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DOT-ENZYME-LINKED IMMUNOSORBENT ASSAY (DOT-ELISA) FOR DETECTION OF PNEUMOCOCCAL POLYSACCHARIDE ANTIGENS IN PLEURAL FLUID EFFUSION SAMPLES. COMPARISON WITH BACTERIAL CULTURE, COUNTERIMMUNOELECTROPHORESIS AND LATEX AGGLUTINATION

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SUMMARY

A dot-enzyme-linked immunosorbent assay (Dot-ELISA) for pneumococcal antigen detection was standardized in view of the need for a rapid and accurate immunodiagnosis of acute pneumococcal pneumonia. A total of 442 pleural fluid effusion samples (PFES) from children with clinical and laboratory diagnoses of acute bacterial pneumonia, plus 38 control PFES from tuberculosis patients and 20 negative control serum samples from healthy children were evaluated by Dot-ELISA. The samples were previously treated with 0.1M EDTA pH 7.5 at 90°C for 10 min and dotted on nitrocellulose membrane. Pneumococcal omniserum diluted at 1:200 was employed in this assay for antigen detection. When compared with standard bacterial culture, counterimmunoelectrophoresis and latex agglutination techniques, the Dot-ELISA results showed relative indices of 0.940 to sensitivity, 0.830 to specificity and 0.760 to agreement. Pneumococcal omniserum proved to be an optimal polyvalent antiserum for the detection of pneumococcal antigen by Dot-ELISA. Dot-ELISA proved to be a practical alternative technique for the diagnosis of pneumococcal pneumonia.

KEYWORDS: Dot-ELISA; Pneumococcal antigen detection; *Streptococcus pneumoniae*, Pleural fluid effusion; Bacterial pneumonia.

INTRODUCTION

Streptococcus pneumoniae is the most frequent bacterial agent of pneumonia, otitis media, and bacteremia, and also the second most common cause of meningitis in infants, children and elderly people [25]. The number and rate of deaths among younger persons have increased in last years due to an association with human immunodeficiency virus (HIV) infections [3]. It is estimated that, worldwide statistical in each year, some 4 million children die from Acute Respiratory Illness (ARI), 80% to 90% from pneumonia. In Americas, pneumonia mortality constitutes an important problem in children under five years and annually, more than 100,000 children under one year old, and more than 40,000 children from one to four years old die from

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pneumonia [6]. Pneumococcal pneumonia is responsible for most cases of ARI and polyvalent pneumococcal polysaccharide vaccine is important in the prevention of pneumococcal diseases. However, it is not used in Brazil and generally indicated in other countries only for high-risk patients [27].

Streptococcus pneumoniae is commonly found as an inhabitant of the upper respiratory tract and has been shown to gain entrance into the blood stream via the lymphatics, when implanted on intact and apparently normal respiratory tract epithelium [17]. These bacteria cause 73% of all bacterial pneumonia infections [1]. Routine blood culture is an accurate but insensitive technique for the detection of *S. pneumoniae* because less than 20% of children with acute bacterial pneumonia are bacteremic [28]. Pleural fluid effusion samples continue to be the material most commonly used for the investigation of the causative agent of acute pneumonia [10, 28].

Antimicrobial agents have been used for prophylatic treatment, usually penicilin, erythromycin, tetracyclin or chloramphenicol, alone or in combination with other antibiotics [26]. Resistance to penicilin among clinical isolates of *S. pneumoniae* is emerging as a serious problem in many geographic locations worldwide, and vancomycin is also indicated as the therapeutic agent of choice, with third-generation cephalosporins being reserved for selected causes in which *in vitro* susceptibility of the infecting bacterial strain is confirmed [2]. Antibiotic resistance has been an enormous problem in developing countries mainly due to self-medication. Thus, antibiotic treatment of respiratory infections is not effective and does not shorten the course of the infection or prevent the development of pneumonia [14]. Antimicrobial therapy reduces the quantity of bacteria in body fluids, thus reducing the usefulness of bacilloscopy by Gram stain and decreasing the yield of positive cultures [9, 24]. Therefore, several immunoassays have been proposed for diagnosing bacterial pneumonia [28] and pneumococcal meningitis [29, 30]. Immunological methods have permitted the diagnosis of acute pneumonia in patients previously submitted to antibiotic therapy [5].

The purpose of the present study was to determine the performance of the dot-enzyme-linked immunosorbent assay (Dot-ELISA) in detecting pneumococcal polysaccharide antigens in pleural fluid samples. Dot-ELISA data were compared with the results of three standardized techniques, i. e., bacterial culture, counterimmunoelectrophoresis and latex agglutination [4].

MATERIAL AND METHODS

A total of 442 pleural fluid samples from children aged 0 to 12 years referred to our laboratory by several hospitals in São Paulo City (1989-1993) with clinical symptoms of acute bacterial pneumonia, were submitted to bacterial cultures (BC), and for pneumococcal antigen detection, to latex agglutination (LA), counterimmunoelectrophoresis (CIE) and Dot-ELISA. A clinical diagnosis was made on the basis of symptoms such as fever, cough, dyspnoea, listlessness, prostration, parenchymal condensation and bronchial or pleural syndromes. Based on these initial symptoms, all children were submitted to chest X-ray. Among the 442 samples 108 were from patients who had been submitted to previous antibiotic therapy. In order to determine the possible occurrence of false-positive results in the immunoassay, 38 pleural fluid samples from tuberculous patients (X-ray and positive bacilloscopy and/or bacterial cultures) were included plus 20 normal serum samples from healthy subjects used as negative controls.

Gold standard assays

Three routine assays such as bacterial cultures (BC), counterimmunoelectrophoresis (CIE) and latex agglutination (LA) were performed as gold standard assays. For bacterial cultures, 1.0 ml of each pleural fluid sample was cultured in brain heart infusion (BHI) base containing 5% rabbit blood, at 37°C for 1 to 7 days. After this 0.5 ml of the cultures were incubated on chocolate-agar medium with BHI base, at 37°C for 48 h. CIE and LA tests for detection of polysaccharide antigens were performed according to routine procedures [4, 28, 29].

Dot-ELISA

Aliquots of 200 µl of pleural fluid samples were treated with 300 µl of 0.1M sodium ethylenediamine tetraacetic acid (EDTA) pH 7.5, and heated, in a water-bath, at 90°C for 10 min. After cooling, the mixture was centrifuged at 2500 rpm for 10 min and the supernatant employed in the immunological tests. For Dot-ELISA, 1 µl of the prepared supernatant undiluted and in serial dilutions of 1:2 to 1:32 in TBS-G [Trizma, 20 mM/NaCl, 500 mM, pH 7.5, containing 1% gelatin EIA grade reagent (Cod. 170-6537, BioRad Laboratories, Richmond, (CA)], was dotted onto a nitrocellulose membrane (Schleicher & Schuell, 0.2 µm). Control serum

samples were submitted to the same procedure. A pooled pneumococcal polysaccharide (1 μg/μl) was also dotted as positive control. After drying for 15 min at room temperature (RT), the nitrocellulose membrane previously cut into strips of 10.5 x 1.0 cm and divided into spaces of 0.5 x 0.5 cm for each dot, was blocked with 2.5 ml of TBS containing 5g% (w/v) gelatin, at RT for 30 min. Each strip washed twice with distilled water and dried between filter-papers was incubated with 2.5 ml of a 1:200 dilution of pneumococcal omniserum (polyvalent antiserum obtained in rabbits, from Statens Seruminstitut, Copenhagen, Denmark) in TBS-G for 2h. After 3 washes (10 min) with TBS containing 0.05% Tween 20 (TBS-T), the material was incubated for 2h with 2.5 ml peroxidase conjugate anti-rabbit IgG (A9169, Sigma Co., St. Louis, USA) diluted 1:1500 in TBS-G. The strips were washed 3 times for 10 min with TBS-T and once with distilled water. The strips were placed in solution of 4-chloro-naphthol (Sigma, Co) in methanol (3 mg/ml) and H_2O_2 (Perhydrol 30%, Merck Co, RJ) (0.6 μl/ml) in TBS. The color development was allowed to occur for 5 min at RT. The reaction was stopped with distilled water. All the steps were allowed with slow stirring.

Statistical analyses

Values of sensitivity, specificity, prevalence, accuracy and positive and negative predictive values of Dot-ELISA were calculated in relation to standardized bacterial culture, counterimmunoelectrophoresis and latex agglutination tests [12]. The Kappa statistics (K) were also evaluated [11] to compare the agreement of Dot-ELISA with each of the other tests (bacterial culture, CIE and LA) and with all three tests. Intra and inter-test reproducibility was evaluated at 95% confidence intervals [11] for all antigen dilutions. In the intra-test, Dot-ELISA was realized fivefold on the same day and in the inter-test, during five different days.

RESULTS

Of the 442 pleural fluid effusions from patients with acute pneumonia submitted to bacterial culture, 40 were positive for *S. pneumoniae*, 21 for *H. influenzae* type b, 3 for *S. aureus* and 378 were negative. In the CIE tests, 131 pleural fluid samples were positive for *S. pneumoniae*, 66 for *H. influenzae* type b and 3 for both bacterial antigens. In the LA tests, 217 samples were positive for *S. pneumoniae*, 92 for *H. influenzae* type b, and 5 for both bacterial antigens.

The Dot-ELISA was positive for 257 pleural fluid samples, 40 of which were coincident with *S. pneumoniae*-positive bacterial culture (15.56%), 131 with CIE-results (51.0%) and 203 with LA-results (79.0%). Of 243 cases of negative Dot-ELISA from the total patients with acute pneumonia, all were *S. pneumoniae*-negative to bacterial culture, 14 were LA-positive (5.8%) and none was CIE-positive.

The samples that were positive for *H. influenzae* type b in the CIE and LA tests were negative for *S. pneumoniae* in Dot-ELISA from the 1:2 dilution. The 3 CIE-positive cases and the 5 LA-positive cases for both bacterial pneumococcal and Hib antigens (cross-reactants) were also negative by Dot-ELISA from the 1:2 dilution. The 3 samples with confirmed bacterial cultures for *S. aureus* were negative in LA and in Dot-ELISA for *S. pneumoniae* when tested from 1:2 dilution. The 38 pleural fluid samples from people with clinical and laboratory diagnoses of tuberculosis as well as the 20 serum samples from healthy children were negative in CIE, LA and Dot-ELISA for pneumococcal antigen detection. Among the samples from patients with confirmed previous antibiotic therapy (108 cases) none of them had positive bacterial culture but 97 (or 89.8%) had positive LA and Dot-ELISA results.

Table 1

Diagnostic performance of dot-enzyme-linked immunosorbent assay (Dot-ELISA), latex agglutination (LA) and counterimmunoelectrophoresis (CIE) in relation to the 500 studied samples.

Evaluating indices	Dot-ELISA	Latex agglutination	Counterimmunoelectrophoresis
Sensitivity	0.940	0.975	0.875
Specificity	0.830	0.613	0.791
Positive predictive value	0.820	0.180	0.267
Negative predictive value	0.942	0.996	0.987
Efficiency	0.880	0.642	0.798

The sensitivity and specificity of Dot-ELISA (Table 1) were 0.940 and 0.830, respectively, compared to the three gold standards taken together (BC + CIE + LA). For CIE these indices were 0.875 (sensitivity) and 0.791 (specificity) and for LA, 0.975 (sensitivity) and 0.613 (specificity). The Kappa (K) agreement coefficient for Dot-ELISA in relation to all three tests (Table 2) was 0.760 (Z_o = 13.38 for $Z_{critical}$ = 1.96).

Twenty five pleural fluid samples were used to evaluate intra and inter-test reproducibility with the titers expressed as $\log_2 T$. Variation indices expressed in terms of standard deviation (SD) were: 0.46, 1.06, 0.50, 0.82 and 0.65 (intra-test) and 0.41, 0.91, 0.46, 0.71 and 0.35 (inter-test), all of them below the accepted control limits of 1.37 (intra-test) and 1.11 (inter-test).

Dot-ELISA for the 442 pleural fluid samples from children with acute bacterial pneumonia yielded an optimal positivity index, 58.2%, in relation to the other methods proposed for the detection of pneumococcal polysaccharide antigens in pleural fluid effusion samples, i. e., 49.1% for LA, 29.6% for CIE and 9.05% for BC (Fig. 1).

DISCUSSION

Several ELISA systems for pneumococcal antibody detection have been developed over the last few years. However, serum levels do not reflect the severity of pneumococcal infection because of the poor antibody responses to type-specific capsular polysaccharides [21]. As to pneumococcal antigen detection, the literature refers almost exclusively to latex agglutination and counterimmunoelectrophoresis [4, 20, 28, 29, 30]. These immunoassays have become very expensive due to the elevated cost of commercial kits for latex agglutination, of cellulose acetate strips and of the concentrated pneumococcal omniserum [22] used in counterimmunoelectrophoresis. This represents a serious problem in developing countries.

ELISA on polyvinyl microtiter plates, capable of detecting pneumococcal antigens in cerebrospinal fluid has been developed with good sensitivity, when monovalent pneumococcal antisera are used [32], but microtiter plates are also very expensive in developing countries. On the other hand, bacterial cultures proved to be a low-sensitivity test due to the common use of previous antibiotic therapy which inhibits bacterial growth in the culture media [9, 24].

When patients with pneumonia are admitted to hospital, antibiotic therapy has often already been initiated by the practitioners and it is difficult to establish the causative agent as the cultures taken are almost invariably negative [13]. KALIN & LUNDBERG [20] had found that only 13% of patients were culture-positive after antibiotic treatment, whereas 56% were still antigen-positive by counterimmunoelectrophoresis. REQUEJO et al. [28] found that 19.13% of patients were blood-culture-positive before antibiotic therapy whereas 18.5% and 44.4% were antigen-positive by CIE and LA, respectively; on the other hand, even after 24 hours of antibiotic therapy, 22.2% (by CIE) and 63.0% (by LA) were antigen-positive and, after 7 days of treatment, 14.8% (by CIE) and 40.7% (by LA) of all patients were still antigen-positive, when the tests were applied using serum samples. These positivities became more elevated, 100.0%, when pleural fluid samples were used as source for capsular antigen detection.

In the present study, a Dot-ELISA using nitrocellulose membrane as support was tested for the detection of pneumococcal polysaccharide antigens. Pleural fluid effusion samples from children with clinical and labo-

Table 2

Kappa statistic index (k) of agreement between Dot-ELISA and the gold standards used for diagnosis, i. e., bacterial culture (BC), counterimmunoelectrophoresis (CIE) and latex agglutination (LA) tests.

Comparison	Agreement index *k*	Confidence interval	Z obtained (Z_o)	Strenght of *k*
Dot-ELISA x BC	0.152	(± 0.032)	2.08	poor
Dot-ELISA x CIE	0.503	(± 0.044)	7.68	moderate
Dot-ELISA x LA	0.734	(± 0.040)	12.92	substantial
Dot-ELISA x (BC+CIE+LA)	0.760	(± 0.040)	13.38	substantial

(*) Z_o significant ($Z_{critical}$ = 1.96, $P < 0.001$) for 95% confidence interval, for all *k* indices.

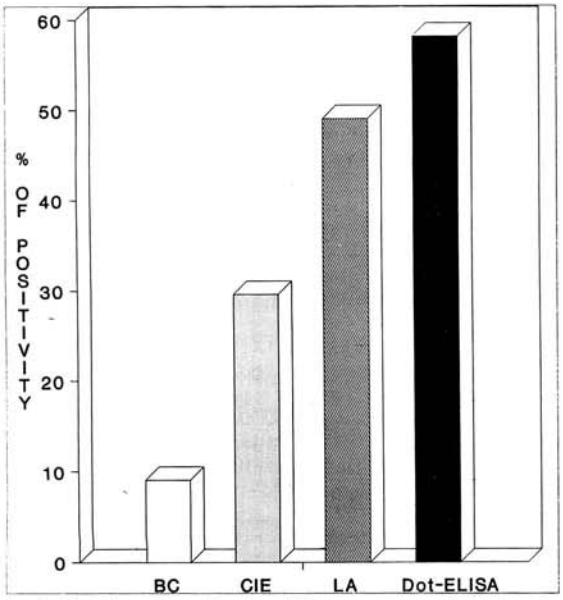

Fig. 1 - Positivity of bacterial culture (BC), counterimmunoelectrophoresis (CIE), latex agglutination (LA) and Dot-ELISA for pneumococcal antigens in pleural fluid samples of 442 patients with acute bacterial pneumonia. The best positivity index was 58.2% for Dot-ELISA, followed in decreasing order by LA (49.1%), CIE (29.6%) and BC (9.05%).

ratory diagnoses of acute bacterial pneumonia were submitted to Dot-ELISA, an immunoassay never used before for pneumococcal polysaccharide antigen detection.

The pneumococcal omniserum employed in the present study reacts against the 83 pneumococcal capsular serotypes and is used for a rapid diagnosis especially in spinal fluid samples [22]. Using counterimmunoelectrophoresis, COONROD & RYTEL [7] showed that reactions of identity occur between purified pneumococcal polysaccharide dissolved in saline solution and pneumococcal polysaccharide antigen present in serum or pleural fluid samples.

About 60 pneumococcal serotypes of pneumococcal meningitis have been identified in Great São Paulo, Brazil, over the last few years [31] and immunoassays have proved to be able to detect pneumococcal antigens in cerebrospinal fluid samples when pneumococcal omniserum is employed [29, 30]. Studies with CIE, LA and enzymatic immunoassays [23] have demonstrated that CIE may detect 20 to 50 ng/ml of pneumococcal, meningococcal and *H. influenzae* polysaccharides, while LA and enzymatic immunoassays may detect 0.3 to 0.6 ng/ml. Pneumococcal capsular antigen detection by immunoassays is a rapid method for establishing a diagnosis in those patients in whom cultures have become negative as well as permitting to define capsular antigen at least 18h earlier than the culture, when positive [18].

Cross-reactions between *S. pneumoniae* and *H. influenzae* type b [19], *Klebsiellae* [16] or *S. aureus* [15] are common sources of false-positive results in immunoassays due to the common epitopes of their polysaccharide antigens. Heat treatment of pleural fluid EDTA was employed to enhance the diagnostic specificity by destroying heat-labile cross-reacting antigens, leaving intact the type-specific polysaccharide [8]. When this procedure was employed in Dot-ELISA together with the use of 5g% (w/v) gelatin in the blocking solution, the immunoassay was found to be more specific for pneumococcal antigen detection.

Dot-ELISA results showed poor agreement with bacterial cultures (k = 0.152) because bacterial growth depends on the presence of viable microorganisms in the body fluids. Agreement with CIE was moderate (k = 0.503) and depended on the presence of a sufficient quantity of soluble antigens in body fluids with electrostatic charges to permit CIE procedure. A substantial agreement was observed with LA (k = 0.734) and with the three (BC + CIE + LA) routine methods (k = 0.760) used as an alternative diagnosis of bacterial pneumonia taken together (Table 2). Comparatively, the diagnosis performance of Dot-ELISA was found to be better than the other two immunodiagnostic methods, since it provided higher sensitivity, specificity and efficiency. Antigen detection still after initiation of therapy even when bacterial cultures are negative, means that sufficient pneumococci were initially present and able to produce the required amount of capsular polysaccharide antigen before losing viability [18].

In view of its execution without the need for reading equipment, and economy of pneumococcal omniserum that may be used at 1:200 dilution, Dot-ELISA proved to be an original and practical alternative procedure for detecting pneumococcal polysaccharide antigens from pleural fluid effusion samples, providing a rapid diagnosis of patients with bacterial pneumonia, even in cases in which pleural fluid is collected after antibiotic therapy. In order to reduce the probability of cross-reactions with other bacterial antigens and to economize reagents, treatment of the pleural fluid samples with EDTA and dilution at 1:2 dilution (cut-off point) in TBS is indicated.

Therefore, Dot-ELISA for antigen detection may be used for the immunodiagnosis of acute bacterial pneumonia in routine hospital laboratories at low cost and with substantial agreement with clinical and standard bacterial culture, counterimmunoelectrophoresis and latex agglutination diagnoses of bacterial pneumonia.

RESUMO

Dot-ELISA para detecção de antígenos polissacarídicos de pneumococos em amostras de líquido pleural. Comparação com cultura bacteriana, contraimunoeletroforese e látex-aglutinação.

Dot-ELISA para detecção de antígenos polissacarídicos de pneumococos foi padronizado em vista da necessidade de se ter um diagnóstico rápido e eficaz para pneumonia pneumocócica aguda. Um total de 480 amostras de líquido pleural sendo 442 de crianças com diagnóstico clínico e laboratorial de pneumonia bacteriana e 38 de pacientes com tuberculose, mais 20 amostras de soros sanguíneos de crianças sadias foram avaliadas no Dot-ELISA. As amostras foram tratadas previamente a 90°C por 10 min com EDTA 0,1 M de pH 7,5 e aplicadas sobre membrana de nitrocelulose. Para a detecção de antígeno pneumocócico foi empregado *omniserum* pneumocócico diluído a 1:200. Os resultados de Dot-ELISA avaliados em comparação com os resultados de cultura bacteriana, contra-imunoeletroforese e látex-aglutinação apresentaram índices de 0,940 para sensibilidade, 0,830 para especificidade e 0,760 para concordância. *Omniserum* pneumocócico mostrou ser um ótimo soro polivalente para a detecção de antígenos pneumocócicos em Dot-ELISA e, essa técnica provou ser uma alternativa prática e eficaz para o diagnóstico de pneumonias pneumocócicas.

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