

DOT-ELISA FOR THE DIAGNOSIS OF NEUROCYSTICERCOSIS

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SUMMARY

The aim of the present study was to standardize and evaluate dot-Enzyme linked immunosorbent assay (Dot-ELISA), a simple and rapid test for the detection of cysticercus antibodies in the serum for the diagnosis of neurocysticercosis (NCC). The antigen used in the study was a complete homogenate of *Cysticercus cellulosae* cysts obtained from infected pigs and dotted on to nitrocellulose membrane. Test sera were collected from the patients of NCC, and control sera from patients with other diseases and healthy students and blood donors of the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) Hospital, Pondicherry, during a study period from 2001 to 2003. Dot-ELISA detected antibodies in 14 of 25 (56%) in clinically suspected cases of NCC, 13 of 23 (56.5%) in CT/MRI proven cases of NCC and 2 of 25 (8%) each in non-cysticercal CNS infection controls and healthy controls. The test showed a sensitivity of 56.25%, specificity of 92%, positive predictive value of 87.09%, and negative predictive value of 70.76%. Results of the present study shows that the Dot-ELISA as a simple test can be used in the field or poorly equipped laboratories for diagnosis of NCC .

KEYWORDS: Neurocysticercosis; ELISA; Dot-ELISA; *Cysticercus* antigen.

INTRODUCTION

Neurocysticercosis (NCC) is an important parasitic disease of the human nervous system and constitutes a public health problem for most of the developing world²³. It has been estimated that the NCC infects approximately 50 million people world-wide and cause at least 50,000 deaths annually². The NCC is endemic in many parts of the world. Taeniasis/cysticercosis is of economic importance in several countries and regions, such as Mexico, Central and South America (except Uruguay and Argentina), Africa, India, Indonesia, Thailand and China^{10,27}. In recent years, an increased incidence of NCC is being reported from America owing to an increase in immigration from endemic regions and also due to improved ease of diagnosis with the imaging techniques. It is common among Hispanic immigrants to the American South west^{4,26,28,31}.

NCC is difficult to diagnose clinically because of its varied clinical presentation and non-specificity. Recently, immunological tests are being increasingly used in adjunct with imaging techniques to aid the diagnosis of NCC. Several immunological tests for demonstration of specific antibodies in the serum and cerebrospinal fluid (CSF) have been devised over the years. The first serological reaction for cysticercosis in the CSF was described by MOSES in the year 1911¹⁸. In most laboratories, indirect haemagglutination (IHA)¹², enzyme-linked immunosorbent assay (ELISA)²⁵, radioimmunoassay (RIA)¹⁶ and most recently the Western blot test³⁴ are being used.

However, the test such as Western blot or plate ELISA although are specific and sensitive, have the disadvantages of being the tests that require expensive equipment, technical expertise, hence difficult to adapt in the field or in the not-so well equipped laboratories. Several authors have hence used dot-enzyme linked immunosorbent assay (Dot-ELISA), a simplified ELISA technique for detection of specific cysticercus antibodies in serum and CSF samples^{9,36,37}. The aim of the present study was to standardize and evaluate dot-Enzyme linked immunosorbent assay (Dot-ELISA), a simple and rapid test for the detection of cysticercus antibodies in the serum for the diagnosis of neurocysticercosis in the less equipped laboratories or in the field situation.

MATERIALS AND METHODS

Patients and controls: Test sera were collected from the patients of neurocysticercosis (NCC), and control sera from patients with other diseases and healthy students and blood donors of the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) Hospital, Pondicherry, during a study period from 2001 to 2003.

The groups included:

Group 1 (strong clinical suspects of NCC): This group included 25 patients with single contrast enhancing ring lesions/single calcified nodular lesion of less than 0.5 cm diameter on CT Scan/MRI presenting

with seizures, hydrocephalus or intracranial hypertension or psychiatric disturbances. This group comprises clinically strongly suspected cases of NCC, a well studied and well accepted clinical entity.

Group 2 (CT/MRI proven cases of NCC): This group included 23 patients with either CT Scan/MRI proven multiple contrast enhancing ring lesions of less than 0.5 cm diameter or multiple nodular calcified lesions of less than 0.5 cm diameter with or without meningoencephalitis. This group comprised clinico-radiologically definite cases of NCC.

Group 3 (Non-cysticercal CNS infection control): This group included 25 patients presenting with single/multiple margin enhancing lesions/calcified polymorphic lesions of more than 0.5 cm diameter with or without meningoencephalitis or microbiologically proven cases of tubercular or cryptococcal meningitis. This group comprised non-cysticercus chronic meningoencephalitis and thus formed an important control group of other infectious diseases.

Group 4 (Healthy controls): This group included 25 healthy adults (blood donors and students) who had not suffered from cysticercosis or any other disease during the study period from 2001 to 2003.

The informed consent was obtained from all human adult participants and from parents or legal guardians of minors. The project was approved by the JIPMER research council.

Serum: Serum samples were collected from all the patients and controls described earlier. Five milliliters of venous blood was also collected from each patient under aseptic precautions and was allowed to clot. The serum was separated and preserved with 0.05 mol/L sodium azide and stored at -20 °C until used.

Preparation of cysticercus antigen: Preparation of *C. cellulosae* porcine complete homogenate antigen was carried out according to the method described by SREENIVASAMURTHY *et al.*³². In this procedure, approximately 100 cysts that were dissected free from pork tissue were washed thrice in PBS (pH 7.2) to remove extraneous matter. The cysts were homogenized in a glass tissue homogenizer with PBS (pH 7.2) containing 0.1 mM phenyl methyl sulphonyl fluoride (PMSF). Homogenization was done under cooling condition. The homogenized tissue suspension was then sonicated eight times at 12 KHz with 30 seconds cooling interval. Each cycle of sonication was for one minute and it was done again under cooling condition in an ice bath. The sonicated material was centrifuged at 40 °C for 30 minutes at 14,000 rpm. The supernatant was collected as the complete homogenate antigen, aliquoted to 1 ml cryoprotected vials and stored at -20 °C.

Detection of cysticercus antibodies in the serum: The circulating cysticercus antibodies were detected in the serum by a plate ELISA and Dot-ELISA.

Plate-enzyme linked immunosorbent assay (ELISA): The presence of cysticercus antibodies in the patient's serum was also detected by a commercially available plate ELISA kit marketed with the brand name UBI, MAGIWEL enzyme immunoassay-cysticercosis (Germany). The tests were carried out according to manufacturer's instructions. Result was noted as per the instructions provided along with the kit.

Dot-enzyme linked immunosorbent assay (Dot - ELISA): The Dot-ELISA depends upon the principle that when test serum is layered on the nitrocellulose membrane bound with the cysticercus antigen, the specific cysticercus antibodies, if present in the serum, will bind to the cysticercus antigen dot. This binding reaction is detected visually by addition of an enzyme-labeled second antibody and subsequent development of the label antibody.

Dot-ELISA was standardized to detect cysticercus antibodies in the serum of NCC patients.

The procedure consists of the following steps:

(i) 2 µl of complete homogenate (CH) porcine cysticercus antigen of 0.72 g% was added to square sized nitrocellulose membrane pasted on plastic strips (3 cm × 0.5 cm). The strips with the antigen were air dried for 30 minutes to 45 minutes. The antigen in the strips was then blocked with a diluent (2% BSA in PBS, pH 7.2) for three hours over a shaker. Washing of the strips was done three times with 0.1% Tween 20 in PBS (pH 7.2) over a period of 15 minutes.

(ii) The strips were incubated at room temperature with 2 µl of patients serum diluted with 20 µl of 0.1% Tween 20 in PBS (pH 7.2), for 90 minutes with continuous shaking.

(iii) Washing of the strips was then repeated three times. Then commercially available rabbit antihuman IgG peroxidase conjugate marketed by BANGALORE GENEI, INDIA was added as (1:1000 dilutions in PBS Tween 20) and incubated for 30 minutes over the shaker. Again washing was repeated three times.

(iv) Then 5 mg of diaminobenzidine substrate in 5 ml of PBS Tween 20 (1:1000 dilutions) was added to the strips followed by 5 µl of hydrogen peroxide. The strips were then incubated for 10 minutes in the dark.

(v) Reaction was stopped with distilled water and the results were read immediately. The results were read as development of a deep reddish brown coloured dot in nitrocellulose membrane was considered as reactive, where as colourless dot was considered as non-reactive.

Statistical analysis of the immunoassays: The statistical analysis was carried out using Epi Info package supplied by the WHO.

The serum samples were tested in a single blind manner. The sources of the specimen (whether they were from the patients or from control subjects) were not known to those performing the different tests with these specimens. The sensitivity, specificity, positive predictive value, and negative predictive value of the tests were calculated according to the method described by GALEN & GAMBINO⁶.

RESULTS

Dot-ELISA: The serum samples, which showed development of brick red coloured dot on nitrocellulose membrane, were considered reactive. The cysticercus antibodies could be demonstrated by Dot-ELISA in the sera of different groups of NCC and controls. Dot-ELISA detected antibodies in 14 of 25 (56%) in clinically suspected cases of NCC, 13 of

23 (56.5%) in CT/MRI proven cases of NCC and 2 of 25 (8%) each in non-cysticercal CNS infection controls and healthy controls.

The sensitivity, specificity, positive predictive value and negative predictive value of the tests by Dot-ELISA were found to be 56.25%, 92%, 87.09% and 68.66% respectively.

Plate ELISA: Commercial microtitre plate ELISA demonstrated a positivity of 9 of 25 (36%) in clinically suspected cases of NCC, 12 of 23 (52.1%) in CT/MRI proven cases of NCC and 1 of 25 (4%) in healthy controls. However, no antibodies were detected in serum of non-cysticercal CNS infection controls.

The sensitivity, specificity, positive predictive value and negative predictive value of the tests by Plate ELISA were found to be 43.75%, 98%, 95.45% and 64.4% respectively.

DISCUSSION

In India, the NCC has been reported widely from different parts of the country as Delhi¹, Uttaranchal region³⁵, Chandigarh¹⁴, Pondicherry^{33,38}. Before the advent of CT/MR era, the frequency of NCC as a cause of epilepsy in India was reported to vary from 2.2% to 9.6%^{11,15}. After the availability of CT/MR imaging, NCC has been found to be the cause in 9% to 18.6% of patients with epilepsy^{7,8}. Varied reports are available regarding the role of NCC in intracranial space occupying lesion. While some workers report that 1% of intracranial space-occupying lesions in children in India are accounted for by parasites²², others show that 17.4% - 29.2% of cases of intracranial space occupying lesions and epilepsy are due to cysticercosis¹⁰. In Bangalore, approximately 26% of the space-occupying lesions of the CNS were found to be caused by cysticercosis¹³. In JIPMER, Pondicherry a total of 21 childhood neurocysticercosis was reported over five years from 1984 to 1989³³.

Demonstration of specific antibodies in the serum is the most common approach for the diagnosis of parasitic diseases. A number of serological tests have been evaluated and used for the detection of specific antibodies in the serum with variable results in the diagnosis of NCC.

The ELISA and EITB are the most frequently used antibody-based tests for the detection of antibodies in the serum⁵. ELISA and EITB were used to detect serum IgG antibodies to *Taenia solium* metacestodes^{17,29} while ELISA also measured IgM antibodies against *Cysticercus* antigen in both serum and CSF samples of patients with active NCC²⁵. The ELISA has been used widely by various authors for the detection of cysticercus antibodies in the serum with variable sensitivities of 50%²⁵, 10.4%¹⁷, 79%³ and 93%³⁰ and specificities of 70%²⁵ and 93%³⁰ for the diagnosis of NCC. Also the ELISA test demonstrated cross reactions with other parasitic diseases as hydatid disease, schistosomiasis and angiostrongyliasis^{3,17}. In the present study, the plate ELISA showed a low sensitivity of 43.75% but a high specificity of 98%.

Dot-ELISA, developed as a modification of the ELISA using nitrocellulose membrane as a carrier of protein, has become increasingly popular as a field test for the diagnosis of many parasitic diseases such as visceral leishmaniasis^{19,20}, malaria, schistosomiasis mansoni and fascioliasis²¹ and cystic echinococcosis²⁴.

Dot-ELISA has also been employed in NCC for the detection of cysticercus antibodies in the serum as well as in the CSF^{9,36,37}. A preliminary study conducted on CSF samples for the presence of specific cysticercus antibodies, with the cysticercus antigen dotted on a new solid phase of synthetic polyester fabric impregnated with a polymerized resin, revealed, 14 out of 15 CSF samples of NCC to have titres ranging from one to 128³⁶. Another study on CSF samples for cysticercus antibody detection, by Dot-ELISA showed sensitivity and specificity of 95.1% and 90.6% respectively for *C. cellulosae* antigen, while showed a sensitivity of 97.6% for *Cysticercus longicollis* antigen and specificities of 96.9% for the membrane – *C. longicollis* (M-Cl) antigen and 100% for the vesicular fluid – *C. longicollis* (VF-Cl) antigen³⁷. For testing serum samples for specific cysticercus antibodies in patients with NCC, Dot-immunogold-silver staining (Dot-IGSS) and Dot-ELISA using *T. solium* antigen have been compared. The average titre of the sera detected by Dot-IGSS was 1:27,470, which was significantly higher than that detected by Dot-ELISA⁹. In the present study, Dot-ELISA using the complete homogenate antigen of *T. solium*, tested only on serum samples for specific cysticercus antibodies was found to be only 56.25% sensitive and 92% specific for diagnosis of NCC. Although the test is of moderate sensitivity, it has potential for wider use for diagnosis of NCC, specially in the field or in poorly equipped laboratories in the absence of imaging facilities such as CT and MR which are highly expensive.

Dot-ELISA as a test for use in the field or poorly equipped laboratories for the diagnosis of NCC offers many advantages. The foremost advantage is that the test is a rapid test, is easy to perform and the results are read visually, eliminating the need for an ELISA reader. Second, the test can be carried out by imparting minimal basic training to locally available technical staff and, it is easy to use. Third, nitrocellulose binds more antigens than microtitre plates. Finally the test is inexpensive because the cost of nitrocellulose membrane is less than that of microtitre plates.

RESUMO

Dot-ELISA no diagnóstico da neurocisticercose

O objetivo do presente estudo foi estandardizar e avaliar o Dot-ELISA, um teste simples e rápido para detectar anticorpos de cisticercos no soro para diagnóstico da neurocisticercose (NCC). O antígeno usado no estudo foi um homogenizado completo de cistos de *Cysticercus cellulosae* obtidos de porcos infectados e marcados sobre a membrana de nitrocelulose. Os soros testados foram coletados de pacientes com NCC e os soros controle de pacientes com outras doenças e estudantes saudáveis e doadores de sangue do "Jawaharlal Institute of Postgraduate Medical Education and Research Hospital", em Pondicherry, durante o período de estudo de 2001 a 2003. Dot-Elisa detectou anticorpos em 14 de 25 (56%) casos suspeitos de NCC, em 13 de 23 (56,5%) em CT/MRI casos provados de NCC e em 2 de 25 (8%) cada em controles de infecções do sistema nervoso não devidas à cisticercose e controles saudáveis. O teste mostrou sensibilidade de 56,25%, especificidade de 92%, valor preditivo positivo de 87,09% e valor preditivo negativo de 70,76%. Resultados do presente estudo mostram que o Dot-ELISA como teste simples pode ser usado em trabalhos de campo ou em laboratórios pobremente equipados para o diagnóstico da NCC.

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