
4. Parrot RH, Brandt OD, Kim HW. Respiratory syncytial virus. In: Lennette EH, ed, *Laboratory diagnosis of viral infections*. New York, Marcel Dekker, Inc, 1985; 453-64.
5. Schmidt NJ, Emmons RW. General principles of laboratory diagnostic methods for viral, rickettsial and chlamydial infections In: Schmidt NJ, Emmons RW, eds, *Diagnostic procedures for viral, rickettsial and chlamydial infections*, 6th ed. Washington DC; American Public Health Association, Inc. 1989; 1-36.
6. Walsh EE, Hall CB. Respiratory syncytial virus. In: Schmidt NJ, Emmons RW, eds, *Diagnostic procedures for viral, rickettsial and chlamydial infections*, 6th ed. Wash-

- ington DC; American Public Health Association Inc. 1989; 693-712.
7. Chanock RM, Kim HW, Vargosk AJ, Johnson KM, Cumming C, Parrot RH. Respiratory syncytial virus. I. Virus recovery and other investigations during 1960 outbreak of bronchiolitis, pneumonia and minor respiratory diseases in children. *JAMA* 1961; 176: 647-53.
8. Parrot RH, Vargosko AJ, Kim HW, *et al.* Respiratory syncytial virus. II. Serologic studies over a 34-month period of children with bronchiolitis, pneumonia, and minor respiratory diseases. *JAMA* 1961; 176: 653-7.
9. Puthavathana P, Wasi C, Kositanont U, *et al.* A hospital-based study of acute viral infections of the respiratory tract in Thai children, with emphasis on laboratory diagnosis. *Rev Infect Dis* 1990; 12; supl 8: S988-S94.
10. Popov-Kraupp TE, Lakits E, Kellner G, Kunz C. Immunglobulin-class specific immune response to respiratory syncytial virus structural proteins in infants, children, and adults. *J Med Virol* 1989; 27: 215-23.
11. Murphy BR, Alling DW, Snyder MH, *et al.* Effect of age and preexisting antibody on serum antibody response of infants and children to the F and G glycoprotein during respiratory syncytial virus infection. *J Clin Microbiol* 1986; 24: 894-8.
12. Watt PJ, Zardis M, Lambden PR. Age related IgG subclass response to respiratory syncytial virus fusion protein in infected infants. *Clin Exp Immunol* 1986; 64: 503-9.
13. Welliver RC, Kaul TN, Patnam TI, Sun M, Riddlesberger K, Ogra OL. The antibody response to primary and secondary infection with respiratory syncytial virus: Kinetics class specific response. *J Pediatr* 1980; 96: 808-13.
14. Erdman DD, Anderson LJ. Monoclonal antibody-based capture enzyme im-

- munoassays for specific serum immunoglobulin G (IgG), IgA, and IgM antibodies to respiratory syncytial virus. *J Clin Microbiol* 1990; 28: 2774-9
15. Medden MM, Herbrink P, Lindeman J, van Dijk CW. Serodiagnosis of respiratory syncytial virus (RSV) infection in children as measured by detection of RSV specific immunoglobulins G, M, and A with enzyme-linked immunosorbent assay. *J Clin Microbiol* 1990; 28: 152-5.
16. Muerman O, Ruuskanen O, Sarkkinen H, Hanninen P, Halonen P. Immunglobulin class-specific antibody response in respiratory syncytial virus infection measured by enzyme immunoassay. *J Med Virol* 1984; 14: 67-72.
17. Murphy BR, Graham BS, Prince GA, *et al.* Serum and nasal-wash immunoglobulin G and A antibody response of infants and children to respiratory syncytial virus F and G glycoproteins following primary infection. *J Clin Microbiol* 1986; 23: 1009-14.
18. Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. *J Infect Dis* 1991; 163: 693-8.
19. Muerman O. Detection of antiviral IgM antibodies and its problems. *Curr Top Microbiol Immunol* 1983; 104: 101-28.
20. Richardson LS, Yolken RH, Belshe RB, Camargo E, Kim HW, Chanck RM. Enzyme-linked immunosorbent assay for measurement of serological response to respiratory syncytial virus infection. *Infect Immun* 1978; 20: 660-4.
21. Goodman JWA. Immunoglobulin structure & function. In: Stites DF, Terr AJ, eds, *Basic and clinical immunology*, 7th ed. Norwalk, Appleton & Lange 1991; 109-21.
22. Morgan - Capner P, Pattison JR. Techniques in clinical virology. In: Mahy BWJ, ed, *Virology: a practical approach*. Oxford, IRL Press Limited, 1985; 237-58.