

DOT-ELISA-IgM IN SALIVA FOR THE DIAGNOSIS OF HUMAN LEPTOSPIROSIS USING POLYESTER FABRIC-RESIN AS SUPPORT (Preliminary Report)

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SUMMARY

In order to improve the diagnosis of human leptospirosis, we standardized the dot-ELISA for the search of specific IgM antibodies in saliva.

Saliva and serum samples were collected simultaneously from 20 patients with the icterohemorrhagic form of the disease, from 10 patients with other pathologies and from 5 negative controls. Leptospire of serovars *icterohaemorrhagiae*, *canicola*, *hebdomadis*, *brasiliensis* and *cynopteri* grown in EMJH medium and mixed together in equal volumes, were used as antigen at individual protein concentration of 0.2 µg/µl.

In the solid phase of the test we used polyester fabric impregnated with N-methylolacrylamide resin. The antigen volume for each test was 1µl, the saliva volume was 8 µl, and the volume of peroxidase-labelled anti-human IgM conjugate was 30 µl. A visual reading was taken after development in freshly prepared chromogen solution.

In contrast to the classic nitrocellulose membrane support, the fabric support is easy to obtain and to handle. Saliva can be collected directly onto the support, a fact that facilitates the method and reduces the expenses and risks related to blood processing.

KEYWORDS: Leptospirosis; Dot-ELISA; Polyester-resin; Solid-phase; Saliva.

INTRODUCTION

Leptospirosis continues to be an important Public Health problem affecting humans and animals, with serious medical and veterinary repercussions in urban regions.

In countries where leptospirosis is endemic, little is known about its true incidence. Because of the pleomorphism of its clinical expressions, a clarifying laboratory diagnosis is required. Indeed, the information available concerns the severe clinical forms of the dis-

ease, such as Weil's syndrome, or the deaths it causes. According to FEIGIN & ANDERSON ⁶, these forms correspond to 5 to 10% of the true incidence of the disease.

In order to facilitate the collection of material for laboratory diagnosis, SILVA et al. ¹⁰ standardized an immunoenzymatic assay (ELISA) for the detection of specific IgM antibodies using saliva as a biological fluid. As a continuation of this research line, we also

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propose a standardization of dot-ELISA for the detection of specific IgM in saliva.

MATERIAL AND METHODS

SAMPLES

Saliva and serum were collected simultaneously from 20 patients in the acute phase of leptospirosis hospitalized at the Instituto de Infectologia Emilio Ribas. The patients presented the icterohemorrhagic form of the disease (Weil's syndrome) and their sera reacted to microscopic agglutination test (MAT) ⁵ and ELISA-IgM ⁸ for leptospirosis.

The negative controls were obtained from 5 apparently healthy individuals with no clinical or epidemiological history of leptospirosis and from 10 individuals with the following pathologies: meningococcal meningitis (1), typhoid fever (2), yellow fever (1), secondary syphilis (4), and malaria (2). The 15 negative controls did not react to MAT or ELISA-IgM and their respective saliva samples did not react to ELISA-IgM ¹⁰.

ANTIGENS

Leptospire of the serovars *brasiliensis*, *canicola*, *cynopteri*, *hebdomadis*, and *icterohaemorrhagiae* were used in the dot-ELISA since they are among the most reactive serovars currently used in MAT in our laboratory for the diagnosis of human leptospirosis ⁹.

The antigen was prepared according to the method described by ADLER et al. ¹ modified by SILVA et al. ⁸. The leptospire were grown on EMJH medium (Difco Lab., USA) at 28°C for 7 days and frozen at -20°C for 7 days. After defrosting and centrifugation at 10,000 xg for 30 min at 4°C, they were washed twice and resuspended to 25% of the original volume in 0.02M phosphate-buffered saline, pH 7.2. After disruption by sonication at 20 KHz, 0.8 mA for 3 periods each of 3 min, the 5 sonicated leptospiral suspensions were mixed in equal proportions of protein concentration (w/v), determined according to BRADFORD ³ and used at final concentration of 0.2 µg/µl in 0.05M bicarbonate buffer, pH 9.6.

DOT-ELISA

Strips of polyester fabric (Rhodia, Brazil) impregnated with N-methylol-acrylamide as described in the literature ^{2, 4, 7, 11} were used in the solid phase of the reaction. The antigen, at a protein concentration of 0.2 µg/µl, was applied in a volume of 1 µl and fixed in an

oven at 27°C for 30 minutes. The remaining reactive sites were blocked with TBS buffer, pH 7.5 containing 5% defatted milk and 0.1% thimerosal for 2 hours at room temperature, with shaking. The membranes were dried, wrapped in plastic film and stored at 4°C until the time for use.

Saliva samples were applied one by one directly to the membranes at a volume of 8 µl and incubated for 30 minutes at room temperature. The membranes were submitted to 3 cycles of washing with 0.85% saline solution containing 0.1% Tween 20 and dried, and 30 µl of peroxidase-labelled anti-human IgM conjugate (Bio-lab-Diagnóstica, Brazil) were applied at 1:100 dilution in TBS buffer, pH 7.5, containing 1% defatted milk. After a new 30-minute cycle of incubation in a moist chamber at room temperature, the membranes were washed again as described above. The strips were soaked in freshly prepared chromogen solution containing 3mg/ml 4-chloro-1-naphthol in methanol P.A. grade (Merck, Brazil), incubated for 10 minutes at room temperature in 5 ml TBS, pH 7.5 containing 5 µl 30% H₂O₂, and washed 3 times in running water.

The positive results were indicated by the development of a well defined purple color in the dot.

RESULTS

The result obtained with the dot-ELISA-IgM using fabric-resin as support showed that 100% of the saliva samples from the 20 patients with leptospirosis presented IgM antibodies (Table 1). The reactive results are documented in Fig. 1, columns 1, 2, 3, 4, and 5, lines A, B, C, and D, providing easy visualization of the results.

No color developed at the sites to which saliva samples from the negative control were applied.

Table 1

Results of the dot-ELISA saliva test using polyester fabric-resin as support in the detection of IgM antibodies specific for human leptospirosis

Group	No. of individuals	Dot-ELISA IgM	
		Reactive	Non-reactive
Patients with leptospirosis	20	100%	-
Control	15	-	100%

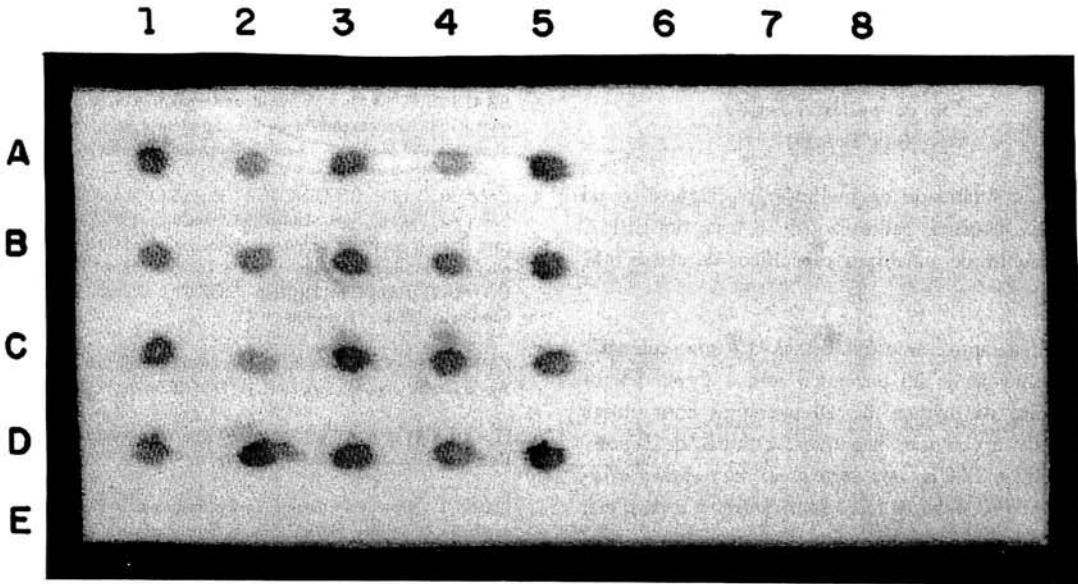


Fig. 1 - Dot-ELISA in polyester fabric-resin for the search of specific IgM antibodies to human leptospirosis in saliva
Columns 1-5: samples from patients with leptospirosis
Columns 6-8: samples from negative control
Saliva dilution 1:1

These results are also presented in Fig. 1, columns 6,7 and 8, lines A, B, C, D and E, showing good specificity.

DISCUSSION

The search for new laboratory methods for ancient human diseases has been a constant goal. In the search, factors such as sensitivity, specificity, practical handling, and time and cost savings have been considered. In addition, obtaining rapid results is of the utmost importance for physicians, permitting a definite diagnosis and the consequent adoption of appropriate therapeutic measures. The set of factors mentioned above is doubtlessly of vital importance for patients, since their survival often may depend on the result of one of these laboratory tests.

The true incidence of human leptospirosis is difficult to establish even because it presents a polymorphic clinical picture, usually requiring laboratory confirmation.

On this basis, we standardized a dot-ELISA for the detection of anti-*Leptospira* IgM antibodies in saliva using the fabric-resin solid phase as support, a material

that is easy to obtain and handle, in contrast to the classical nitrocellulose membrane support.

The support used here has free methylol groups permitting covalent bonds with amino and hydroxyl groups of proteins and sugars present in the antigen extract, with consequent high stability for the assay of several samples^{2, 4, 7, 11}.

The results obtained showed that the test is sensitive and discriminatory and can be used for the laboratory diagnosis of human leptospirosis.

The use of saliva, which can be collected directly onto the previously sensitized fabric-resin support, saves the cost of hypodermic needles, syringes, glass tubes, asepsis material, and the time and equipment needed to obtain serum. It should also be pointed out that the use of saliva prevents the possible risks related to blood collection and processing for the professional who handles blood. In addition to the facts related to the ease and rapidity of saliva collection, the easier execution of the test should also be pointed out. This simpler test can be carried out in less complex laboratories or under field conditions, with rapid results obtained by visual reading without the use of sophisticated equipment

RESUMO

Dot-ELISA-IgM em saliva para diagnóstico da leptospirose humana, empregando como suporte tecido de poliéster-resina

(Nota Prévia)

Com a finalidade de melhorar o diagnóstico da leptospirose humana, padronizou-se o teste dot-ELISA para a pesquisa de anticorpos específicos da classe IgM na saliva.

Empregaram-se amostras de saliva e soro coletadas simultaneamente de 20 pacientes com a forma ictero-hemorrágica da doença, de 10 pacientes com outras patologias e 5 controles negativos. Culturas de *Leptospira* em meio EMJH, dos sorovares: *icterohaemorrhagiae*, *canicola*, *hebdomadis*, *brasiliensis* e *cynopteri*, foram utilizadas como antígeno, na concentração proteica individual de 0,2 µg/µl, misturadas em volumes iguais.

Na fase sólida do teste empregou-se tecido de poliéster impregnado com resina de N-metilol-acrilamida. O volume do antígeno para cada teste foi de 1µl, o de saliva 8µl, o de conjugado anti-IgM humana marcada com peroxidase, de 30µl. A leitura foi visual, após revelação em solução cromógena de preparo recente.

O suporte de tecido é de fácil obtenção e manuseio, diferente do clássico de membrana de nitrocelulose. A saliva pode ser coletada diretamente sobre o suporte, o que facilita o método e diminui gastos e riscos inerentes à coleta e processamento do sangue.

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