ARTIGO

# AVALIAÇÃO DA IMUNIDADE HUMORAL ESPECÍFICA PARA O VÍRUS DO SARAMPO, COMPARANDO QUATRO TESTES DE DIAGNÓSTICO

## *EVALUATION SPECIFIC HUMORAL IMMUNITY OF THE MEASLES VIRUS COMPARING FOUR DIAGNOSTIC TESTS*

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### RESUMO

*Amostras de soro de casos suspeitos de sarampo, recebidas após uma campanha de vacinação em massa, apresentaram 20,8% de seus resultados negativos para IgG de sarampo, através de dois ensaios imunoenzimáticos (EIE) (um comercial/Behring e outro produzido pelo CDC/ Atlanta). Estas amostras negativas foram testadas por inibição da hemaglutinação (HI) e neutralização por redução de placa (PRNT) e os resultados foram estratificados conforme faixa etária e estado vacinal. Os EIEs (Behring e CDC) e HI apresentaram 100% de especificidade e 0,90; 0,89 e 0,93 de sensibilidade respectivamente, quando comparados com PRNT. Crianças acima de 12 meses de idade apresentaram taxas maiores de resultados falso negativos por EIEs. Crianças verdadeiramente soronegativas foram predominantemente abaixo de 9 meses de idade. Os resultados do PRNT foram consistentes com o estado vacinal em 81% das crianças cujos dados estavam disponíveis. Recomenda-se a utilização do PRNT como um teste complementar ao EIE, em avaliações de campanhas de vacinação para avaliação de taxas de soroconversão e para confirmação de resultados negativos por EIE em amostras clínicas.*


***Palavras chave***: *Sarampo; Teste de Neutralização por Redução de Placa; Ensaio Imunoenzimático; Teste Inibição da Hemaglutinação.*


### ABSTRACT

*Sera samples from suspected measles cases received after a mass vaccination campaign, showed 20.8% IgG negative for measles from two enzyme immune assays (EIA) (one commercial/ Behring and another "home made" by CDC/Atlanta). These negative samples were tested by Hemagglutination Inhibition (HI) and Plaque Reduction Neutralization Test (PRNT) and the results were stratified according to age groups and vaccination status. The EIAs (Behring and CDC) and HI presented 100% specificity and 0.90, 0.89, 0.93 sensibility, respectively, when compared with PRNT. Children of 12 months or over had higher rates of false negative results in the EIAs. The true seronegative children were predominantly under 9 months of age. PRNT results were consistent with vaccination status in 81% of the children for whom data were available. PRNT should be used as a complementary test to EIA, in vaccination campaigns for the evaluation of seroconversion rates and for confirmation of negative EIA results in clinical samples.*


***Keywords***: *Measles; Plaque Reduction Neutralization Test; Enzyme-immune assay; Hemagglutination Inhibition Test.*

(1) Trabalho realizado no Instituto Oswaldo Cruz, durante a realização da tese de mestrado do Instituto de Microbiologia Professor Paulo de Góes/UFRJ.

## INTRODUCTION

The methods used in serological diagnosis are important in confirming the diagnosis of virus diseases and in determining incidence patterns (Black, 1989b). Several techniques have been developed for the demonstration of measles virus-specific immunoglobulins in serum. Traditionally, the most commonly used quantitative assays are the neutralization test (NT) and the hemagglutination inhibition test (HI). More recently, qualitative immunoenzymatic assays (EIAs) (World Health Organization, 1994; Bellini & Rota, 1995) have been introduced and these tests are now most commonly used for diagnostic purposes. The NT is regarded as the most effective and reliable test for the quantitative evaluation of immunity to measles virus (de Sousa *et al.*, 1991; Nates et al., 1994). HI is also useful for measles serology, giving results that correlate well with those of the NT. This test however, is less sensitive than NT and presents limitations, requiring fresh monkey erythrocytes and pretreatment of serum samples (Black, 1989a and b; Njayou & Balla, 1990; Diaz-Ortega *et al*, 1994). The qualitative EIAs are available as commercial and "home-made" kits and are widely used because of their rapidity, sensitivity and inherent practicability (Weigle et al, 1984; de Sousa *et al.*, 1991; Erdman *et al*, 1991; Ratman et al, 1995).

Measles outbreaks continue to occur even after a significant increase in vaccine coverage (de Quadros *et al.*, 1996). In Brazil, measles continued to represent an important cause of morbidity and mortality, even after the establishment of the National Immunization Program (PNI) in 1973, when coverage was low and heterogeneous (Grupo Técnico do Sarampo, 1994). In 1992, the Health Ministry carried out a National Vaccination Campaign against measles virus, covering 48 million children from 9 months to 14 years of age, achieving a coverage rate superior to 95%. After the mass vaccination campaign, the surveillance of measles was conducted using laboratory tests for serum IgG and IgM, performed on all suspected cases. During 1992 - 1993 the measles National Reference Center in Rio de Janeiro, Brazil, received 1124 serum samples, of which 234 (20,8%) had no measles-specific IgG detectable by EIA. This proportion was considered too high for a post-vaccination period, and thus, the samples were submitted to two additional laboratory tests, namely, haemagglutination inhibition (HI), and plaque reduction neutralization (PRNT) tests. Comparison of the results was performed using PRNT as a reference. The results were stratified by age group and vaccination status, to determine the effect of these variables.

## MATERIALS AND METHODS

**Serum Specimens**: Were collected from 1124 suspected measles cases in the states of Rio de Janeiro and Espírito Santo. The health care units sent samples to the National Measles Reference Center (IOC/FIOCRUZ) in 1992 and 1993. When the serum samples were collected, duplicate records were prepared for each case including the patient's name, date on which the exanthem started, date of sample collection and date of the last dose of the vaccine. One copy of this record accompanied the sample to the laboratory while the second was sent to the Secretary of Health. In many cases, these records were incomplete and the laboratory, rather than the health care unit, notified the Secretary of Health. Accordingly, complete data was not available for all of the cases analysed.

Of the 1124 samples received, 234 (20.8%) were negative by EIA and 149 of these, of which sufficient sample remained, were re-tested by four test methods in parallel with 147 of the EIA positive samples. The four test methods were carried out as described below.

**CDC-EIA**: (Erdman *et al.*, 1991; Hummel et al., 1992). Briefly, this microplate test employs the measles nucleoprotein expressed in baculovirus as capture antigen and the non-infected host cells, Spodoptera frugiperda (Sf9), as the control. Sera are diluted 1:100 in PBS pH7.2 containing 0.5%w/vgelatin, 0.15%Tween20, 4% normal goat serum and 4% uninfected Spodoptera frugiperda (Sf9) cell lysate. After reaction with the capture antigen, bound antibody is detected using an anti-human IgG antibody conjugated to peroxidase (Kirkegaard & Perry Laboratories, Inc.) and bound enzyme detected using tetramethyl benzidine as substrate.

**Commercial-EIA**: The Enzygnost Measles EIA (Behringwerke AG Diagnostica) antibody detection test was used exactly as specified in the protocol for the detection of measles specific IgG. This test employs whole virus as the capture antigen in microplates and non-infected cells as control. Sera were prediluted in sample buffer and applied to the microplate. Anti-human IgG conjugated to peroxidase was then added and tetramethyl benzidine was used as substrate.

**Hemagglutination Inhibition Test**: This test was carried out according to the protocol of Gershon & Krugman (1979). Serum samples were prepared by inactivation at 56°C for 30 minutes followed by adsorption with washed monkey erythrocytes and treatment with kaolin. Phosphate buffered saline (pH 7.2) was used as diluent throughout and all reagents in microplates were used in 50ul volumes. Serial twofold

dilutions of the samples were prepared in U well microplates and 4 units of measles haemagglutinin added to all of the wells. Negative controls without haemagglutinin and positive controls without antibody were included in each test. Monkey erythrocytes at 0.5% v/v were then added and the plates incubated at 37°C for 1 hour. The serum titer was determined as the reciprocal of the highest serum dilution giving complete inhibition of haemagglutination. Samples with titers of less than four were considered negative.

**Plaque Reduction Neutralization Test**: This test was carried out according to Whitle *et al.* (1984). Throughout this test, medium 199 supplemented with 5% v/v fetal bovine serum and 40ug/ml of gentamicin was used as diluent. After inactivation at 56° C, serial twofold dilutions of test sera and a standard preparation (1/10 to 1/1280) were prepared in 50ul volumes in cell culture microplates. Schwarz measles virus was then diluted to contain 30 pfu per 50ul and added to all of the wells. After incubation at 37°C for one hour, Vero cells were added (8 x $10^4$ per 50ul) to all of the wells. Vero cells were allowed to adsorb to the wells for three hours at 37°C following which the liquid medium was discarded. Medium containing 1% w/v carboxymethylcellulose was then added at 100ul per well and the plates re-incubated at 37°C for 7days. Cells were then fixed with formalin, washed and stained with crystal violet. Measles plaques were counted in all wells and the dilution of each sample giving a 50% reduction in plaque numbers was calculated and converted to International Units (IU) based on the known potency of the standard antibody preparation. Samples with titers greater than 120 mUI/ml were considered positives.

**Data Analysis**: The test results were compared so as to determine (1) proportional agreement between EIAs, (2) EIAs' sensitivity and specificity estimates using results from PRNT as reference (Fleiss,1982). Those probabilities were calculated for the whole batch on the basis of results obtained in sub-samples according to the scheme below. Ninety five percent confidence intervals were constructed for the estimates (Fletcher*et al.*, 1996).

The calculation was done considering the 1124 serum samples sent to the laboratory during the years 1992/93. Initially, these samples were tested by one of the immunoenzymatic assays, the CDC-EIA or the commercial-EIA, resulting in 234 negative samples and 890 positive ones. From these negative samples, 149 could be tested by PRNT and HI test, resulting in positive and/or negative samples by one of them. From the 890 positives, 147 were tested by the PRNT and HI, and confirmed to be positive. The sensitivity and specificity of immunoenzymatic assays were derived from the results obtained, adjusting fordiferences in the proportions of specimens retested in each original group (EIA positive and EIA negative), according to the scheme below.

## RESULTS

CDC-EIA and commercial-EIA results agreed in 82% of the specimens originally seronegative (Table 1).

Disagreement was asymetrical: from 147 specimens negative to CDC-EIA, 18 (12.1%) had positive and 7 (4.8%) had equivocal results to commercial-EIA.

Sera originally positive to CDC-EIA and the commercial-EIA were all positive by HI and PRNT. However, the HI and PRNT tests were positive in a substantial proportion (20.4% and 48.9%, respectively) of specimens originally negative to CDC-EIA (Table 2).

These apparent false negatives appeared to be more frequent among children of more than 1 year of age compared to those under 1 year (37.2% and 4.1%, respectively) according to HI test, and 62.8% vs. 24.5%, according to PRNT. Caution in the interpretation of age differences is necessary however, since data were available for only 60% of the individuals.

Results with commercial-EIA were somewhat closer to PRNT and HI than those of CDC-EIA (Table 2). Again, false-negatives were more frequent among children with 1 or more years of age compared to those under 1 year: 19.2%nvs. 2.0% according to HI test, and 53.8% vs. 24.5%, according to PRNT.

The PRNT detects antibody at concentrations of 50 mIU/ml and considering this level, only 4 samples were negative by PRNT and the difference between this test and the other three tests (HI and the two EIAs,) was large. Antibody concentrations of >120 mIU/ml are reported to be required for protection from classic measles illness (Chen et al; 1990) and considering this value there were 74 negative samples by PRNT reducing the difference between PRNT and the other three tests. PRNT results were consistent with vaccination status in 81% of the 73 children for whom data were available. Many of the negative PRNT samples were from unvaccinated children under 9 months of age.

EIA sensitivity estimates, adjusting for proportions tested in each original group are presented in table 3.

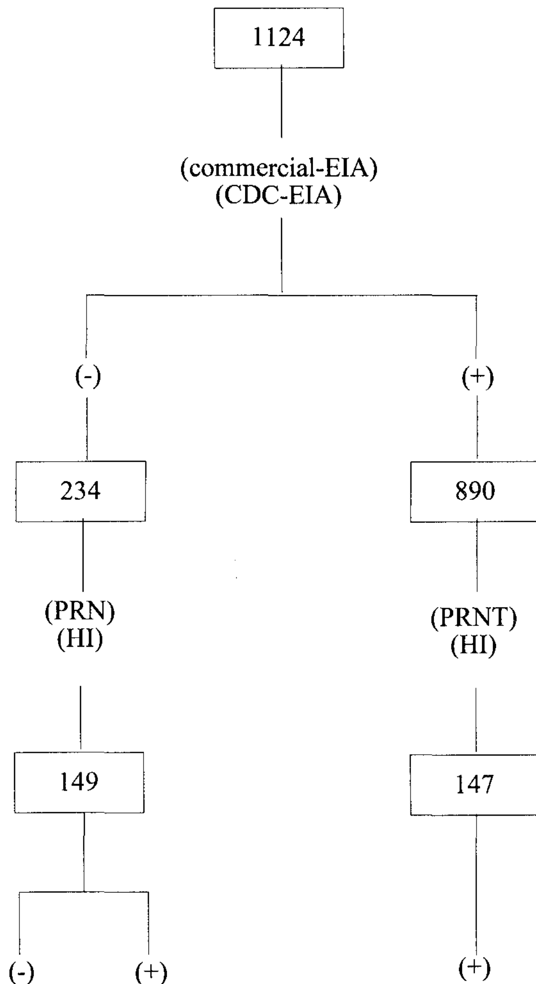


**Figure 1.** Sampling scheme

**Table 1.** Comparison between CDC-EIA and commercial-EIA in 149 sera originated from suspected measles cases.

| **Commercial-EIA** | | | | |
|---|---|---|---|---|
| | Positive | Negative | Equivocal | Total |
| CDC-EIA Positive | 0 | 1 | 1 | 2 |
| Negative | 18 | 122 | 7 | 147 |
| Total | 18 | 123 | 8 | 149 |

Those estimates take into account the performance of the EIAs shown in table 2, and sampling scheme in figure 1. They assume that sera known to be positive or negative to PRNT, were submitted to EIA's. The proportion of PRNT- positive specimens found to be positive to EIA was called sensitivity, whereas the proportion of PRNT-negative specimens found to be negative to EIA was called specificity. Specificity turned out to be 100% for both EIAs, meaning that no false-positive results were found.

## DISCUSSION

The immunoenzymatic assays (EIAs) are available as commercial kits or the technology is standardized in research laboratories. They can use the whole virus as the capture antigen in which case antibodies to all of the surface proteins of the virus are detected (Rossier *et al*, 1991; Chui *et al*, 1991; Hummel *et al.*, 1992; Hesketh *et al.*, 1997). Where recombinant proteins are used, such as the nucleoprotein in the CDC ELISA, only antibodies to the specific protein are detected. Comparing the results obtained with the commercial-EIA from Behring and the EIA standardized by CDC (CDC-EIA), we verified that the commercial-EIA presented the same specificity as the CDC-EIA, with slightly higher sensitivity. CDC-EIA uses SF9 cells in the adsorption of the serum and as cell control for capture. We observed that when using certain lots of SF9, specificity is decreased, with high values for optical density in the cell control that made interpretation of the results difficult. Hummel *et al.* (1992), compared the test of CDC-EIA with a commercial-EIA (Measelisa II. Whittaker Bioproducts, Inc. Walkersville, Md.), and a neutralization test. This study demonstrated that the CDC test showed better correlation with neutralizing antibodies, than that observed using the commercial-EIA. Hesketh *et al.* (1997) compared nine commercial-EIAs for measles specific IgG with HI and a plaque reduction neutralization (PRNT), and found that the Behring-EIA performed better, qualitatively, than the Whittaker Measelisa II.

Our data show that the HI test is a little more sensitive than the EIAs conducted according to the protocol provided. The samples that were positive by HI and negative by the EIAs, had low titters for HI, and this is probably the reason why they were not detected by the EIAs. Weigle et al (1984) and de Souza *et al.* (1991), using EIA standardized in their laboratories, demonstrated a better performance of EIA compared to HI, where PRNT was used the reference point. They found EIA equivalent to PRNT, in terms of sensitivity and specificity, being a good alternative to the PRNT which is time consuming in its processing and reading. Hesketh *et al* (1997) demonstrated that some commercial-EIAs, including one from Behring, presented high sensitivity and relatively low specificity when compared with HI and PRNT.

Chen *et al.* (1990) demonstrated that a "home made" EIA, was less effective than PRNT in the detection

of low levels of antibodies. Using commercial kits, it is not always possible to know the technology used in virus purification and adsorption to the microplate, which can alter the conformation of epitopes on the virion surface. Other variables include the source and concentration of the virus used for HI and EIA and the type of enzyme conjugate and substrate used. In addition, the dilution of serum tested and different ways of defining the cut off point for the reaction represent further sources of variation in the results (Diaz-Ortega et al, 1994).

Sensitivity and specificity of CDC and commercial-EIA's are attributes of the tests although they may be influenced by the spectrum of infection/ disease (severity, timing, etc). Of interest, is the performance of the tests expressed by the probability that EIA-positive individuals are really infected (positive predictive value). Predictive values (positive or negative) depend on the prevelence of infection/disease (measles infection rates may vary widely in different settings), as well on the accuracy of the test (Fletcher et al, 1996). With such a high specificity showed by EIA, a positive result ensures measles infection (positive predictive value of 100%). On the other hand, negative EIAs included false results, that is, the predictive value of negative EIA gets worse as sensitivity of the test decreases.

HI and PRNT tests were used to evaluate the use of different diagnosis techniques, through the testing of sera originally negative in the CDC and commercial-EIAs. The results suggest that children below 12 months of age were more likely to be seronegative than older children. Children of 12 months or greater had higher rates of false negative results in the EIAs. In this age range measles vaccination plays an important role in antibodies levels, particularly in view of mass vaccination campaigns conducted prior to the time these specimens were obtained. Our data are limited in this regard, but seem to indicate that most children giving false negative results by EIA had been vaccinated while most true seronegative children had not been vaccinated. These true seronegative children were predominantly below 9 months of age and while maternal antibodies could be present at low levels, very few of those children were seropositive by PRNT. A possible explanation for this is that vaccinated mothers present lower levels of antibody than that resulting from natural infection thus giving less maternal antibodies in their offspring. Oliveira *et al.* (1996) conducted a seroepidemiological study of children and young adults of one to nineteen years of age in the municipality of Niterói, in the state of Rio de Janeiro. In this study, a large number of negative samples were found by the CDC-EIA in the 1 to 4 years old age group and a marked increase in the percentage positive was demonstrated by PRNT. In the 5 to 19 year age group, fewer seronegatives were found by EIA and more than 98% were positive by PRNT. The work of Oliveira et al. (1996) was a seroepidemiological study, in a city of homogeneous and high measles vaccine coverage. This is markedly different from the present work in which samples from suspected measles cases were used, coming from a variety of cities in the states of Rio de Janeiro and Espírito Santo. In these states, measles vaccination coverage was inhomogeneous.

**Table 2.** HI and PRNT test results in subsamples of specimens previously tested with CDC-EIA and commercial-EIA.

| | Commercial-EIA | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Pos. | (%) | Neg. | (%) | Pos. | (%) | Neg. | (%) |
| Commercial-EIA seronegative* (n=131) | 14 | (10.7) | 117 | (89.3) | 57 | (43.5) | 74 | (56.5) |
| CDC-EIA seronegative (n=147) | 30 | (20.4) | 117 | (79.6) | 72 | (49.0) | 75 | (51.0) |

*includes 8 specimens with inconclusive tests results

**Table 3.** Sensitivity (95% of confidence limits) of CDC and commercial-EIAs and HI to measles antibodies detected by PRNT.

| **Reference test** | | |
|---|---|---|
| Tests | PRNT | |
| CDC-EIA | 0.89 | (0.94 - 0.83) |
| Comercial-EIA | 0.90 | (0.95 - 0.85) |
| HI | 0.93 | (0.97 - 0.89) |

From the second half of the 1980's, Brasil has played an outstanding role as one of the developing countries which obtained the best results in the control of diseases that can be prevented by vaccination. This has been conducted in spite of a large population and territory, important regional differences in the level of

socioeconomic development, and metropolitan areas with high levels of poverty (Waldman & Camargo, 1996). However, in 1996 measles outbreaks occured in the states of Santa Catarina and São Paulo, spreading throughout the country in 1997. Brasil predicts measles elimination in the beginning of the year 2001.

Confirmed cases of measles have been observed without the characteristic signs and symptoms, rendering the clinical diagnosis difficult. For this reason, the disease can be confused with other exanthematic diseases (Modlin, 1984). Therefore, it is possible that the measles virus circulates at some level, even in vaccinated populations (World Health Organization, 1994). The detection of measles IgG can be undertaken by a number of assays (Hesketh *et al.*, 1997) and has been suggested as the means to of accompany and evaluate vaccination campaigns in several Brazilian areas and in different age groups.

We recommend the use of PRNT as a complementary test to EIA, in the evaluation of vaccination campaigns for the evaluation of seroconversion rates and for confirmation of negative EIA results in clinical samples. This procedure would provide a sound basis for seroepidemiological studies and for the evaluation of population immunity, leading to improved control of the disease.

## ACKNOWLEDGEMENTS

A acknowledge Dr. Willian Bellini, measles section, CDC/Atlanta for kindly supply the CDC/EIA reagents and Dr. Marilda Mendonça Siqueira for the orientation na corrections during this work. I tank Dr. José Nelson dos Santos Silva Couceiro for the correction of the thesis' text in his co-orientation. I also thanks Dr. Jussara Pereira do Nascimento for the idea of this work, for its title and for the explanation about HI test. I tank researcher George F. Mann for teaching the PRNT and corrections of the English and researcher Marcos da Silva Freire for completing George Mann's teaching of PRNT, accomplished in Biomanguinhos, and for the reagents he supplied. I acknowledge Dr. Luiz Antonio Bastos Camacho for data analysis support. Finally also tank Secretaries of Health from Countries and States of Rio de Janeiro e Espirito Santo for epidemiological data. This work was partially supported by COLAB/CENESPI/FNS - Brasil. I received a fellowship from CAPES intermediated by Teacher Paulo de Góes Microbiology Institute/ URFJ.

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