Rev. Inst. Med. trop. São Paulo
36 (2): 139-147, março-abril, 1994

MEASLES SERODIAGNOSIS: STANDARDIZATION AND EVALUATION OF A DOT-ELISA

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SUMMARY

A Dot-ELISA using a measles virus (MV) antigen obtained by sodium deoxycholate treatment was standardized and evaluated for IgM and IgG antibody detection in measles patients and measles-vaccinated subjects. A total of 192 serum samples were studied, comprising 47 from patients with acute and convalescent measles, 55 from 9-month old children prior to measles vaccination and 41 from children of the same age after vaccination, and 49 from patients with unrelated diseases. The diagnostic performances of the IgG Dot-ELISA and IgG immunofluorescence test (IFT) were found to be close, varying from 0.97 to 1.00 in sensitivity and the specificities were maximum (1.00). Nevertheless, the sensitivity of the IgM Dot-ELISA (0.85) was higher than that (0.63) of the IgM IFT, although both assays had comparably high (1.00) specificities. The IgM Dot-ELISA in particular proved to be more sensitive in relation to other assays studied by revealing antibodies in 80.0% (12/15) of vaccinated children on the 15th day after immunization. In contrast the IgM IFT, failed to detect antibodies in the same group of vaccinated children. The stability of the MV antigen was longer than that of the IFT antigen, and the reproducibility of the Dot-Elisa was satisfactory.

KEYWORDS: Dot-ELISA; Measles, vaccination; Serologic diagnosis.

INTRODUCTION

The incidence of measles has been considerably reduced in the world after vaccination started to be practised in Public Health programmes. However, this viral disease still constitutes a major cause of morbidity and mortality in developing countries, and unexpected measles outbreaks among preschool children in inner cities have been reported in recent years [4]. Moreover, data provided by an epidemiologic study of a measles outbreak among U. S. schoolchildren have indicated that the primary vaccine failure added to a possible waning immunity could represent the major factor in measles epidemics [17].

During the outbreaks, the laboratory confirmation of clinical measles and the identification of susceptible individuals for vaccination are relevant measures to control the disease.

Several serologic techniques are available to obtain this information, such as haemagglutination inhibition, neutralization, immunofluorescence and immunoenzy-

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matic. Despite their good serodiagnostic performances, the aspects related to the technical complexity, use of unstable, need for special equipment and skilled personnel constrain their use in seroepidemiologic studies to be conducted in developing countries.

Preliminary findings have shown that the Dot-ELISA is sensitive, practical and economic for the detection and titration of antibodies to measles virus [12], but requires further investigation.

On this basis, the present study was conducted to standardize the Dot-ELISA to establish the optimal conditions for the detection of IgG and IgM antibodies to measles, and to assess the serodiagnostic performance and characteristics of the assay for the evaluation of measles vaccination efficacy, in comparison to the IF test.

MATERIAL AND METHODS

Measles Virus (MV) Antigens

The Toyoshima strain Measles virus (MV) was grown in Vero cell culture (American Type Culture Collection, USA) containing minimum essential medium (MEM) supplemented with 2% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2µg/mL amphotericin B. When an extensive cytopathic effect was observed after incubation at 37°C, the cells were dislodged by scraping the surface of the bottle with glass beads. The cells were then washed 3 times with 0.01M phosphate buffered saline pH 7.2 (PBS), and centrifuged at 200g for 10 min. The sediment was resuspended in PBS in a volume corresponding to a 20-fold reduction of the original volume.

The uninfected cells were similarly processed to provide a control antigen for checking specificity.

Three types of MV antigen were obtained using different detergents as follows:

a) ***Treatment with an anionic detergent*** - An equal volume of 0.2% sodium deoxycholate (Sigma Chemical Co., St. Louis, Mo., USA) in PBS was added to the infected and uninfected cell suspensions [5]. After stirring at 4°C for 30 min., the cells were sonicated (1mA) in the cold for 3 min., and clarified by centrifugation (220g) at 4°C for 15 min. The supernatant was again centrifuged (28,000g) at 4°C for 30 min. The antigen extract thus obtained (MVa) and its control (Ca) were aliquotted (0.1 ml) and stored at -70°C.

b) ***Treatment with an amphoteric detergent*** - A volume of infected and uninfected cell suspensions was treated with 5 volumes of 8mM CHAPS 3[(3-cholamidopropyl) dimethylammonio] - 1- propanesulphonate) (Sigma Chemical Co., St. Louis, Mo., USA) in PBS and stirred at 20°C for 30 min. [11, 14]. The same procedure described for the former antigen preparation was then followed. This antigen was called MVb and its control, Cb.

c) ***Treatment with a non-ionic detergent*** - A volume of both infected and uninfected cell suspensions was treated with 5 volume of 0.5% Nonidet (NP40) (Sigma Chemical Co., St. Louis, Mo., USA) in PBS and stirred at 4°C for 30 min.[14] The same procedure described for the MVa antigen was then followed, including the control antigen Cc.

d) ***Treatment with PBS*** - The infected and uninfected cells were processed in PBS with no detergent, as described in **a**, and the control Cd antigen was prepared.

The protein contents of the antigen extracts were determined according to BRADFORD [3] and LOWRY *et al.* [15].

Serum Sample

A total of 192 serum samples from 3 groups of patients were studied. Group I included 47 sera from 22 patients with acute and convalescent clinical diagnosis of measles confirmed by seroconversion (IgG-IFT). Two serum samples were collected from each patient, except for 3 patients of which 3 serum samples were obtained. Group IIa consisted of 29 sera from 9-month old children without clinical or serologic evidence of measles, or a history of past measles vaccination. Group IIb consisted of 49 sera from patients with unrelated active infections and with well-defined clinical and laboratory data for their respective diagnoses, such as rubella (5), syphilis (4), acute (2) and chronic (3) toxoplasmosis, infectious mononucleosis (3) *Streptococcus pyogenes* infection (5), and autoimmune disease with high rheumatoid factor (27). Group III comprised 67 serum samples from 26 9-month old children. Twenty six of these serum samples were obtained prior to measles vaccination, and 15 and 26 samples were collected 15 and 30 days after vaccination, respectively.

Also, 3 standard sera were included in all tests. The standard positive serum for IgG antibodies to measles virus (SG) was prepared by pooling the sera with high

(2,560), intermediate (320 to 160) and low (20 to 40) titers in the IgG-IF test. These were obtained from patients with past measles infections and with no presence of IgM antibodies. The standard positive serum for IgM antibodies to measles virus (SM) was prepared with a pool of sera from measles patients showing symptomatology, seroconversion in the IgG-IF test and IgM-IF titers ranging from 80 to 160. The standard negative serum (SN) consisted of a pool of sera from clinically healthy and serologically negative children with no evidence of past measles and no previous vaccination. All sera were aliquotted in about 1ml volume and stored at-20°C.

Dot-ELISA Technique

The optimal conditions for Dot-ELISA were standardized here according to the technical procedures described by HAWKES [10] and modified by BENNETT & YEOMAN [2] with some modifications. Briefly, the base of a microtiter 96-well plastic plate was cut and this bottomless tray was placed on a nitrocellulose membrane (Millipore, 0.45 µm pore size) of the same size and 1 µl antigen extract was dotted through each hole.

After drying at 37°C for 10 min., the membrane free proteinbinding sites were blocked with 5% skim milk in Tris buffer solution (0.02M Tris and 0.5 NaCl) (TBS) at 4°C overnight with constant shaking.

In the detection of IgM antibodies, the rheumatoid factor was previously removed by treating the sera with human gamma globulin aggregates (RF-Absorbent - Behring, Germany). Then, the rheumatoid factor-free serum samples were serially diluted in TBS containing 1% skim milk from 1:10 to 1:20,480 for IgG antibody detection and from 1:20 to 1:640 for IgM detection, in a volume of 50 µl, using a microtiter plastic plate (96 wells). After removing excess blocking solution, the membrane was set with dotted antigens facing down on the plastic plate wells containing serum dilutions. The membrane and plate were well sealed with parafilm (American Co., Greenwich, USA), wrapped with 4 to 6 filter paper layers and an acrylic top plate with 4 spring-type paper clamps to fasten firmly whole the system. The system was then inverted to allow the serum dilutions to react with dotted antigens, and incubated at room temperature for 4 h in a shaker. The membrane was then washed 3 times (10 min per wash) in TBS with 0.05% NP40 under constant shaking.

The membrane was placed on a microtiter plate containing peroxidase conjugate dilutions of 1:2,000 for anti-human IgG (gamma-chain) and of 1:400 for anti-human IgM (mi-chain) supplied commercially (Biolab-Mérieux, Brazil), after checking their monospecificities by immunoelectrophoresis. The same system was incubated at room temperature for 2 h. Three washing cycles were processed as before, and dots were vizualized after a 30-min treatment with a substrate containing 18 mg 4-chloro-1-naphtol. (Sigma Chemical Co., St Louis, MO., U.S.A.) previously dissolved in 6ml methanol, 30 ml TBS and 60µl 30% H202. The reaction was stopped by adding distilled water with 3 subsequent changes. The membrane was treated with 10% glycerin in distilled water and dried on filter paper. Positive results displayed blue coloured dots, varying from 1+ to 4+ while negative results consisted of no staining or very faint blue shadows.

Immunofluorescence Test (IFT)

The IgG and IgM-IFT were performed as previously described [20].

Statistical analysis

The geometric mean titer(GMT) was estimated after transforming the titer in $\log_{10}$.

To determine the Dot-ELISA and IFT diagnostic performances, sensitivity, specificity were calculated [8] and Kappa index agreement between the test results as described [7, 13, 16].

TABLE 1

Measles virus antigen extracts and reactivity of standard positive serum in DOT-ELISA.

Antigen Extract 2 µg/µl protein obtained with detergent	Positive standard for IgG antibody (SG) Titer							
	10	20	40	80	160	320	640	1280
DOC	4+	4+	4+	4+	3+	3+	3+	2+
CHAPS	4+	4+	4+	4+	3+	3+	2+	2+
NP40	3+	2+	1+	-	-	-	-	-
PBS	4+	4+	4+	4+	3+	2+	1+	-

RESULTS

The MV antigen treated with the anionic detergent, DOC, gave better results in Dot-ELISA for the detection of either IgG or IgM antibodies than antigens obtained with CHAPS, NP40 and PBS.

Table 1 illustrates the reactivity of standard SG serum with these different types of MV antigens. The standard SN serum gave no false-positive results when tested against these antigens, and the standard SG, SM and SN sera all showed negative results against control antigens Ca, Cb, Cc and Cd, simultaneously prepared with uninfected Vero cells by the same procedure as used for the infected cells.

The effect of antigen concentration was determined by varying the concentration of DOC-MV antigen from 0.1mg/ml to 4mg/ml. Sensitive results could be observed at the concentration of 2mg/ml for the detection of both specific antibody isotypes.

To block free-protein binding sites on the nitrocellulose membrane, 1% to 5% bovine serum albumin (BSA) was also assessed along with 1% to 5% skim milk; 5% skim milk was the better blocking solution, particularly after boiling for 10 minutes and filtration since no background staining was seen. The selected time for this blocking process was 2h, at room temperature, although after a longer period of time (18 h) at 4°C, the results were similar.

To fix the MV antigen on the nitrocellulose membrane, periods of time varying from 10 to 30 min., at room temperature or 37°C, were tried. Sensitive results were obtained after incubating the antigen at 37°C for 10 min.

The serum samples were incubated with antigen for a period of time varying from 1 to 4h, at room temperature, as well as 24 h at 4°C. Best results could be observed after 4 h incubation, while 1 to 2 h incubations gave poor results. The peroxidase conjugate incubation period of 1 to 2 h for the detection of different antibody isotypes provided satisfactory results.

When two chromogen solutions, 4 chloro-naphtol and diaminebenzidine, were assayed only the former provided reliable results with no background staining.

The cutoff 20 titer was chosen for IgM Dot-ELISA because it permitted the separation of patients with measles from those with no previous measles infection and/or vaccination with the highest possible efficiency. However, the cutoff titer for IgG Dot-ELISA was 10, permitting the discrimination of patients with measles active and past infections including vaccinated from patients with no previous measles infection and no vaccination. Also, the cutoff titers for both IgM and IgG-IFT were found to be 5, confirming previous findings [20].

Table 2 and 3 show the diagnostic features of the serologic techniques studied in terms of sensitivity, specificity, kappa index(K) as well as the degree of K agreement. The kappa indices proved to be significant since the Z values obtained ranged from 4.391 to 8.978, with a critical value of Z = 1.96 at the 95% level. Although the results of IgG Dot-ELISA were comparable to those of the IgG-IFT, the IgM Dot-ELISA showed more sensitive results than the IgM-IFT.

The geometric mean titer (GMT) of IgG antibodies detected by Dot-ELISA was close to that given by IFT, and the GMT of IgM antibodies was similar in both assays. However, the GMT of the former antibodies was significantly higher than that of the latter (Table 2).

As illustrated in Fig. 1, the higher sensitivity of IgM Dot-ELISA is also reflected in the antibody profile developed by measles patients from the day onset of symptoms to 25 days thereafter. This assay revealed more infected patients than IgM IFT, except from the 6th

TABLE 2

Diagnostic performance of IgG DOT-ELISA and IgG IFT in the study of 76 serum samples from patients with measles infection (47) and with no previous measles infection and/or vaccination (29).

Test (GMT)	Specificity	Sensitivity	Kappa Index (K)	Rank for (K)
IgG DOT-ELISA	(29/29)	(47/47)		
(1.633)	1.00	1.00	1.00	Almost perfect
IgG IFT	(29/29)	(46/47)		
(1.274)	1.00	0.97	0.97	Almost perfect

TABLE 3

Diagnostic performance of IgM DOT-ELISA and IFT in the study of 125 serum from patients with measles infection (47) and with no previous measles infection and/or vacination (29) and from patients with no related diseases (49).

Test (GMT)	Specificity	Sensitivity	Kappa Index (KI)	Rank for (RI)
IgM DOT-ELISA	(78/78)	(40/47)		
(36)	1.00	0.85	0.81	Almost perfect
IgM IFT	(78/78)	(30/47)		
(10)	1.00	0.63	0.57	Moderate

to the 10 th day after the onset of measles symptoms, when both assays displayed the same positivity (90.9%).

As to the vaccinated children the Dot-ELISA again presented more sensitive results in comparison to IFT (Fig. 2). The Dot-ELISA was able to demonstrate IgM antibodies in 80% (12/18) of children 15 days after immunization (GMT=21.87) and in 92.3% (24/26) 30 days afterwards (GMT=30.52). In contrast the IFT revealed no IgM antibodies during the same period of measles infection, i. e. the antibody titers were lower than the stimated cutoff (GTM=2.45). Also, the IgG antibodies were detected in 46.7% of children on the 15th day post-vaccination by Dot-ELISA, in contrast to IFT which showed negative results.

The antigens dotted on membranes were stored in sealed plastic bags at 4°C and the antigens were stable for at least 6 months as tested against standard positive and negative sera.

The reproducibility of IgM and IgG Dot-ELISA was checked with a panel of 4 positive and one negative sera in 20 assays carried out on different days, considering titer variations of ± 1 dilution to be non-significant. According to this criterion, 100% titer agreement was observed in the total of 100 tests.

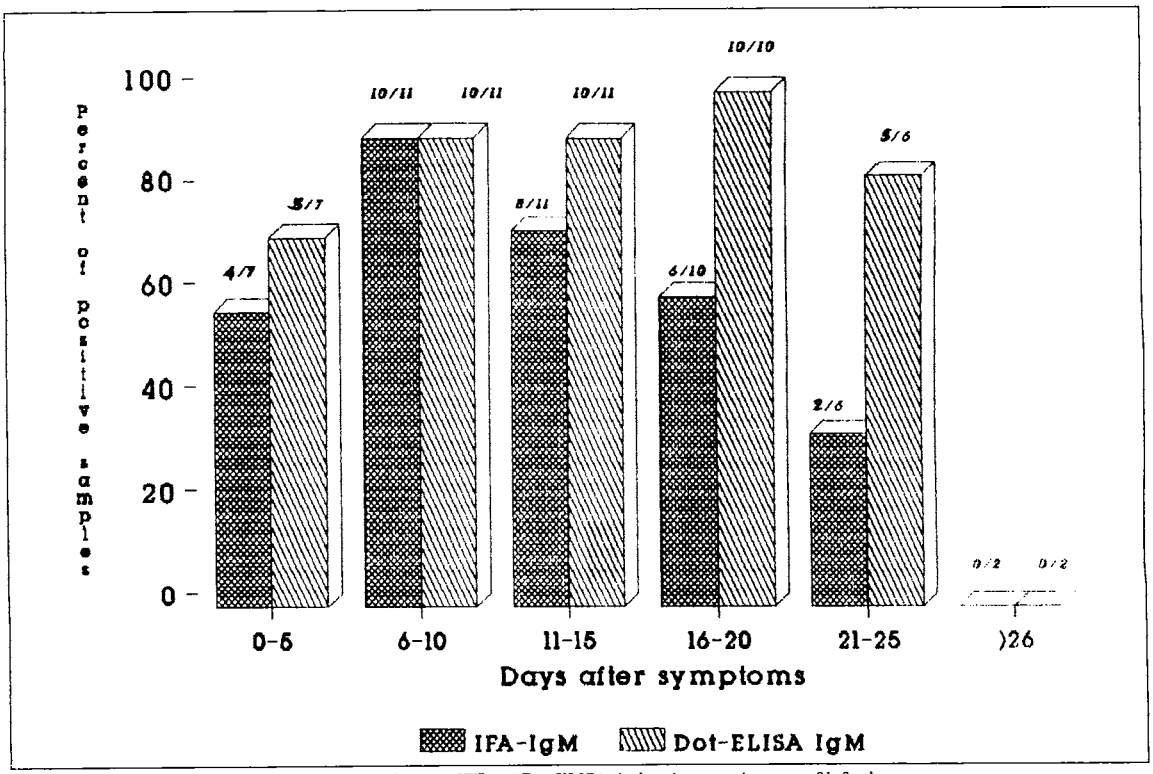

Fig. 1 Detection of specific IgM Ab in measles patients by IFT and Dot-ELISA during the natural course of infection.

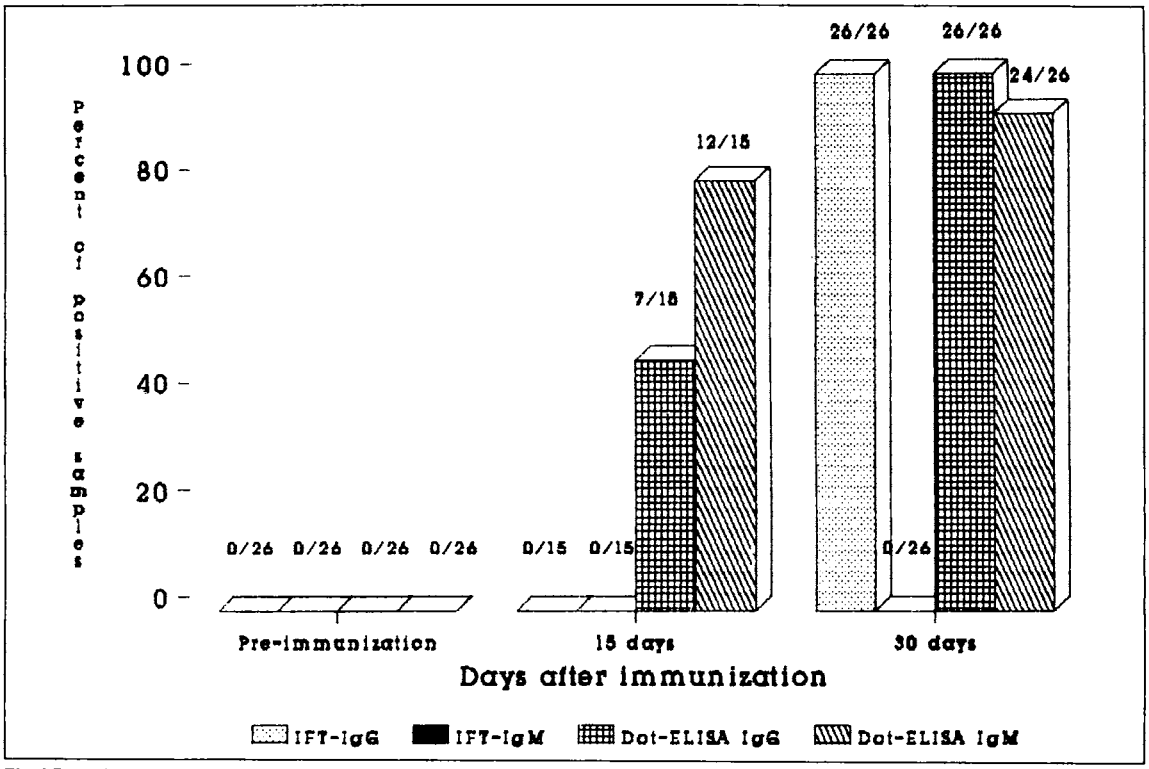

Fig. 2 Detection of IgM IgG measles antibodies in 26 immunized children.

DISCUSSION

In the first step of this study we performed the standardization of Dot-ELISA since the available data are very scarce and the studies done on measles serodiagnosis by this technique have uitlized antigens from commercial sources produced either for ELISA [1] or for HI assay [12]. Thus, the factors influencing Dot-ELISA such as type of antigen, blocking solution, incubation time, temperature and chromogen solution were studied here in detail.

To better solubilize MV and to provide reproducible, stable, and sensitive results, three types of detergent were assessed: Doc (anionic), CHAPS (amphoteric) and NP-40 (non-ionic). Doc yielded a better antigen in terms of these desired features, followed by CHAPS.

The antigen treated with NP40, however, spread on the nitrocellulose membrane affecting the sensitivity of test results. This finding confirms that reported by CREMER *et al.* [5] working with MV antigen for ELISA, although other detergents were not studied by these authors.

Detergents such as NP-40 and CHAPS have been utilized with success in the solubilization of cell membrane antigens such as MHC-like glycoproteins 6 and thyroid plasma membrane [9].

Under our working conditions Doc-solubilized MV antigen displayed longer stability, at least 3 times longer than the IFT antigen, a crucial characteristic for an assay to be useful in serodiagnosis and in seroepidemiologic surveys.

As to the blocking solution, 5% skim milk proved to be better than BSA in our system, confirming the previous finding of KALTER *et al* [12]. However if BSA (5%) is used, it seems that more proteins are needed, for example supplementing with 5% fetal calf serum, as seen in the MV system described by AEPPLI *et al.* [1].

The time for on the nitrocellulose membrane sensitization with MV antigen was 10 min at 37°C, a period shorter than that reported by KALTER *et al* [12], which was 30 min. It is conceivable that when the antigen is fixed on the membrane at less than 37°C, a longer period of time is required, for sensitization.

The incubation period needed for serum samples to react with dotted antigen was 4 h, and the period needed for both anti-human IgG and IgM peroxidase conjugates to bind to the antibodies was 2 h, at room temperature. These periods are longer than those standardized for other antigen systems in which the nitrocellulose membranes are differently processed by immersing them in serum and conjugate dilutions. However, BENNETT & YEOMAN [2], working with hybridoma clones, reported incubation periods similar to those used by us for the serum and peroxidase conjugates.

As chromogen 4-Chloro-naphtol was chosen based on the absence of background staining. This observation agrees with those reported by KALTER *et al.* [12] and AEPPLI *et al.* [1], in studies on the MV antigen system.

In the detection of IgM antibodies by Dot-ELISA, the interference of rheumatoid factor, was seen and this was eliminated by absorbing the sera with human gamma globulin aggregates similarly to that described for ELISA[19] and for IFT.

In the second step of this study, the diagnostic performance of the standardized Dot-ELISA was investigated in active measles patients, and also to establish the antibody profiles developed in active infections and in vaccinated children in comparison with IFT.

The diagnostic features of the Dot-ELISA resembled those of the IFT in the detection of IgG antibodies to measles.

In a note, KALTER *et al.* [12] also referred to IgG Dot-ELISA being as sensitive as the HI test in the diagnosis of measles, as well as in the immunity evaluation of monkeys after vaccination; however, they did not include control groups in their study.

In the present study, the IgG Dot-ELISA was found to present slightly better diagnostic features than the IgG line immunobinding assay (LIBA), an assay closely related to Dot-ELISA described by AEPPLI *et al.* [1] also for measles diagnosis, which yielded 0.970 sensitivity and 0.944 specificity in a comparative study with ELISA.

With respect to IgM Dot-ELISA, our data indicate its good performance in comparison to IgM IFT. Probably, the Doc-solubilized MV antigen on a nitrocellulose membrane exposes more antigenic epitopes than the viral particle antigen from the IFT. The IgM Dot-ELISA in addition, demonstrated similar sensitivity and higher specificity when compared with IgM ELISA [18]. The sensitivity of IgM capture ELISA [6], was slight lower but its specificity was similar.

The IgG IFT utilized here as reference test presented a better diagnostic performance than the test described by CREMER *et al.* [5]. However, the IgM IFT showed lower sensitivity, despite its closer specificity when compared to those reported by ROSSIER *et al.* [19].

In the course of measles infection, the Dot-ELISA was more sensitive than IFT. The IgM antibodies were detected in almost all patients 6 days after the onset of symptoms showing a peak 16 to 20 days afterwards and these antibodies were still present in a larger proportion of patients by the 25th day. Thereby, IFT and Dot-ELISA can provide descrepant results depending on the time when the serum sample was colleted. The Dot ELISA IgM antibody profile agrees with those observed by ERDMAN *et al.* [6] and by ROSSIER *et al.* [19], working with IgM capture ELISA and IgM-ELISA, respectively.

The data in Figure 1 are useful to establish a criterion for the assay to be selected, and also for the most convenient time for colleting patient serum. Recently, suspected cases of measles with a clinically undefined rash have become common among previously vaccinated children. Thus, the Dot-ELISA seems to be adequate for use even after such a rash, contributing to the elucidation and detection of cases not diagnosed by IFT.

In vaccinated children IgM antibodies were detected 15 days after immunization by Dot-ELISA, while both IgM and IgG antibodies were detected 30 days later. These data are consistent with those reported by ERDMAN *et al.* [6] who detected IgM antibodies in 97% vaccinated children and IgG antibodies in 100% vaccinated children 3 weeks after immunization by capture ELISA.

In contrast, the IFT detected only IgG antibodies and no IgM antibodies in the vaccinated children studied 30 days after immunization. Since the IgM antibody levels detected (GMT=30.5) in Dot ELISA were nearing the stipulated cutoff titers (20) the negative results for

the same antibodies (GMT=2.45, < cutoff 5) in IFT might be considered as an expected event.

Our findings indicate that IgM Dot-ELISA is more sensitive than IgM IFT, requiring no special equipment and being applicable to any laboratory for measles diagnosis and for the assessment of immunity status in vaccinated populations.

RESUMO

Diagnóstico sorológico do sarampo: padronização e avaliação do teste Dot-ELISA.

A técnica de Dot-ELISA (DE) para detecção de anticorpos IgM e IgG anti vírus do sarampo foi padronizada e avaliada utilizando-se antígeno viral obtido por tratamento com desoxicolato de sódio (DOC).

Foram estudadas 192 amostras de soros, compreendendo 47 amostras de 22 pacientes com sarampo nas fases aguda e convalescente, 55 amostras de soros de crianças antes da vacinação, tendo 9 meses de idade, 41 amostras de soros de crianças da mesma idade colhidas após vacinação e 49 amostras de soros de pacientes com outras patologias.

O desempenho diagnóstico da técnica de Dot-ELISA-IgG foi semelhante ao de Imunofluorescência indireta (IFI) IgG cujos índices de sensibilidade variaram de 0,97 a 1,00 e os de especificidade sendo de valor máximo, 1,00. Contudo, a sensibilidade da técnica de Dot-ELISA IgM(0,85) foi mais alta que a de IFT IgM (0,63), embora ambos os ensaios apresentassem especificidades máximas (1,00).

A técnica de Dot-ELISA IgM em particular mostrou-se mais sensível em relação a IFT, revelando anticorpos em 80% das crianças vacinadas (12/15), 15 dias após a imunização. Ao contrário, IFT IgM falhou em detectar anticorpos no mesmo grupo de crianças vacinadas. A estabilidade do antígeno viral obtido com detergente (DOC) foi maior que a do antígeno de IFT, e a reprodutibilidade da técnica de Dot-ELISA foi satisfatória.

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Recebido para publicação em 12/08/1993.
Aceito para publicação em 24/09/1993.