Comparison of dot-ELISA and standard ELISA for detection of *Neisseria meningitidis* outer membrane complex-specific antibodies

ABSTRACT

Dot-ELISA using the outer membrane complex antigens of *Neisseria meningitidis* as a target was standardized for rapid detection of meningococcal-specific antibodies in human serum. We investigated the level of meningococcal-specific IgG, IgA, and IgM in serum using dot-ELISA with outer membrane antigens prepared from *Neisseria meningitidis* serotype B:4.19:P1.15,3,7,9 (a strain isolated from a Brazilian epidemic). The dot-ELISA is based on the same principles as the standard ELISA and is useful for detection of anti-*N. meningitidis* B antibodies in serum of patients with meningococcal infections. For the assay, outer membrane complexes (OMCs) were absorbed by nitrocellulose membrane and blocked with a 5% skim milk solution. Serum samples were drawn upon hospital admission and during convalescence from patients with meningococcal septicemia, and single samples were drawn from uninfected controls. We retrospectively examined a total of 57 serum samples: 35 from patients infected with *N. meningitidis* B, 12 from patients infected with *Haemophilus influenzae* b, and 10 from health individuals. When performed at room temperature, dot-ELISA took approximately four hours to perform, and the optimum antigen concentration was 0.42 μg per dot. The specificity of IgG, IgM, and IgA demonstrates that dot-ELISA using OMCs from *N. meningitidis* B as a target is suitable for serologic verification of clinically suspected meningococcal disease in patients and for titer determination of antibodies produced during different phases of natural infection. Furthermore, the sensitivity of dot-ELISA was comparable to that of standard ELISA. Overall, dot-ELISA is simple to perform, rapid, and low cost. Further validation of the test as a screening tool is required.

Keywords: *Neisseria meningitidis*, dot-ELISA, ELISA, antibodies, outer membrane complex.

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INTRODUCTION

Bacterial meningitis remains a serious threat to global health and annually accounts for an estimated 170,000 deaths worldwide. Even with the availability of antimicrobial therapy and sophisticated intensive care, the case fatality rate remains 5-10% in industrialized countries and is even higher in the developing world. Between 10-20% of survivors develop permanent sequelae, such as epilepsy, mental retardation, or sense-neural deafness. Three species (*Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*) are responsible for most cases of bacterial meningitis that occur after the neonatal period. Since the introduction of *H. influenzae* type b (Hib) conjugate vaccine, *N. meningitidis* and *S. pneumoniae* have become the most common cause of bacterial meningitis in the world. Moreover, *N. meningitidis* is the only bacterium capable of generating epidemics of meningitis.[1]

Between 2000 and 2006, there were 74,449 reported cases of meningitis in the state of São Paulo; however, the microbiological etiology was determined in only 24.7% of these cases. Among these, 8,710 cases (11.7%) were diagnosed with meningococcal meningitis, 3,497 (4.7%) with pneumococcal meningitis, and 489 (0.6%) with meningitis caused by Hib. An additional 14,990 cases (20%) were considered to be possibly caused by a bacterial infection; however the etiologic agent was not identified.[2]

Dot-ELISA has been used to detect a variety of bacterial and protozoal antigens.[3-6] Coll *et al.*, 1998, developed a dot-ELISA that uses monoclonal antibodies for detection of

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serogroup B meningococcal antigens in cerebrospinal fluid that has become a valuable tool in the detection of meningococci. Furthermore, in 1989 Oprandy & Sippel[7] used a monoclonal antibody that was immobilized on a nitrocellulose membrane for an antigen capture assay. The detection of a *N. meningitidis* serogroup A polysaccharide using this assay was more sensitive than enzyme-based immunoassays.[7] Dot-ELISA using meningococcal outer membrane complexes (OMCs) as the antigen has been developed and used to investigate the isotypes of antibodies produced in patients with meningococcal disease caused by serogroup B. However, dot-ELISA using the outer membrane complex has not been evaluated for antibodies detection of in human serum from patients infected with *N. meningitidis*. Therefore, the present study was conducted to standardize and evaluate dot-ELISA in order to establish the optimal conditions for the detection of *N. meningitidis*-specific IgG, IgM, and IgA in sera during natural infection. Dot-ELISA was compared with standard ELISA.

MATERIAL AND METHODS

Clinical samples

A total of 35 serum samples from 15 individuals with a clinical diagnosis of bacterial meningitis were analyzed. The clinical diagnosis was made on the basis of symptoms including fever, headache, vomiting, neck muscle rigidity, cerebral dysfunction, and toxemia.[8,9] The cerebrospinal fluid (CSF) samples were assayed using counterimmunoelectrophoresis (CIE) in the Immunology Section of the Adolfo Lutz Institute. CSF was collected for immunodiagnosis and bacterial culture according to the standard technique, as previously described.[10,11]

Counterimmunoelectrophoresis

CIE was performed on cellulose strips (Cellogel; Chemetron, Italy) that had been stored in a 40% methanol solution.[11] Before use, the strips were washed in 0.05 M sodium veronal buffer, pH 8.6 (Merck, Rio de Janeiro) and dried between filter papers. For each test, 7 µL of antiserum was applied at the anode and 10 µL of the treated sample was applied at the cathode, with the sample wells 0.6 cm apart. CIE was carried out for 10 min with a 15-20 mA electric current using sodium veronal buffer. After electrophoresis, the strips were washed six times for 10 min in 0.15 M NaCl, stained in 50% methanol containing 0.5% (w/v) amido black, and destained in 50% methanol containing 5% (v/v) acetic acid.

Bacterial culture

All CSF samples were cultured for bacterial growth and for serotyping at the Bacteriology Section of Adolfo Lutz Institute.[12] Serum samples were collected from patients in the acute phase of the disease (upon admission to the hospital; day 0), during the convalescent phase (day 10), and during the late convalescent phase (4 weeks).[8,9] All of the serum samples were collected in 1998 and were stored at -70° C until use. To determine the specificity and cross-reactivity of the tests, 12 samples obtained from patients in the acute phase of *Haemophilus influenzae* b infection and 10 samples from healthy individuals were also examined. A *N. meningitidis* outer membrane complex from strain B:4.19:P1.15,3,7,9, which was isolated from the cerebrospinal fluid of a patient infected in the 1998 Brazilian epidemic, was used as antigen source. Since 1986, serogroup B *N meningitidis* has been responsible for approximately 80% of the meningococcal disease in Brazil.[13] The serotype was determined in the Bacteriological Section of Adolfo Lutz Institute. Bacteria were grown on brain heart infusion agar, and 10 mL of the culture was transferred to 500 mL of tryptic soy broth (Difco Laboratories, Detroit, MI) and incubated at 37° C in a rotary shaker (120 rpm) for 18 hours.

Isolation of outer membrane complex

The bacteria were harvested by centrifugation at 10,000´g for 15 min. The outer membrane complexes were extracted in 5 mL of buffer solution (0.1 M sodium acetate and 0.2 M LiCl, pH 5.8) per gram of cells (wet weight) by shaking with 2 mm diameter glass beads in a gyratory water bath at 45° C for 2 hours.[14] The purified OMCs was obtained by removing cells by centrifugation at 12,000 g for 20 min. The supernatant was dialyzed overnight in 0.15 M NaCl, and protein concentration was determined as previously described.[15]

SDS-PAGE and by immunoblot

The antigen used in dot-ELISA was analyzed by SDS-PAGE in a 13% acrylamide gel, as described.[16] Approximately 20 µg of the OMC antigen was stained with Coomassie brilliant blue, and 2 µg was used for lipopolysaccharide determination (detected by silver staining), as described .[17]

The murine monoclonal antibodies against LPS determinants (WBE12-C10-C10, L3,7,9-specific; 6E7-10, L8-specific; and 3G3-1-8C, L1-specific; all generously supplied by Dr. Zollinger, Walter Reed Army Institute of Research, Washington, DC) were used to analyze the immunotypes present in the strains isolated from the 15 patients. Based on the antigenicity of the LPS, *N. meningitidis* can be divided into 12 immunotypes. Immunotypes L1 to L8 were found among the Group B and C strains[18,19] and immunotypes L9 to L12 were in the Group A strains.[20,21] The apparent molecular weight of the separate cell com-

ponents was determined by comparing their migration with those of molecular weight markers (Pharmacia, Uppsala, Sweden).

Dot-ELISA

The membrane antigen–specific antibody titer was quantified using an enzymatic dot-ELISA. All operations were carried out at room temperature and all incubations were performed on a shaking platform to provide gentle agitation. A nitrocellulose membrane (BioRad, Richmond, CA) was cut to fit the top of a 96-well plate and 1 μL of the OMCs sample (0.21–0.84 μg) was carefully placed as a dot on the membrane in a position corresponding to the center of each well. The nitrocellulose membrane was blocked with 5% nonfat dry milk in TBS (0.01 M Tris-HCl, 0.14 M NaCl). This solution was pre-heated in a 100°C water bath for 5 min to inactivate the endogenous milk proteases and then filtered. In previous experiments, we optimized the incubation time with blocking solution: no differences in background reactivity were seen when the blocking solution was incubated for 1 hour, 2 hours, or overnight. After blocking the membranes, 100 μL of serum samples serially diluted in 2.5% nonfat milk in TBS were added to each well and incubated for 2 hours. The nitrocellulose membrane was blotted in order to remove excess moisture and placed on the microtiter plate. The nitrocellulose membrane was sealed with a layer of parafilm by rolling a pipet across the surface. Sheets of Whatman 3 MM paper were added, and the lid was clamped down with spring-type clamps. At the end of the first incubation, the nitrocellulose membrane was removed and rapidly washed in PBS with 0.05% NP-40 (Shell Química do Brasil, São Paulo, Brazil) for 1 min with shaking and then washed in several changes of the same buffer for 30 min. The washing solution was removed and 50 μL of diluted horseradish peroxidase–conjugated anti-human IgG, IgM, or IgA antibody (Sigma, St Louis, MO) were added to each well and incubated for 2 hours. The optimum dilution of the secondary antibody was found to be 1:500 for anti-IgG, 1:1000 for anti-IgM, and 1:500 for anti-IgA. The conditions of the secondary antibody preparation and evaluation have been described previously.[22] The secondary antibody was removed and the membrane was washed as described above. The chromogen 4-chloro-1-naphthol (Sigma) was dissolved in a (stock solution, 3 mg/mL; Merck, Darmstadt, Germany) and stored in a dark bottle at room temperature for up to 10 days. Immediately before use, 10 mL of PBS, 10 μL of 30% H_2O_2, and 2 mL of the chromogen stock solution were mixed together and incubated for 20 min. The chromogen solution was removed and the membrane was washed, as described above. Samples, in which blue dots developed, when compared with the negative control sera and the antigen and secondary antibody controls, were considered to be positive. The optimal antigen concentration for dot-ELISA was determined using three concentrations of antigens (0.21, 0.42, and 0.84 μg/dot) and tested with positive sera and negative control sera.

ELISA

A standard ELISA was performed for meningococcal-specific antibodies. The OMCs from strain B:4.19:P1.15,3,7,9 was plated in triplicate in microdilution plates (Nunc), as described by Harthug *et al.*[23] with some modification. A polystyrene immunoplate (Nunc) with 96 wells was coated with 100 uL of OMCs per well, corresponding to 2 ug of protein per mL in 0.1 M Tris buffer, pH 8.5, and incubated for 18 hours. All incubations were performed at 37° C. The plates were stored at 4° C for up to 2 weeks and were washed four times in phosphate-buffered saline, pH 7.4, with 0.05% Tween 20 and 0.02% sodium azide immediately before use. Two serum samples that showed strong immune responses to OMCs antigens were used as internal standards. All serum samples were diluted 1:200 in phosphate-buffered saline, pH 7.4, with 0.05% Tween 20, 0.1% bovine serum albumin, and 0.02% sodium azide and analyzed in triplicate. Diluted serum (100 μL) was added to each well, incubated for 3 hours, and then washed as described above. A horseradish peroxidase–conjugated goat anti-human IgA, IgM, or IgG (Sigma Chemical Co., St Louis, MO) detection system was used to detect the bound antibodies. Incubations were carried out at 37º C for 1 hour in all steps, except for that with the substrate (15 min). The reaction was interrupted with 4 N H2SO4. The absorbance was obtained at 450 nm in a plate reader (SLT-Spectra, US).

RESULTS

The electrophoretic profile of the *N. meningitidis* antigens (both protein and lipopolysaccharide) used in the assay is shown in Figure 1A. The proteins are mainly 20 to 120 kDa in weight and the expression of L3,7 determinants could be seen when monoclonal antibodies were used.[24] We tested the serum from patients with clinically and laboratory proven *N. meningitidis* serotype B and healthy individuals using the dot-ELISA (Figure 1B). Positive reactions (blue dots) developed against antigens spots that were incubated with dilutions of serum from patients with *N. meningitidis* serotype B, whereas spots incubated with the serum from healthy individuals were negative or below the cut-off for IgG, IgM, or IgA. To test the reproducibility of dot-ELISA for detection of antigen-specific IgG, IgM, and IgA each serum sample was tested in duplicate against meningococcal antigens, and the optimal agreement between duplicates was obtained. The mean level of antigen-specific IgG, IgM, and IgA were significantly higher in patients with

active meningitis than in normal subjects. The reactivity of several standard positive and negative control serum samples for meningococcal antigens (0.42 µg antigens) suggested the possibility of using serum samples diluted more than 1:100. Importantly, while the serum samples from patients with *H. influenzae* b presented cross-reactivity, this cross-reactivity decreased with the dilution of the serum samples. As seen in Table 1A, the IgM and IgA presented a lower degree of cross-reactivity when compared with the IgG in the same serum sample.

Figure 1:

(A) The SDS-PAGE of the native outer membrane complex of representative *N. meningitidis* B strains isolated from patients are shown in lanes 1, 4, 7, and 10 after Coomassie blue staining. Lanes 2, 5, 8, and 11 demonstrate the analysis of LPS variants by PAGE and silver staining, and lanes 3,6,9, and 12 are immunoblots. The immunoblots were sequentially reacted with three different monoclonal antibodies. The band at position (a), (b), and (c) reacted with the WBE12-C10 (L3,7,9-specific; 5.9 kDa), the 3G3-1-8C (L1-specific; 4.8 kDa), and the 6E7-10 (L8-specific; 3.6 kDa) monoclonal antibody, respectively. The outer membrane complex of the strain) B:4.19:P1.15,3,7,9 shown in lanes 1 was used in the dot-ELISA and the ELISA assay.

(B) Dot-ELISA for the detection of *N. meningitis* B outer membrane complex–specific IgG in patient serum at a 1:100 (A) to 1:12,800 (H) dilution demonstrating the sensitivity of the assay. For each spot, 0.42 µg of the OMC was spotted onto a nitrocellulose membrane. Lanes 1 through 10 demonstrate the immunoreactivity of patient serum in different phases of the disease. Serum samples were collected from patients in the acute phase (upon admission to the hospital; day 0), during the convalescent phase (day 10), and during the late convalescent phase (4 weeks). Lanes 11 and 12 are serum samples from normal controls. The color intensity was judged visually, and the intensities were assigned values on an arbitrary scale (0, +, ++, +++, or ++++) in reference to the negative control (assigned a value of 0).The dots are blue in color.

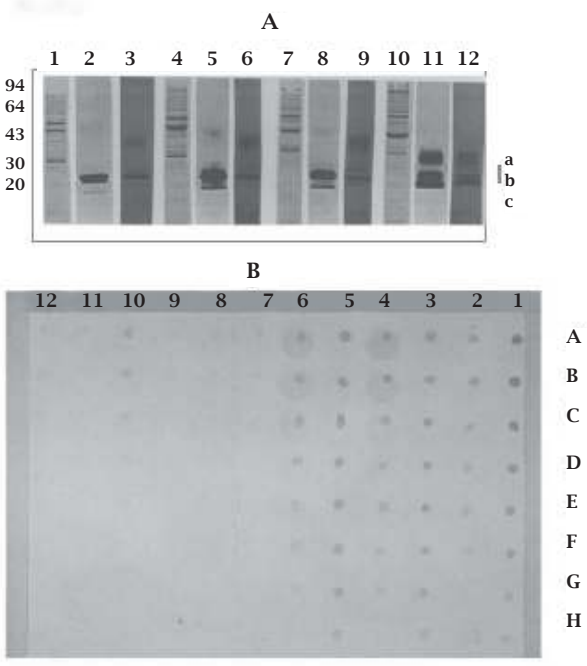

Table 1. Performance of assays for detecting antibodies in this sample studies

A- ELISA						
Isotypes	**Cut-off titer**	**Sensitivity***	**Sensitivity***	**Preditive positive**	**Value negative**	**Efficiency**
IgG	1:200	98.1	98.5	89.1	93.5	91.9
IgM	1:200	86.4	100	100	92.2	94.9
IgA	1:100	89.1	100	100	93.9	95.9
ELISA						
IgG	1:100	91.9	100	100	99.9	83.8
IgM	1:100	92.3	100	100	100	93.8
IgA	1:100	88.3	100	100	95.3	99.7

*(%)

B - Comparison between Standard ELISA IgG absorbance and dot-ELISA positivity

Dot-ELISA Scale	ELISA samples A 492nm	Tested
+4	1.87-1.68	10
+3	1.00-1.12	10
+2	0.80-0.65	10
+1	0.36-0.55	5
0	0.05-0.34	0

To obtain a higher efficiency for dot-ELISA, cut-off values were selected to better discriminate between meningitis patients and the normal controls (Table 1A). Furthermore, the longitudinal titer of the different antibody isotypes during the course of meningococcal infection was determined. The IgG titer was low at admission ($p < 0.05$) and increased progressively thereafter. IgM titer was greater than IgG titer and also increased in later stages of infection. IgA titer was higher than average during early convalescence ($p < 0.05$) and decreased during the convalescent stage. The color intensity was judged visually, and the intensities were assigned values on an arbitrary scale (0, +, ++, +++, or ++++) in reference to the negative control (assigned a value of 0). For determination of the sensitivity, specificity, and positive and negative predictive values of dot-ELISA, a standard ELISA was carried out and analyzed. Our results demonstrated a good correlation between the visual grading of dot-ELISA and ELISA absorbance (Table 1B).

The antibodies measured in this study were most likely directed against the 20–120 kDa proteins and the L3,7 LPS date not shown.

DISCUSSION

LPS is an important component of the outer membrane of meningococcal bacteria. *Neisseria* produces one to six different LPS molecules (3,200 to 7,200 Da) that can be characterized by their electrophoretic mobility in SDS-PAGE.[22] The difference in mobility appears to be due to the heterogeneity of the chemical composition of oligosaccharides. Antibodies directed against strain-specific epitopes and different outer membrane proteins of *N. meningitidis* B have been detected in serum from convalescent patients.[5,6] In this study, a dot-ELISA using the OMCs derived from *N. meningitidis* serotype B was found to be a specific technique for establishing the level of antigen-specific IgG, IgM, and IgA present in serum of patients during the different stages of infection. It also has technical advantages, such as at the use of minute amounts of antigens, antibody conjugates, and a chromogenic solution.

The procedure of dot-ELISA has a number of advantages over the standard ELISA currently performed in laboratory. The nitrocellulose membrane is capable of binding more antigens than the microtiter plates.[25] Therefore, only a small amount of the OMCs is required as an antigen for the test. Additionally, the reaction on nitrocellulose membrane is viewed against the membrane white background, making it much easier to identify a positive or negative reaction. Another advantage of dot-ELISA is that it does not require spectrophotometric readings, which are required in classic plate- or cuvette-based ELISAs, an important consideration for laboratories in developing countries. Most immunologic assays, including the dot-ELISA, are influenced by both the antibody concentration and affinity. However, dot-ELISA appears to be one of the more sensitive and least affinity-dependent procedures.[3] In contrast with antibody response against serotype B polysaccharide, which has been shown to be mediated mostly by IgM, the response against the OMCs antigens using dot-ELISA seems to be mediated by other isotypes, such as IgG and IgA. This assay was also useful in the longitudinal analysis of the antigen-specific immunoglobulin titers for use in immunological studies of this severe disease. OMC-specific IgM was detected in all *N. meningitidis* patients, without any *H. influenzae* b cross-reactivity. This suggests that this assay will be suitable for use in hospital laboratories, especially when the bacterial culture of CSF samples produce a negative result. Application of this technique in patient diagnosis and screening is valid because of the test easy execution and the characteristics described above. The sensitivity of dot-ELISA and standard ELISA was similar, as previously reported.[26] Dot-ELISA could be used for screening meningococcal disease in less well-equipped laboratories. *N. meningitidis*-specific IgG, IgM, and IgA were detected in the serum from all 35 sera investigated using dot-ELISA with good sensitivity.

A multianalyte Dot-enzyme-linked immunosorbent assay (Dot-ELISA-Multi) with *Trypanosoma cruzi* epimastigote alkaline extract (EAE), trypomastigote excreted–secreted antigen (TESA), recombinant protein derived from 19-kDa C-terminal region of the *Plasmodium vivax* merozoite surface protein 1 (PvMSP119), *Plasmodium falciparum* ZwittergentR extract (Pf-Zw), and *Treponema pallidum* ZwittergentR extract (Tp-Zw) was standardize and evaluated as a method for surveying IgG-specific antibodies in Chagas disease, malaria, and syphilis in a single test.[27]

In conclusion, dot-ELISA presented here is very useful and easy to handle; it uses minute amounts of antigen, can measure the level of antibodies IgG, IgM and IgA in *Neisseria meningitidis* infection with facility, and the results can be seen directly by the naked eye.

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