Development of an Enzyme-Linked Immunosorbent Assay for Immunoglobulin M Antibodies against Measles Virus

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Measles is a highly contagious respiratory virus infection, with typical clinical symptoms including maculopapular rash, fever, cough, coryza, and conjunctivitis. Despite implementation of widespread vaccination programs throughout the world, the rates of global morbidity and mortality are still considerable. This study was performed to design a reliable indirect enzyme-linked immunosorbent assay (ELISA) to measure measles-specific immunoglobulin M (IgM). First, human IgM was purified, and then an anti-IgM antibody was produced in rabbits and purified in a multistep process. The rabbit IgG against human IgM was conjugated to peroxidase. Measles virus-infected Vero cells produced viral antigen. One hundred serum samples from infants of 9 to 18 months of age, mostly vaccinated, were evaluated for determining the presence of specific IgM antibodies against measles virus. The samples were also evaluated for neutralizing antibodies against measles virus by a microneutralization test (MNT). By comparing the results of the ELISA with those of MNT, it was demonstrated that ELISA had a sensitivity and specificity of 100 and 92%, respectively. On the other hand, when the results obtained by our ELISA system were compared with those of an imported measles virus IgM ELISA kit (EIAgens; Adaltis Italia SPrA, Bologna, Italy), a high level of agreement was shown (k = 0.926).

MATERIALS AND METHODS

Human sera. One hundred serum samples (51 from males and 49 from females) were collected during the years 1999 and 2000 from healthy Iranian children 9 to 18 months old living in rural areas of north Iran. Most of the subjects had received at least one dose of measles A1K-C vaccine (RAZI Serum and Vaccine Institute, Tehran, Iran), which is given as a monovalent measles vaccine in two doses to 9- and 15-month-old children. Twenty-eight percent of the serum samples were taken from 16- to 18-month-old children who had received two doses of measles vaccine. Blood was collected from children 5 to 7 days after measles vaccination. The sera were stored at −20°C until use.

Cells and virus. Monolayer cultures of HeLa and Vero cell lines were grown in Eagle minimal essential medium (EMEM; Sigma, St. Louis, Mo.) containing 10% or 2% fetal bovine serum (Gibco) for growth and maintenance, respectively, and antibiotics including 100 U of penicillin, 100 μg of streptomycin, 50 μg of kanamycin, and 1 μg of amphotericin B (Fungizone; Gibco)/ml. The Edmonston B strain of measles virus was obtained from the Razi Serum and Vaccine Production Institute. Serial passages of virus were performed to achieve a high-titer virus.

Microneutralization assay. HeLa cells growing as a monolayer in 96-well tissue culture plates (Falcon) were inoculated with measles virus-serum mixtures containing increasing dilutions of each heat-inactivated serum sample (50 μl of virus at 1/2 to 1/256 dilutions) and a constant amount of virus (50 μl of virus at 100.50% tissue culture infective doses). Before inoculation, the virus-serum mixtures were incubated for 1 h at 37°C. Fifty microliters of each serum-virus mixture was added to wells of microplates, and, after an adsorption period of 1 h at 37°C, 200 μl of EMEM was added. Each serum dilution was examined in duplicate. For each plate, a cellular control consisting only of EMEM and a viral control containing 50 μl of virus suspension were included. Known positive- and negative-control sera were run accordingly. The tests were read when in virus control wells more than 75% of cells showed measles cytopathic effect, which usually took 4 to 5 days (11, 18).

Purification of human IgM. An IgM-rich fraction was prepared with polyethylene glycol 6000 (PEG-6000). Our initial experiments indicated that precipitation of IgM could best occur in an 8% PEG solution (Sigma), while the smallest amount of IgM was precipitated in a 3% PEG solution. Therefore, the following two-step procedure was followed in order to eliminate unwanted proteins and
prepare an IgM-rich fraction. First, serum was diluted 2.5-fold in 20 mM potassium phosphate buffer, pH 7.4, and was placed in a magnetic stirrer. Then 40% PEG solution was added dropwise to achieve a final concentration of 3% PEG in the mixture. After 1/2 h of gentle stirring, the mixture was centrifuged at 3,000 × g for 15 min and the supernatant was collected. In the second step, a 40% PEG solution was added as in step 1 to reach a final concentration of 8%. The solution was incubated overnight in a refrigerator, and IgM-rich precipitants were separated by centrifugation and kept at 4°C.

**Gel filtration.** Gel filtration was done on a Sepharose CL-6B column (Pharmacia, Piscataway, N.J.). After the column was prepared and washed with potassium phosphate buffer (20 mM, pH 7.4, containing 0.5 M sodium chloride; Sigma), the IgM-rich precipitant was dissolved in buffer and loaded onto the column. Absorbance of collected fractions was measured at 280 nm. IgM-containing fractions were specified by the dual-diffusion (Ouchterlony) method. To further purify IgM, IgM-containing fractions were precipitated with PEG and gel filtration chromatography was repeated.

**SDS-PAGE.** The degree of purity of collected Ig fractions was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, it was done as described by Laemmli (10) in 10% acrylamide gels on a vertical slab electrophoresis unit. Protein samples dissolved in sample buffer were boiled for 5 min before electrophoresis at a constant voltage of 150 V. The gel was stained with brilliant blue R-250 (Sigma).

**Immunoprecipitation with IgM.** Two hundred fifty microliters of prepared IgM (1 mg/ml) in phosphate-buffered saline (PBS; pH 7.4) was emulsified with 0.25 ml of Freund’s complete adjuvant (Sigma) and inoculated intramuscularly into 4- to 6-month-old New Zealand White rabbits (Razi Serum and Vaccine Production Institute). The second and third inoculations were performed on days 21 and 35 with Freund’s incomplete adjuvant (Sigma), and the fourth inoculation was on day 45 without any adjuvant. The degree of immunization in rabbits was evaluated by the double-radial-diffusion method. Protein concentration was quantified by a Coomassie dye binding assay, as described by Bradford (2), with bovine serum albumin (BSA) as the standard.

**Conjugation of rabbit anti-human IgM antibody.** The gamma globulin fractions of immunized rabbit sera were precipitated by half saturation of the serum with ammonium sulfate in 0.1 M phosphate buffer, pH 7.2 to 7.4. Anion-exchange chromatography was done on a DEAE-Sepharose CL-6B column (Pharmacia). First, gamma globulin sediment was dialyzed against sodium phosphate buffer at 4°C for 24 h. After centrifugation of the dialyzed solution, the supernatant was adjusted to a protein concentration of 10 mg/ml and passed through the column. IgG-rich fractions were collected and precipitated by ammonium sulfate. Unwanted antibodies were adsorbed by affinity chromatography using IgA and IgG as ligands.

**Preparation of antigen.** Monolayers of Vero cells were inoculated with Edmonston B measles virus. Upon observation of 75 to 100% cytopathic effect, the culture medium was discarded and infected cells were washed with cold PBS. The cell suspension was then centrifuged at 700 × g for 20 min. The pellet in 1/500 of the initial volume was freeze-thawed three times at −70°C. Cells were removed by centrifugation at 800 × g. Virus-containing supernatants were collected in small plastic vials and kept at −70°C.

**Indirect ELISA.** Checkboard titration was used to determine the optimal concentration of antigen, serum, and conjugate. One hundred microliters of prepared viral antigen, which was diluted 1:100 in PBS, was added to each well of a microplate (Grainer) and incubated at 37°C for 24 h. After the wells were washed with PBS-Tween (0.05% Tween 20) three times followed by tapping, 200 μl of blocking solution (PBS–0.5% Tween 20) was added, followed by incubation at 37°C for 1 h. After a washing step, 100 μl of each serum specimen diluted 1:100 in diluting buffer containing PBS, 1% BSA, and 0.05% EDTA was added to specified wells. The microplates were incubated at 37°C for 1 h. The washing step was repeated, 100 μl of prepared horseradish peroxidase-conjugated antibody against human IgM at a 1:2,500 dilution was added to each well, and incubation as described above was repeated. Upon a final wash, 100 μl of OPD (O-phenylenediamine) substrate (0.05% OPD in phosphate-citrate buffer containing 0.01% H2O2) was added for 30 min in the dark. For stopping the reaction 50 μl of 5% sulfuric acid (Sigma) was added to each well, and absorbance was read at 492 nm by an ELISA reader (laboratory system).

**Statistical analysis.** The results obtained with the constructed ELISA were compared with those of a microneutralization assay, as the “gold standard.” Based on the results showing different amounts of sensitivity and specificity for various cut points and related receiver operating characteristic curves, a cut point with optimum sensitivity and specificity was finally chosen for in-house ELISA. In addition, the results of ELISA were compared with those of a commercial ELISA kit (EIAGen), a cross-tabulation table was made, and agreement between the two tests was assessed.

**RESULTS**

**Microneutralization test.** The results of the microneutralization test demonstrated that about 88% of the children were immune. Previous studies had shown that titers of 1:8 or higher in this test can be considered positive (15).

**Purification of human IgM.** Figure 1 demonstrates the result of gel filtration. Double diffusion indicated that the second peak contains IgM.

**Purification of rabbit anti-human IgM.** Figure 2 shows the results of SDS-PAGE for determining the degree of purity of IgG, which was purified by ion-exchange chromatography followed by affinity chromatography. The first lane is the molecular weight marker, and, in the second lane, two bands of 25 to 26 kDa and 49 to 50 kDa, which are IgG light and heavy chains, respectively, can be observed.

**Indirect ELISA.** Based on results of statistical analysis of data, an optical density (OD) of 0.350 was chosen as the cutoff for the developed ELISA. The sensitivity and specificity of this test were calculated to be 100 and 92%, respectively. Based on the results of ELISA, 91% of 9- to 18-month-old children had the measles virus IgM-specific antibody. This was an acceptable result considering the fact that most of these children

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**FIG. 1.** Gel filtration of the human IgM-rich fraction in a Sepharose CL-6B column.
were vaccinated. Statistical analysis demonstrated that there are significant correlation between antibody titers in the microneutralization test and OD values obtained with the developed ELISA kit ($R = 0.949, P < 0.001$; Fig. 3). Furthermore, based on the agreement test, a high level of agreement between the in-house ELISA and the imported ELISA kit was observed ($k = 0.926, P < 0.0001$).

**DISCUSSION**

The ultimate goal of a measles control program is to stop the indigenous circulation of measles virus. Monitoring the success of such programs requires a sensitive surveillance system. Measles virus-specific IgM serology is the standard test for the rapid laboratory diagnosis of measles (7, 13). The hemagglutination inhibition test is commonly used to measure measles antibodies for the determination of immune status. However, this test has been shown to be less sensitive than the plaque neutralization test and ELISA (15). In this study, the results of the microneutralization test showed that 88% of vaccinated children had measles antibody titers of 1:8 and higher. One of the important difficulties in measuring IgM by ELISA is the presence of RF, which causes false-positive results (6).

To purify rabbit anti-human IgM-specific IgG, ion-exchange chromatography was used. One of the important points to be considered while using affinity chromatography is the choice of an appropriate ligand to which unwanted antibodies could be absorbed. Since our preparation contained both anti-human IgA and IgG (mostly anti-Fab fraction), we excluded these antibodies from our preparation by passing it through an affinity column containing human IgA and IgG as ligands. Double diffusion and SDS-PAGE indicated that IgG was highly purified and IgM specific.

Since a measles outbreak exists in a community whenever a single case of measles is confirmed, laboratory confirmation should be available through small diagnostic laboratories. A recent study in Egypt which compared reverse transcription-PCR, virus isolation, and IgM ELISA concluded that serological methods are sufficient for a laboratory diagnosis of measles virus infection; measurement of measles virus-specific IgM alone could diagnose 95% of the patients (5).

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**REFERENCES**


