Evaluation of the MTT lymphocyte proliferation assay for the diagnosis of neurocysticercosis


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A B S T R A C T

Neurocysticercosis (NCC) is caused by the larval form of the pork tapeworm Taenia solium when lodged in the central nervous system (CNS). Clinical diagnosis of NCC is complicated due to its polymorphic manifestations with no specific signs or symptoms. A wide range of serological assays and neuroimaging modalities are used for its diagnosis. The aim of the present study was to evaluate the MTT assay for the diagnosis of NCC and to determine its sensitivity, specificity and accuracy. MTT assay was based upon the cellular reduction of the tetrazolium salt by the proliferating cells and quantification of the colored product. Total 59 patients with NCC-related active epilepsy (AE), 30 with AE other than NCC (disease controls) and 64 healthy volunteers were enrolled for the study. Lymphocytes were freshly isolated from the enrolled subjects and cultured on cyst fluid antigen coated tissue culture plates. MTT assay was performed according to the standard protocol. The mean values of proliferation index (PI) with cyst fluid antigens were 2.13 ± 0.72, 0.622 ± 0.31 and 0.71 ± 0.36 for NCC patients, disease controls and healthy volunteers respectively. PI values for NCC patients were higher than the cut-off value (mean of controls + 2 standard deviations; 1.31). The sensitivity, specificity and accuracy of the MTT assay for the diagnosis of NCC were 87.93%, 94.68% and 91.5% respectively. For single cyst infection the sensitivity of the assay was found to be 86.4%. The present study shows that MTT is an adaptable technique which can be used for diagnosis of NCC.

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1. Introduction

Neurocysticercosis (NCC) is the most common helminthic parasitic infection of the central nervous system (CNS), caused by the larval form of the pork tapeworm Taenia solium. It is endemic in most developing countries of Asia, Latin America, Central and South Africa (Cruz et al., 1999; Garcia-Noval et al., 1996; White, 1997) and is also being increasingly reported in the developed countries due to the migration of persons with the disease or T. solium carriers. Clinical diagnosis of NCC is intricate due to its polymorphic manifestations with no specific signs or symptoms (Carpio et al., 1998; Del Brutto et al., 2001; Rahalkar et al., 2000). Approximately 50 million people worldwide are infected (Pardini et al., 2001) and it is the single most common cause of acquired epileptic seizures in the developing world where prevalence rates of active epilepsy are twice those in developed countries.

A wide range of serological assays have been used in diagnostic and epidemiological studies to detect anticysticercal antibodies in serum/cerebrospinal fluid (CSF). These assays however, lack either specificity or sensitivity for human cysticercosis. Such tests include ELISA for the detection of antibodies in the serum; however this is associated with high rates of false-positive and false-negative results due to cross-reactivity from heterologous infection (Ramos-Kuri et al., 1992). An enzyme-linked immuno-electrotransfer blot (EITB) was also developed as serological test for the diagnosis of NCC at the Centre for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. The test uses lentil lectin purified glycoproteins which are specific for human cysticercosis, with reported a sensitivity of 98% and a specificity of 100% (Tsang et al., 1989). It is commercially available and is also included as a major criterion for the diagnosis of NCC (Del Brutto et al., 2001), besides ELISA performed with CSF. EITB was claimed to be the most reliable serological test for the diagnosis of NCC but several studies showed variable sensitivities. In a study on patients having a single cyst, the sensitivity of EITB was found to be 28% (Wilson et al., 1991) and a recent study from India demonstrated that only 26.1% patients with NCC were positive by EITB (Rajshhekar et al., 2006). Neuroimaging modalities such as computed tomography (CT) and magnetic resonance imaging (MRI) are the most commonly
used techniques for diagnosing NCC, which have greatly improved the
diagnosis of NCC. But these techniques are expensive and generally
not available in disease endemic areas of most developing countries.
Recently, lymphocyte transformation test (LTT) was developed as an
immunodiagnostic modality for the diagnosis of NCC from our centre
with a sensitivity of 93.75% and a specificity of 96.20% (Prasad et al.,
2008).

The MTT assay was used as an alternative to thymidine based LTT.
In this assay, mitochondrial enzyme activity of the proliferating cells
reduces yellow tetrazolium salt \((3-(4,5\text{-dimethylthiazol-2-yl})-2, 5-
diphénylethényl tetrozoïd bromide)\) to purple colored formazan, and the
intensity of color development is detected by colorimetric method.
The aim of the present study was to develop and evaluate the MTT, a
non-radioactive assay for the diagnosis of NCC and to determine its
sensitivity, specificity and accuracy.

2. Materials and methods

2.1. Study subjects

A total of 153 individuals were enrolled for the study from the rural pig farming community of the Mohanthalganj block, Lucknow
district India. The distribution of the patients was as follows: patients
with NCC-related active epilepsy \((n = 59)\), age/gender matched
patients with active epilepsy other than NCC \((n = 30)\), and healthy volunteers \((n = 64)\). Institutional ethics com-
mittee approved the study and informed consent was obtained from
all the patients or their nearest kin (in case of minors).

2.2. Diagnosis of NCC-related active epilepsy and seizures other
than NCC

A definitive or probable diagnosis of NCC was made on the basis of
proposed clinical, radiological and epidemiological criteria (Del Brutto
et al., 2001). All the subjects included in the study underwent T1 and
T2 weighted magnetic resonance imaging (MRI) examination of the
brain. Active epilepsy was defined in a patient who had 2 or more
episodes of seizures, one of which had occurred in the previous 5 years,
regardless of antiepileptic drug treatment. Patients who fulfilled the
criteria of either definitive or probable diagnosis were considered to
have NCC-related active epilepsy and enrolled for the study.

None of the NCC patients and controls received corticosteroid or
anti helmimthic therapy at least 12 weeks prior to sample collection.

2.3. Antigen preparation

*Taenia solium* cysts were collected from the pig muscles and cyst
fluid was aspirated. The collected fluid was sonicated at 20 kHz for
4 min of 1 min pulse each and centrifuged at 200,000 g for 2 h.
Supernatant was collected and phenyl methyl sulfonyl fluoride
(Sigma, St. Louis, MO) was added to a final concentration of \(4 \times 10^{-1} \text{ mmol/L}\). The protein was estimated by modified Lowry’s method
and the supernatant was stored at \(-80°C\) till further use.

2.4. Isolation of peripheral blood lymphocytes

Blood samples were collected from all the subjects in heparin
 tubes and diluted in 1:1 ratio with 1 M phosphate buffered saline
(PBS). The lymphocytes were isolated according to the protocol
followed for Ficoll–Hypaque (Research grade; Pharmacia, Uppsala,
Sweden) density gradient method as described in our earlier study
(Prasad et al., 2008). In brief, 3 ml of Ficoll–Hypaque was taken in a
15 ml tube and 10 ml of diluted blood was loaded without disturbing
the interface and centrifuged at 1800 g for 30 min. Lymphocyte ring
was separated and washed thrice with PBS. Cell viability was checked
by trypan blue (Gibco, Auckland, New Zealand) staining and cells
were counted using haemocytometer as per standard procedure. Cells
were finally suspended in RPMI-1640 supplemented with 10% fetal
calf serum (FCS) (Gibco, Auckland, New Zealand).

2.5. MTT assay

Cell proliferation was tested using a \(3-(4,5\text{-dimethylthiazol-2-yl})-2, 5-
diphényl tetrozoïd bromide (MTT)\) assay (MTT assay kit,
Cayman, USA). For the assay, lymphocytes were freshly isolated and
plated in 96-well flat bottom tissue culture plate at a concentration of
\(1 \times 10^{5}\) cells/well containing 200 µl of RPMI-1640 (supplemented
with 10% FCS) tissue culture medium. For a test sample, cells were
cultured with 20 µg of cyst fluid antigen, as a positive control cells
were cultured with 2.5 µg of phytohaemagglutinin (PHA) and for
negative control cells were cultured without cyst fluid antigen or PHA.
The optimal concentration of cyst fluid antigen and PHA were chosen
empirically by titration. At 72 h of culture, 10 µl of MTT solution was
added per well. After 3 h of incubation, colored crystals of formazan
were dissolved with a 100 µl of dissolving solution. Plates were kept
on orbital shaker for 5 min and optical density (O.D.) was read on a
multiwell scanning spectrophotometer (ELISA reader) at 570 nm.

2.6. Proliferation index and cut-off value

Proliferation index (PI) was calculated by [O.D. of test sample – O.D.
of negative control]/O.D. of negative control]. Cut-off value for the
diagnosis of NCC-related active epilepsy was defined as the PI more than
the mean PI of all controls + 2 standard deviations (SDs) (Prasad et al.,
2008; Nyati et al., 2010). The sensitivity, specificity and accuracy of the
test were calculated using the following equations:

\[
\text{Sensitivity} = \frac{\text{no. of true positives in tests}}{\text{total true positives}} \times 100
\]

\[
\text{Specificity} = \frac{\text{no. of true negatives in tests}}{\text{total true negatives}} \times 100
\]

\[
\text{Accuracy (performance index)} = \frac{\text{no. of true positives and true negatives in test}}{\text{all tested samples}} \times 100.
\]

2.7. Statistical analysis

Data was analyzed with SPSS statistical software version 16.0
(SPSS, Chicago, IL). The mean values of all the groups were compared
by independent t-test, and \(p \leq 0.05\) was considered significant.

3. Results

3.1. Diagnosis of NCC

Definitive or probable diagnosis of NCC related with active epilepsy
was made in 59 patients. Single cyst, two and multiple (more than
two) cysts were detected on MRI in 37, 7 and 15 patients respectively.

3.2. MTT assay

The mean values of proliferation index with cyst fluid antigens were
\(2.13 \pm 0.72\), \(0.622 \pm 0.31\) and \(0.71 \pm 0.36\) for NCC patients,
disease and healthy controls respectively. In patients with NCC, the
PI values were significantly higher than both the control groups
\((p<0.001)\) (Fig. 1). The mean PI value of both the control groups was
\(0.65 \pm 0.33\), therefore the value above 1.31 was considered for the
diagnosis of NCC. The mean PI values with PHA were comparable in
both the groups with no signifcant difference. The PI values with PHA
were \(4.30 \pm 1.91\), \(4.97 \pm 1.12\) and \(4.86 \pm 1.13\) for NCC patients,
healthy controls and disease controls respectively. Out of 59 NCC
patients, cells from 8 patients have PI value less than the cut-off whereas 2 disease and 3 healthy controls have PI value above the cut-off. Of 8 MTT negative NCC patients, 5 patients had single cyst and 3 had multiple cysts. The sensitivity, specificity and accuracy of the assay for the diagnosis of NCC-related active epilepsy were 87.93%, 94.68% and 91.5% respectively. The mean PI values for single cyst, two and multiple cysts infection were 2.01 ± 0.72, 2.12 ± 0.24 and 2.16 ± 0.89 respectively (Fig. 2). For single cyst infection, the sensitivity of the assay was 86.4%. On the basis of different stages of parasites in patients with NCC group, the mean PI values for viable, degenerating, calcified/ healed and multiple stages were 1.66 ± 0.37, 2.26 ± 0.86, 1.76 ± 0.59, 2.19 ± 0.60 and 2.02 ± 0.80 respectively with no significant difference (p ≥ 0.05) (data not shown).

4. Discussion

In the present study, the MTT assay using cyst fluid antigens was evaluated for its efficiency for the diagnosis of NCC-related epilepsy in the rural pig farming community. The results were compared between NCC patients and two groups of controls. The sensitivity, specificity and accuracy of the assay were found to be 87.93%, 94.68% and 91.5% respectively for the diagnosis of NCC and for solitary cysticercus granuloma the sensitivity was 86.4%. Neuroimaging by magnetic resonance imaging (MRI) was used as a gold standard against which the assay was compared.

The MTT tetrazolium salt colorimetric assay was initially described to measure mammalian cell survival and proliferation (Mossman, 1983). It is a quantitative colorimetric method which relies upon the cellular reduction of tetrazolium salts by dehydrogenase enzymes of metabolically active cells into highly colored formazan products. Since its first description, the assay was further explored to extend its application in various fields. It has been used successfully in studies of chemosensitivity (Arnould, and Dubois, 1999), radio sensitivity (Price and McMiller, 1990), cell stimulation in immunology (Nikes and Otto, 1990), cytokine production (Leslie and Hay, 1989), immunocytotoxic activity (Ferrari et al., 1990), to evaluate the viability of filarial parasite (Comely and Turner, 1990), as clonogenic assay (Shimoyama et al., 1989), in hematological malignancies (Hayon et al., 2003) and for testing chemotherapeutic drugs (Ulukaya et al., 2008).

Serological tests such as ELISA/EITB were also developed for diagnosing NCC but these methods are inadequate because of the natural history of NCC. These tests are normally used as an adjunct along with neuroimaging techniques to confirm the diagnosis. The evolving nature of the CNS lesions, variation in the stage and number of lesions, the intensity of infection and measurable antibody levels vs. CNS invasion affect the serological testing. The sensitivity of ELISA varied from 43% to 93% (Biswas et al., 2004). Recent studies show that the detection of antibodies by ELISA can be stage specific and also depends upon the source of antigens (Sahu et al., 2009; Rodriguez et al., 2009). EITB is a very specific test for NCC but various reports demonstrate that there is a variation in the sensitivity and specificity of the EITB (Prabhakaran et al., 2004; Singh et al., 1999). A study on patients with definitive NCC suggested 50% and 63% sensitivity of EITB in serum and CSF respectively (Sanchez et al., 1999). Besides these, molecular methods like polymerase chain reaction based assays for the detection of T. solium eggs; proglutelides and cysts have been described (Gonzalez et al., 2005). However the problem with this tool is the same as with ELISA; none has been standardized for endemic communities.

The results from the present study illustrate that MTT can be used as the diagnostic test for NCC using cyst fluid antigens. The assay measures the lymphocyte proliferation after stimulating them with cyst fluid antigens. The mean values of PI with cyst fluid antigens were 2.13 ± 0.72, 0.622 ± 0.31 and 0.71 ± 0.36 for NCC patients, disease controls and healthy controls respectively. The lymphocytes pre-exposed to cyst fluid antigens in the patients, proliferate rapidly compared to disease and healthy controls when challenged with the same antigen in vitro (Restrepo et al., 2001). In contrast, it has been reported earlier that mononuclear cells from NCC patients show antigen specific suppression when compared with a control group (Buono et al., 2001) and the probable reasons are the higher concentration of antigen used, composition of the antigen or the corticosteroid treatment. Further, Correa et al. (1989) have reported a decrease in the CD4+ /CD8+ ratio in NCC patients suggesting the involvement of CD8+ cells in immune suppression. An animal study demonstrated that an increase and/or decrease in CD8+ cells depends on the evolutive phase of the disease (Meeusen et al., 1989) while some investigators confirmed that CD4+ T cells are involved in the suppression of immune system in experimental model (Sciutto et al., 1995; Robinson et al., 1997). However, the data from the present study showed that there was no apparent variation in lymphocyte proliferation among NCC patients and control groups with PHA. The mean PI values with PHA were 4.30 ± 1.91, 4.97 ± 1.12 and 4.86 ± 1.13 for NCC patients, healthy controls and disease controls respectively having no significant difference (p > 0.05). This shows that patients with NCC have an intact peripheral cellular immune response in the absence of concurrent corticosteroid treatment and these results are consistent with the previous studies (Blanca et al., 2001; Prasad et al., 2008).
Earlier LTT, standardized from our centre for diagnosis of NCC has the sensitivity and specificity of 93.75% and 96.2% respectively. For single cyst infection the sensitivity of LTT was 87.5% (Prasad et al., 2008). However, the test requires trained manpower, specialized equipments such as scintillation counter, radioactive ³H– thymidine and can only be performed at reference laboratories. Comparative result shows that LTT is more sensitive than MTT assay for diagnosis of NCC even though MTT assay have several advantages over LTT. MTT is non-radioisotopic assay, which can be easily performed and quantified. The method is technically and practically simple and the results can be obtained rapidly. It has replaced LTT in various applications such as for quantitating macrophase cytotoxicity (Ferrari et al., 1990), to measure proliferation and cell death of human peripheral blood lymphocytes (Weichert et al., 1991) and for detection and quantitation of murine and human IL-4 and IL-2 (Gieni et al., 1995). However, the performance of MTT will be interpreted with caution since patients with toxemia and other parasitic infections had not been included in the study.

5. Conclusion

The present study shows that MTT is a simple and reliable technique with reasonable sensitivity and specificity for the diagnosis of NCC. However, the assay needs further validation in different patient populations like toxemia and other parasitic infections and also in different geographical areas with a large sample size for its broader application in the diagnosis of NCC.

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References

Arnould, R., Dubois, J., 1999. Comparison of two cytotoxicity assays, tetrazolium derivative reduction (MTT) and tritiated thymidine uptake on the malignant cell lines using chemicalotherapeutic agents. Cancer Res. 10, 145.