

APPLICATION OF DOT IMMUNOASSAY (DIA) FOR DETECTION AND TITRATION OF MEASLES VIRUS ANTIBODIES

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ABSTRACT

Measles is one of the most contagious human diseases. Although mass vaccination programs have reduced the incidence of this disease, measles is still an important cause of morbidity and mortality among children in developing countries. Therefore the use of sensitive techniques to evaluate vaccine efficacy and level of immunity among members of susceptible communities is crucial. Serum neutralization test (SNT) and Dot immunoassay (DIA) are among the best methods utilized for evaluating measles virus antibodies. In this study, DIA was applied for detection and titration of measles virus antibodies. This test was developed for the first time in Iran in the Virology Department of the School of Medical Sciences, Tarbiat Modarres University.

Viral antigen was first prepared and titrated. Then human IgG was isolated by affinity chromatography. Anti-human immunoglobulin was prepared by immunizing rabbits with human IgG and was later conjugated with peroxidase. DIA was applied using these reagents.

The results indicated that the specificity and sensitivity of DIA in comparison with SNT was 96% and 89%, respectively. This study demonstrated that DIA is a rapid and simple test which can be applied for the detection of mass immunity against the measles virus.

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INTRODUCTION

Measles virus is a member of the morbillivirus genus and belong to the *Paramyxoviridae* family of viruses. Spherical virions consist of an envelope and helical nucleocapsid. The nucleocapsid contains single-strand RNA with negative polarity and codes for all of the viral structural and non-structural proteins. Hemagglutinin (H) and fusion (F) glycoproteins are embedded in the envelope. HA gly-

coprotein contains specific regions which bind receptors on the host cellular membrane. The fusion protein is responsible for the syncytial formation and hemolytic activity of the measles virus.^{1,2} Measles virus is sensitive to high temperature, and its infectivity can be neutralized after 30-60 min. at 56°C or 10 minutes at 60°C. The virus can be kept at -70°C in growth medium containing specific proteinaceous substances.

It can be cultured in many different cell lines and depending on the cell line, cytopathic effect (CPE) is usually observed as multinucleated giant cells or spindle-shaped cells.³

Infection occurs via the upper respiratory tract. The

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Measles Antibody Detection with Dot Immunoassay

virion multiplies in the local lymph nodes. Symptoms of disease appear as the virus spreads throughout the body by cells of the reticuloendothelial system. After the incubation period, there is an abrupt onset of illness with fever, sneezing, conjunctivitis, rhinitis and cough followed by the appearance of characteristic Koplik spots in the mouth and a maculopapular rash all over the body. Upon appearance of the rash, symptoms subside and ultimately, life-long immunity is achieved. Common complications include otitis media, complications of conjunctivitis such as keratitis, pneumonia and encephalitis.^{3,4}

After natural infection or vaccination, protecting antibodies against H and F proteins can be detected. These antibodies neutralize free virions circulating in the blood. They can pass through the placenta and confer immunity to measles in the first months of life.⁵ Complete eradication of the virus and recovery is achieved by cell-mediated immune mechanisms. However, detection of measles antibodies in blood is usually the method of choice for determining the presence of immunity against the disease.

MATERIAL AND METHODS

Antigen

Antigen was prepared by inoculating a vaccine strain (Edmonston B strain) of measles virus into HeLa cells. In order to increase virus titer, repeated passage of positive cultures was performed and infected cells were subsequently transferred to a large tissue culture flask (Roux Flask). When CPE appeared, infected cells were frozen and thawed once and the contents were centrifuged. The virus-containing supernatant was collected into small vials and kept at -70°C until use. Control antigen was also prepared in parallel from uninfected cultures. Virus titer was detected by determining the Tissue Culture Infective Dose₅₀ (TCID₅₀). This method measures the titer of virus which can infect 50% of inoculated cells.⁷ Briefly, different dilutions (semi-logarithmic) of virus preparation in tissue culture medium were inoculated into tissue culture tubes (for each virus dilution, four tubes were considered). Only tissue culture medium was inoculated into control tubes. Tubes were incubated at 37°C

and checked daily for presence of CPE. Virus titer was calculated to be 10^{5.4} TCID₅₀ in 0.1 mL of culture supernatant.

Anti-human conjugated antibody

Human immunoglobulin was isolated by affinity chromatography,⁸ and the purity of the preparation was determined by cellulose acetate electrophoresis and its concentration was calculated. Rabbits were injected intramuscularly with various doses of human immunoglobulins. In the first injection, complete Freund's adjuvant and in the subsequent injections incomplete Freund's adjuvant was used along with viral antigen.⁹ The second injection was followed 1 month after the first injection and the boosters were given at 15 day intervals. Blood was collected before each injection and the titer of anti-human immunoglobulin produced in rabbits was measured by ELISA. Antibody production increased until 75 days after the first injection and did not change significantly afterwards. At this time, final blood collection was done and antibodies were separated from serum by affinity chromatography. Cellulose acetate electrophoresis was used to demonstrate the degree of purity of antibody and double diffusion in gel was applied to characterize the type of antibody produced.^{10,11}

Antihuman antibody was conjugated with peroxidase (Sigma) by the help of sodium periodate (Sigma), as described by Wilson and Nakane.⁹ This procedure yielded various mixtures from which conjugate had to be purified by passage through Sephadex G-200 column.¹² The conjugate was later concentrated by using polyethylene glycol (PEG-8000) and its titer was determined by ELISA.

Specimens

111 cord blood specimens were collected. Sera were separated and kept at -20°C until use.

Serum neutralization test (SNT)

In this assay, antibodies present in test serum are reacted with viral antigens to neutralize their infectivity. This would prevent the appearance of CPE upon inoculation of viruses into cell cultures. HeLa cells were grown into 96-well microtiter plates, sera were first inactivated at 56°C

Table I. Comparison of measles virus antibody titer in 45 serum specimens by SNT and DIA.

| SNT \ DIA | DIA | | | | | | | | |
|-----------|-------|-------|-------|-------|--------|--------|--------|---------|---------|
| | 1/100 | 1/200 | 1/400 | 1/800 | 1/1600 | 1/2300 | 1/6300 | 1/12800 | 1/25600 |
| 1:2 | 1 | | | | 1 | | | | |
| 1:4 | | 2 | 2 | | | | | | |
| 1:8 | | | 2 | 2 | 1 | | | | |
| 1:16 | 2 | | | 1 | 3 | 2 | | | |
| 1:32 | 1 | | | 2 | 2 | 3 | | | |
| 1:64 | 1 | | | | 1 | 1 | 3 | | |
| 1:128 | | | | | | | 2 | 2 | 3 |
| 1:256 | | | | | | | 1 | 2 | 2 |

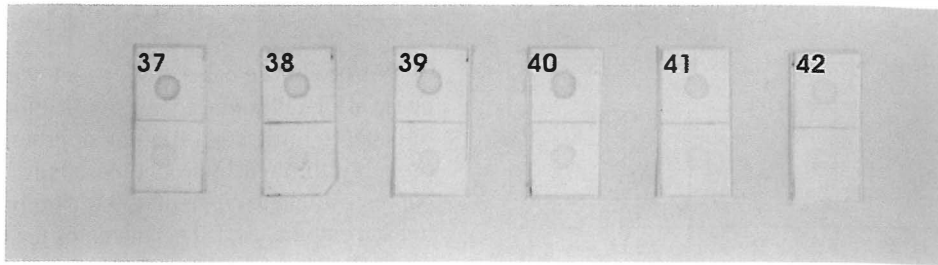


Fig. 1. Detection of measles virus antibody titer in a representative serum specimen by DIA. On each piece, viral antigens are dotted on the top and the cell culture control is dotted on the bottom.

for 30 min. to inactivate non-specific inhibitors. Multiple 1:2 dilutions of specimens were prepared (1/2 to 1/256). Each serum dilution was mixed with the same volume of 100 TCID₅₀ viral antigen preparations and kept at 37°C for 1 hr for neutralization to occur. After the incubation period, 50µL of each serum-virus mixture was inoculated into 2 wells of microtiter plates. 4 wells were considered as negative controls consisting only of tissue culture medium and 4 wells were considered as positive control consisting of viral antigens without test serum. Microplates were kept at 37°C and checked daily for the appearance of CPE by an inverted microscope. The endpoint was considered as the period necessary for the appearance of viral CPE in positive control wells while negative control wells were intact. The highest dilution of serum which could prevent CPE in cells was considered as the serum titer.¹³

Dot immunoassay (DIA)

DIA is a modified EIA in which nitrocellulose paper is used in place of microtiter plates as a solid phase. The test procedure was performed as follows:

1. Preparation of solid phase: First, 1×1 cm squares were drawn on nitrocellulose (NC) paper and cut into pieces. They were then washed in double-distilled water for 5 minutes and dried on several filter papers at RT.
2. Dotting: 3 µL of prepared viral antigen was dotted on the center of each square. After drying, NC paper was incubated for 5-20 minutes in the refrigerator.
3. Blocking: The NC sheet was later submerged into 0.5% gelatin solution to block unreacted protein-binding sites.
4. Washing: The NC paper was then washed 3 times, each time for 5 minutes, with PBS-Tween (PBS containing 0.05% Tween -20).
5. Primary incubation: Serum was diluted in PBS-Tween and incubated with prepared NC paper for 2 hrs at RT on a shaker.
6. Washing was performed as described in step 4.
7. Secondary incubation: The NC papers were then submerged in a pre-determined dilution of anti-human immunoglobulin conjugated with peroxidase and put on a shaker for 90 minutes at RT.

8. Washing was again performed as described in step 4.

9. Development: The substrate was prepared in the dark and allowed to react with NC paper for 1-2 minutes until a distinguishable color development on positive control was observed. Diaminobenzidine (DAB) was used as a substrate for peroxidase in this study.

10. The papers were then washed in tap water for several minutes and allowed to dry on filter paper at RT in the dark.^{6,14}

By following the described method, anti-measles antibody titers were determined in serum specimens and the results were compared with those of SNT as the reference method.

The use of whole blood in DIA

Whole blood may also be used in DIA. Blood is dried on filter paper and used in place of serum. In this study, 10 µL of whole blood specimens collected from volunteers were placed on small pieces of Whatman Filter Paper and allowed to dry. The papers were then put in plastic bags and kept at 4°C until use. Before DIA, each piece of blood-containing paper was kept in 500 µL of buffer. The blood solution in buffer was considered as 1: 100, and serum dilution and subsequent dilutions were made afterwards. Comparison of results obtained with whole blood and serum demonstrated that the results were comparable.¹⁶

RESULTS

Checker-board titration was performed in order to determine the optimum virus concentration needed to be put on solid phase in DIA. Different dilutions of viral antigen and control (cellular antigen alone) were prepared, dotted on solid phase and incubated with a positive control serum. The results of DIA indicated that the use of undiluted antigen or antigen at 1:2 dilution resulted in a color intensity which would give better results with various dilutions of serum.

To measure measles antibodies by DIA, the same specimens used in SN were examined. Figure 1 shows results of DIA for a representative serum specimen. Serial dilutions were prepared from 1: 100, left to right. This specimen has

Measles Antibody Detection with Dot Immunoassay

Table II. The comparison of positive and negative sera by DIA and SNT.

| DIA \ SNT | SNT | | SUM |
|-----------|-----|---|-----|
| | + | - | |
| + | 98 | 1 | 99 |
| - | 4 | 8 | 12 |
| SUM | 102 | 9 | 111 |

reacted non-specifically with cell culture control. However, the color change is much lower than that of viral antigens. At one serum dilution, the color intensity is the same for antigen and control. Serum titer was considered as one dilution above this titer. Thus, the measles antibody titer in this serum is 1: 1600. 45 sera were evaluated by DIA as described and results were also compared with those of SNT (Table I). Table I demonstrates a direct relationship between measles virus antibody titers determined by DIA and SNT. Antibody titers by DIA were shown to be consistently more than those of SNT. The results indicated that the immune titer was 1: 4 by SNT,¹⁷ which was equivalent to 1: 200 to 1: 400 by DIA. Four positive serum specimens by SNT were detected as negative by DIA (false negative) and one negative serum specimen in SNT was detected as positive by DIA (false positive).

Based on the above findings, the sensitivity and specificity of DIA was determined by using 111 sera at 1: 200 dilution. Figure 2 demonstrates the results of 4 sera tested by DIA for the presence of measles virus antibodies at a dilution of 1: 200. A specimen was considered as positive when the color change it produced was more than that observed for negative control serum and similar to or less than that observed for positive control serum. The sensitivity and specificity of DIA as compared with SNT (as a reference method) was considered to be 96% and 89%, respectively (Table II).

DISCUSSION

Nitrocellulose paper (NC) which was used as the solid phase in DIA is produced by esterification of thin-cut pieces of paper in nitric acid. Binding of proteins to this paper is probably through hydrophobic bonds. For this purpose, papers with an average of 450 to 500 million 0.45 μ holes in each cm² are used. This increases the binding surface of nitrocellulose and enhances its absorption capacity in comparison with polystyrene microtiter plates. Thus, very little amounts of antigen are needed to perform DIA. The binding of antigens to NC membrane is very stable and antigen-coated NC may be kept at 4°C for long periods of time.^{18,19} Another advantage of DIA is the possibility of evaluating the presence of antibodies against several different antigens which are dotted on one single NC strip.²⁰ The authors have used measles and rubella antigens on one strip and obtained good results (data not shown).

Some investigators believe that the sensitivity of DIA is equal to or more than that of SN or hemagglutination inhibition.¹⁵ Others have shown that the sensitivity of DIA is even more than that of ELISA. This high sensitivity rate may cause false positive results which is specifically important in evaluating post-vaccination immunity. To avoid this, it is possible to use higher serum dilutions or to reduce the incubation time needed for interaction of serum and viral antigen.²¹

In this study, SNT was applied to measure the sensitivity and specificity of DIA. Our results indicated that DIA has comparable specificity and sensitivity. The specificity can be measured more precisely if more seronegative specimens can be collected. In order to use DIA to evaluate measles virus antibodies in large communities, certain modifications are recommended. For example, antigens have to be purified and improvements may be seen by changing the incubation period. These could lead to increased specificity of DIA.

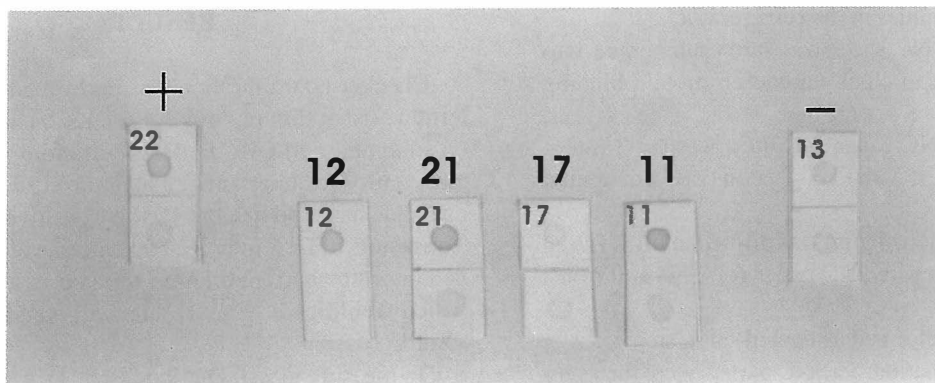


Fig. 2. Detection of immune sera in several specimens tested. On each piece, viral antigens are dotted on the top and the cell culture control is dotted on the bottom. Numbers are related to representative sera tested.

In summary, DIA is a simple and rapid technique which does not require specialized instruments. It can be used to evaluate the level of immunity against measles virus antibodies in place of more complicated methods.

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