Medical Journal of the
Islamic Republic of Iran

Volume 15
Number 3
Fall 1380
November 2001

APPLICATION AND ADAPTATION OF DOT IMMUNOASSAY TO DETECT AND MEASURE RUBELLA VIRUS ANTIBODY

T. BAMDAD,* M.H. ROUSTAI,* H. SOLIMANDJAHI,* AND M. MALEKANEH**

*From the Department of *Virology, Faculty of Medical Sciences, Tarbiat Modarres University, P.O. Box 14115-111, Tehran, and the **Department of Biochemistry, Faculty of Medical Sciences, Birjand University of Medical Sciences, Birdjand, I.R. Iran.*

ABSTRACT

A dot immunobinding assay (DIA) was used for a quantitative and qualitative assay of rubella antibody. Purified antigen and conjugated anti-human immunoglobulin (RAHIg) were prepared. Nitrocellulose paper dotted with the antigen was added to serially diluted sera or blood samples. The reacting antibodies were visualized by a peroxidase system. Development of a colored insoluble substrate was taken as a positive result. The adapted DIA was applied to test 105 serum samples. The sensitivity and specificity and immune titer of DIA were compared with hemagglutination inhibition (HI) test.

MJIRI, Vol. 15, No. 3, 171-174, 2001.

Keywords: Rubella virus, dot immunoassay (DIA), nitrocellulose paper, hemagglutination inhibition (HI) test, enzyme immunoassay.

INTRODUCTION

Rubella is an infectious viral disease which is endemic in Iran. Fetal infection with rubella virus is a known cause of congenital birth defects and mental retardation. Based on the WHO report, there are outbreaks leading to congenital rubella syndrome (CRS) epidemics occurring every 4-7 years in countries without any national rubella control program.[1] In order to reduce the rate of CRS it is necessary to know the immune status of women at childbearing age and detect active infections with the rubella virus, especially during the first trimester of pregnancy. A number of serology tests are available for detecting the antibody response to rubella virus, among which HI and ELISA are more routine. DIA was used in this research to detect and measure antibodies against the rubella virus. This test is easier and more rapid than the above mentioned tests and a large number of sera can be tested in short time.[2] It provides a very suitable and economic method for serological survey of rubella especially in developing countries.

MATERIAL AND METHODS

Cells

Baby hamster kidney-21 (BHK-21) cells were propagated as a monolayer using minimum essential medium (MEM) supplemented with 5% heat inactivated calf serum (HICS).

Antigen preparation

Rubella virus, strain RA27/3 obtained from Razi research institute (Karaj, Iran) was propagated in the cells. Inoculated cells were added with MEM+1% HICS and incubated at 35°C for 4 days, after which the medium was harvested and glycin buffer (pH 9.5) was added to the cells. The cells were then incubated at 35°C for 30 hours. The buffer containing viral antigen was harvested. The harvested virus was added with EDTA and Tris base (final concentration 0.01 M), and centrifuged at 6000g for 30 min at 4°C. The supernatant was concentrated up to 400 times using an ultrafilter. The concentrate was

layered onto a 60%-24% discontinuous sucrose density gradient in 0.05 M Tris, 0.15 M NaCl, 0.005 M EDTA, pH 7.4 and centrifuged at 9800 g for 2h at 4°C. Control antigen was prepared using an identical procedure with uninfected BHK-21 cell supernatant. The antigen was stored at -70°C until further use.

Conjugated rabbit antibody

Human Ig (HIg) was precipitated with 33% saturated ammonium sulfate and purified using DEAE-cellulose column (phosphate buffer, 0.01 M, pH 8).[4] Two rabbits were each inoculated with 50 μg of HIg with complete Freund's adjuvant. Four further injections were given to the rabbits at 14 day intervals using incomplete Freund's adjuvant. A control rabbit was also injected in a similar way, using PBS with the adjuvant. The anti-human antibody titer was measured with a direct ELISA before each injection. Fifteen days after the last injection all rabbits were bled and the obtained sera kept at -20°C for later use. RAHIg was purified using the above mentioned method (phosphate buffer, 17.5 mM, pH 6.3),[4] and it's purity was determined. The purifed RAHIg was conjugated to HRP using the method explained by Wilson and Nakene.[5] A direct ELISA test was done to calculate the best working dilution of the conjugated antibody.

Serum samples

One-hundred and five cord sera were obtained. Some blood samples were also collected on filter papers (Whatman paper, No. 1) from the same individuals.

DIA

Nitrocellulose papers (pore size 0.45) were divided to squares (1 × 1), washed with distilled water and then dried. Two squares were each coated with 3.5μg of virus and control antigen separately. Spots were allowed to dry at room temperature (RT) and then the filters were soaked in PBS containing 0.5% gelatin in order that the remaining binding sites become saturated. The paper was then washed with PBS-tween (PBS and 0.05% tween) for half an hour, three times. Serum dilutions (1/400, 1/800, 1/1600,...) were prepared in PBS-tween and added to coated papers, for two hours at RT, after which all papers were washed with PBS-tween. The papers were then incubated with conjugated RAHIg for half an hour at RT and washed as mentioned above. The reaction was detected by adding substrate (DAB- 3, 3 diaminobenzidine) to the obtained complex for 2-3 minutes and then the reaction stopped by tap water. The same procedure was done for whole blood collected on Whatman filter papers. The filters containing dried bloods were soaked in PBS-tween resulting in a serum dilution of approximately 1/400, and further dilutions of 1/800, 1/1600, ... were prepared from that, and were tested as mentioned above.

HI test

HI test is standard assay for rubella specific antibody. The titer antibody to rubella virus in each serum sample was determined by this test.[6]

RESULTS

In DIA the presence of antigen-specific antibodies in the examined sera was revealed by the formation of color spots on the white nitrocellulose paper. The intensity of the color reaction was dependent on the amount of antibodies present in each dilution. In our experiments, none of the sera showed nonspecific reactions with uninfected BHK-21 cell control antigen. The antibody titer was therefore determined by the last dilution of each serum giving a positive reaction.[7] Figure 1 shows the antibody titer against rubella virus in one serum sample. The upper row illustrates papers coated with virus antigen and the lower row belongs to control. The result indicates that the titer of the serum antibody is 1/6400.

Taking HI as the standard test, the results of DIA obtained from 105 sera samples were compared with those of HI test. Antibody titers obtained from DIA and HI are compared in Table I. Considering different cut-off points, the sensitivity and specificity of DIA were estimated. Using the Roc curve,[8] the best DIA sensitivity and specificity obtained in 1/800 dilution of sera was 94% and 85%, respectively (Table II).

Table I. Comparison of antibody titers obtained from HI and DIA.

HI \ DIA	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200
<1/8	2*	2	2	1					
1/16		1	3	6	1				
1/32			3	6	9	6			
1/64					2	9	10		
1/128							7	12	5
1/256							3	11	4

*The figures in the table demonstrate the number of positive sera in the specified dilution.

Table II. Sensitivity and specificity of DIA in different dilutions of sera as the immune titer.

DIA dilution	Sensitivity (%)	Specificity (%)
1/200	100	28
1/400	99	57
1/800	92	85
1/1600	80	100

Results obtained from whole blood were in agreement with those of serum samples.

DISCUSSION

The screening of rubella antibodies in sera is routinely done using HI test and/or ELISA.

We have applied a DIA test for detecting and measuring rubella anitbodies. In this test rubella antigen is dotted onto nitrocellulose paper, which is used as the antigen carrier in the DIA test.

In addition to qualitative assays, DIA can be used for detection of antibody in samples. Based on our experiments the titer of antibody equal to 1/8 in HI corresponds with that of 1/800 in DIA. Therefore, for quantitative assays sera can be diluted to 1/800 and tested with DIA. Appearance of visible color on the paper is taken as immunity of the individual to rubella.

DIA benefits simplicity, needs a tiny amount of the agent, can be applied for a large number of samples simultaneously,[9,10] and can determine the specific antibody content of the tested sera to different antigens (unpublished data). It does not require sophisticated equipment. The test is easy to perform, the antigen coated nitrocellulose filter can be stored at 4°C without any loss of activity for several weeks, and the possibility of using whole blood collected on filter paper makes this test suitable for large studies in unfavorable conditions. Our findings are in agreement with those of Condorelli and Ziegler.[11]

It is worth mentioning that purified rubella antigen should be used in this test to obtain good results. Crube antigen can not distinguish between virus and control coated papers in color exactly.

DIA can detect tiny amounts of antibodies against rubella virus proteins, while HI normally shows hemagglutinating antibodies, which are important protective antibodies and correspond with immunity. Because HI antibodies are not produced in a small percentage of people,[12] the positive results obtained with DIA in these people might be the false ones. By using subunit antigens or synthetic peptides one can solve this problem.[13]

Although the specificity of DIA used in this study was not very satisfactory (85%), it should be mentioned that the number of negative sera tested was too low (7 sera) and if we had had more negative samples, the specificity of DIA would have been better.

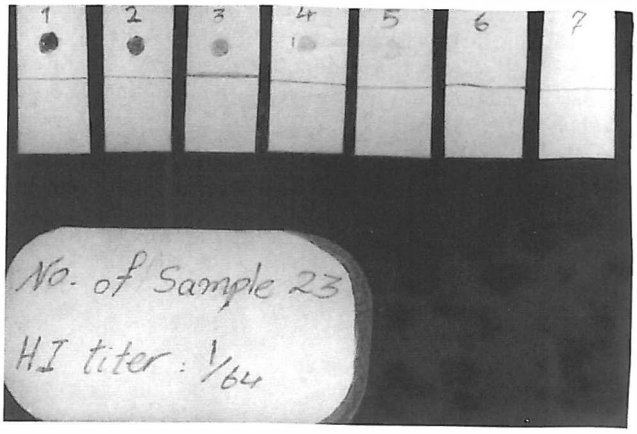

Fig. 1. Titration of serum antibodies against rubella virus by DIA. Dilutions of the serum are 1/400, 1/800, 1/1600, ... , 1/25600. The last dilution showing visible color was taken as the titer of serum antibody. Note that the upper row contains virus antigen and the lower one is the control.

ACKNOWLEDGEMENT

We would like to thank Dr. A. Shafii of the Razi Research Institute for his valuable help.

REFERENCES

1. Cutts FT, Robertson SE, Diaz-Ortega JL, Samuel R: Control of rubella and congenital rubella syndrome (CRS) in developing countries, part 1: burden of disease from CRS. Bulletin of WHO 75: 55, 1997.
2. Heberling RL, Kalter SS: Rapid dot-immunobinding assay on nitrocellulose for viral antibodies. J Clin Microbiol 23: 109, 1986.
3. Gravell M, Dorsett PH, Gutenson O, Ley AC: Detection of antibody to rubella virus by enzyme-linked immunosorbent assay. J Infect Dis 136: S300, 1977.
4. Tigssen P: Practice and theory of enzyme immunoassay. Ist ed., (8th print), Netherlands: Elsevier, p. 101, 1993.
5. Wilson MB, Nakane PK: Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In: Knapp W, Holubar K, Wick G, (eds.), Immunofluorescence and related staining techniques. Elsevier/North Holland Biomedical Press, p. 215, 1978.
6. Schmidt NJ, Lennette EH: Variables of the rubella hemagglutination-inhibition test system and their effect on antigen and antibody titers. Appl Microbiol 19: 491, 1970.
7. Aeppli RE, Bargetzi MJ, Binz H: Diagnostic screening of multiple antigen-antibody reactions in a new single assay on nitrocellulose: the line immunobinding assay. J Immunol Methods 120: 93, 1989.

8. Dawson-Saunders B, Trapp RC: Basic and clinical biostatistics. translated by Sarafraz A, Ghaffar-Zadegan K, lst ed.. (lst print), Mashhad University, p. 579, 1994.
9. Hawkes R, Niday E, Gordon J: A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem 119: 142, 1982.
10. Heberling RL, Kalter SS, Smith JS, Hildebrand DG: Serodiagnosis of rabies by dot immunobinding assay. J Clin Microbiol 25: 1262, 1987.
11. Condorelli F, Ziegler T: Dot immunobinding assay for simultaneous detection of specific immunoglobulin G antibodies to measles virus, mumps virus, and rubella virus. J Clin Microbiol 31: 719, 1993.
12. Wolisky JS: Rubella. In: Fields BN, Knipe DM, Howky PM (eds.), Fields Virology. Lippincott-Raven, p. 899, 1995.
13. Mitchell LA, Zhang T, Ho M: Characterization of rubella virus specific antibody responses by using a new synthetic peptide-based enzyme-linked immunosorbent assay: J Clin Microbiol 30: 1841, 1992.