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RESEARCH ARTICLE

Real Time Loop Mediated Isothermal Amplification Assay of Urine for Diagnosis of Neurocysticercosis: A Preliminary Observation Study

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ABSTRACT

Objectives: Neurocysticercosis (NCC) is one of the commonly Neglected tropical disease worldwide. Improvement in Living conditions with better diagnostics can reduce the incidence of this disease. The burden of NCC is high in areas with poor socio-economic development. Despite high prevalence in India, the diagnostic challenge remains especially when differentiating from tuberculosis which is also common in the same setting. We describe a novel and rapid diagnostic method for NCC, which might add to our diagnostic Repertoire.

Methods: It was prospective case control study involving consecutive patients of definite and probable NCC at a tertiary teaching hospital in Northern India. LAMP assay in urine was performed in all the patients. LAMP amplified target *Taenia solium cox*1 gene at 60ºC in 120min. The results were compared with 24 controls. The specificity, sensitivity, positive predictive value and negative value were calculated using a 2X 2 contingency table.

Results: Total of 58 patients recruited, 53 were definitive NCC and 5 had probable NCC based on Del Brutto criteria and 24 volunteers were taken as control all of them underwent urine LAMP of *T. solium*. *T. solium cox*1 gene was detected in 60% of Urine samples in patients of NCC, overall specificity of LAMP assay was 92%. The negative predictive value and positive predictive value of real time LAMP assay was 50% and 95%.

Conclusions: Conclusion: Real time urine LAMP assay for *T. solium* gene offers noninvasive, cost effective and rapid method to detect Taenia parasite in patients, in Addition to available investigations. Specially in resource limited setting of endemic countries. *J Microbiol Infect Dis 2020; 10(3):154-159.*

Keywords: *Loop mediated isothermal amplification, Taenia solium, cox1 gene, Neurocysticercosis, Urine*

INTRODUCTION

Neurocysticercosis (NCC), resulting from infection of human brain by larval stage of tapeworm *Taenia solium*, has been a major public health concern in Asia, Africa, and Latin America [1], affecting millions of people in these countries. NCC remains the principal reason for higher rate of epilepsy observed in these regions compared to the western population [2].

At present, diagnosis of NCC is based primarily on neuroimaging complemented by immunological tests documenting presence of cysticercal antigens or antibodies [3] according to the revised diagnostic criteria for NCC [3].

Among the various immunological tests, the most commonly used ones include enzyme linked immunoelectro transfer blot (EITB) and enzyme linked immunosorbent assay (ELISA) in serum and CSF (cerebrospinal fluid). Major disadvantages of EITB are limited availability, high cost and low sensitivity in patients with single cysts which constitute approximately 60-70% of all patients with NCC [4]. Anticysticercal antibody-based tests may yield false positive results compared to detection of cysticercal antigens. These antigen-based tests were found to be useful for follow up of patients of NCC, but has low sensitivity for initial diagnosis [3]. Thus, there is a need to develop immunodiagnostic

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tests with high sensitivity, high specificity, low cost and wider applicability.

Recently Loop mediated isothermal amplification assay or LAMP, is a relatively inexpensive, easy to perform tests which has been found to be useful in detection of various infections [5,6]. A major problem with conventional LAMP is that its interpretation is based on detection of turbidity in reaction tube and may yield false positive results. To obviate this problem, real time LAMP assay is developed which is based on detection of florescence in the reaction tube and thus avoids false positives [7,8].

Urine based screening tests are novel methods for diagnosis of NCC. In this study, we have evaluated utility of Real time LAMP assay (based on detection of, *T. solium cox*1 gene, in urine samples of patients) in diagnosis of NCC.

Aims and objectives

To evaluate utility of Real time LAMP assay of urine (i.e. detection of *T. solium cox*1 subunit gene) in diagnosis of NCC.

METHODS

Our research enrolled 58 patients of NCC, who were consecutively attending Neurology clinic in a tertiary care in Northern India. They were diagnosed with NCC, based on Del Brutto criteria [9]. There was a total of 53 definite and 5 probable cases of NCC according to the said criteria. Individual consent to enroll in this research was obtained from all patients and the subjects were enrolled and the research was approved by the Ethics Committee of the institute. After enrollment a detailed demographic and medical history and examination was noted according to a predesigned study specific Performa. MRI (magnetic resonance imaging) of brain along with (susceptibility weighted imaging) SWI and (constructive interference in steady state) CISS-3D sequences were done. Patients requiring cysticidal therapy were treated as per standard treatment guidelines. A urine analysis for excretion of *COX*1 of *T. solium* gene was done by using LAMP assay. Details have been given below. The results were then compared with 24 controls. For the LAMP assay 15 ml of urine samples, individually from all patients and control subjects, were collected in sterile disposable plastic containers respectively. Urine samples were centrifuged at 3500 rpm for 20 minutes and the supernatant was discarded till 1.5 ml of urine remained in tube. The samples were stored at-20 ^{0}C till further use. Extraction of DNA was carried out using “spin column kit (DNeasy blood and tissue kit, Qiagen)” and extracted DNA in elution buffer, was again stored at -20 ^{0}C for future use.

Real time LAMP assay was performed as described previously by Nkouawa et al [10] and Goyal et al [11] with modifications. LAMP assay results for some patients are shown in Figure 1. DNA from cysticerci cysts, obtained from flesh of infected pigs, which was brought from slaughter houses and pig farms was used as positive control, while sterile “molecular grade water” was used as negative control. These results were compared with 24 control samples.

Real time LAMP assay sensitivity: Sensitivity of assay was calculated to be 1 fg (femtogram) of extracted DNA in tested samples.

RESULTS

In current study 58 patients of NCC were included. Of which a majority i.e. 50 (86.2%) patients had intraparenchymal cysts alone, whereas 8 (13.7%) had both intraparenchymal and extraparenchymal brain cysts. The distribution of patients with solitary and multiple NCC was equal i.e. 50% each. The most common symptom was seizures (79.3%) followed by headache (56.9%), while altered sensorium was seen only in 5 (8.6%) of patients. Majority of cysts were in colloidal-nodular stage (58.6%), followed by vesicular (8.6%) and calcified stage (5.2%). 6.9% of patients had racemose cysts and multistage cysts were seen in 20% of patients. These results are exhibited in Table 1.

Real time LAMP (RT- LAMP) assay of urine: RT-LAMP method detected *cox*1 *T. solium* gene in total of 60.3% of all urine samples of NCC patients. The gene was detected in 58% of total patients with intraparenchymal cysts only and in 75% of total patients with both extra and intra parenchymal cysts. *T. solium cox*1 gene was present in 8.3% of control subjects by the LAMP assay, citing a possibility of endemicity and exposure to the parasite in the general population. The overall sensitivity of RT- LAMP assay for detection of *cox*1 gene was 60.3% in urine samples collected from NCC patients.

Table 1. Baseline characteristics of study population.

Groups	Patients with NCC (n=58)			Controls (n=24)	
Parameter	All Cases, No. of patients (%age)	IP – NCC, (N=50), n (%age)	EP – NCC, EP+IP-NCC, (n=8), n (%age)	No. of patients (%age)	P (cases vs. controls)
Age, y, median (Interquartile range)	26 (16.5%)	24.5 (17.7%)	31.5 (10.75)	34.5(8)	0.05
Gender (Male)	37 (63.8%)	32 (64%)	5 (62.5%)	18 (75%)	0.3
Headache	33 (56.9%)	27(54%)	6(75%)	-	-
Altered Sensorium	5 (8.6%)	2 (4%)	3 (37.5%)	-	-
Seizures	46 (79.3%)	40(80%)	6 (75%)	-	-
Number of solitary cysts on neuroimaging	29 (50%)	27 (54%)	2 (25%)	-	-
Number of multiple cysts on neuroimaging	29 (50%)	23 (46%)	6 (75%)	-	-
Type of cysts on neuroimaging					
Only vesicular	5 (8.6%) Solitary ir 05	5 (10%) Solitary in 05	0(0)	-	-
Only colloidal/ nodular	34 (58.6%) Solitary in 23	32 (64%) Solitary in 21	2 (25%) Solitary in 2	-	-
Only calcified	3 (5.2%) Solitary ir 1	3 (6%) Solitary in 1	0(0)	-	-
Racemose cysts	4 (6.9%)	1 (2%)	3 (37.5%)	-	-
Multiple stage	12 (20.7%)	9 (18%)	3 (37.5%)	-	-

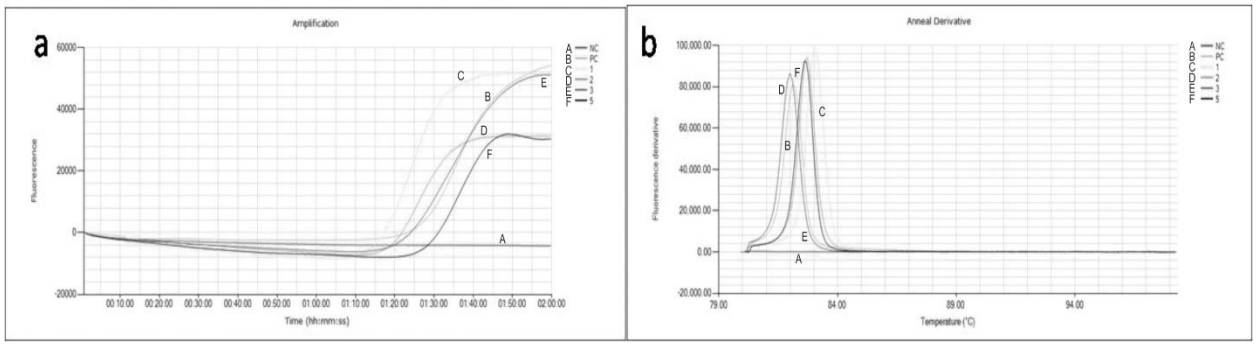

Figure 1. Real time LAMP assay of urine for *Taenia solium cox*1 gene. Figure a: Showing plot of florescence versus time of incubation. Note negative control (nc) is represented by A, which does not show any florescence. Positive control (po) by B and patients (1, 2, 3, 5) represented by C, D, E, F show positive florescence which usually starts after an incubation time varying between 1:15 hours to 1:30 hours; Figure b: showing plot of florescence versus annealing temperature. Note Florescence starts at a temperature of around 80 ^{0}C and peaks between 80 ^{0}C -84 ^{0}C.

The overall specificity of RT- LAMP assay was 91.7% in both subgroups. The RT- LAMP assay was 58.6% and 62.1% sensitive in patients with solitary and multiple cysts respectively, while its specificity was 91.7% in both solitary and multiple NCC as well as in various subtypes of NCC. LAMP assay sensitivity was low in patients with calcified cysts (66.7%), colloidal nodular cysts (55.9%) and racemose (50%), and while it was 100% in patients with multiple cysts (Table 2). The NPV (negative predictive value) ranged between 48-50% whereas the PPV (positive predictive value) of RT- LAMP method was close to 95% for NCC- both solitary and multiple NCC (Table 3).

Table 2. LAMP results and validity of the assay (Urine).

LAMP assay for *Taenia solium* cox1 gene	Patients (n=58) All cases (n=58) No. of patients (%age)	IP-NCC(n=50) No. of patients (%age)	EP+IP-NCC, n=8 No. of patients (%age)	Controls (n=24)	P
Positive	35 (60.3%)	29 (58%)	6 (75%)	2 (8.33)	<0.0001
Negative	23 (39.7%)	21 (42%)	2 (25%)	22 (91.67)	<0.0001
Sensitivity (95% CI)	60.3% (46.6% - 72.9%)	58% (43.2%- 71.8%)	75% (34.9%- 96.85)	-	-
Specificity (95% CI)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	-	-
Patients with solitary cysts (n=29)					
Sensitivity (95% CI)	58.6% (38.9%- 76.5%)	59.3% (38.8%- 77.6%)	50% (1.3%- 98.7%)	-	-
Specificity (95% CI)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	-	-
Patients with multiple cysts (n=29)					
Sensitivity (95% CI)	62.1% (42.3% - 79.3%)	56.5% (34.5%- 76.8%)	83.3% (35.9% - 99.6%)	-	-
Specificity (95% CI)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	-	-
Patients with vesicular NCC only (n=5)					
Sensitivity (95% CI)	80% (28.4%- 99.5%)	80% (28.4%- 99.5%)	NA	-	-
Specificity (95% CI)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	NA	-	-
Patients with colloidal/ nodular NCC only (n=34)					
Sensitivity (95% CI)	55.9% (37.9%- 72.8%)	56.2% (37.7%- 73.6%)	50% (1.3%- 98.7%)	-	-
Specificity (95% CI)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	-	-
Patients with calcified cysts only (n=3)					
Sensitivity (95% CI)	66.7% (9.4%- 99.2%)	66.7% (9.4%- 99.2%)	NA	-	-
Specificity (95% CI)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	NA	-	-
Patients with multiple stage NCC (n=12)					
Sensitivity (95% CI)	100% (39.7%- 100%)	100% (2.5%- 100%)	100% (29.2%- 100%)	-	-
Specificity (95% CI)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	-	-
Patients with racemose cysts (n=4)					
Sensitivity (95% CI)	50% (21.1%- 78.9%)	44.4% (13.7%- 78.8%)	66.7% (9.4% - 99.1%)	-	-
Specificity (95% CI)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	91.7% (73%- 98.9%)	-	-

Table 3. LAMP assay in NCC- Positive and negative predictive value in present study.

Parameter with regards to NCC	Positive predictive value	Negative predictive value
Any NCC (all cases)	94.6%	48.9%
Solitary NCC	94.4%	47.8%
Multiple NCC	94.7%	50%
Vesicular NCC only	66.7%	95.6%
Colloidal nodular NCC only	90.5%	59.5%
Calcified NCC only	50%	95.6%
Presence of racemose cysts	50%	91.7%
Multiple stage lesions	85.7%	100%

DISCUSSION

NCC appears to be a major health concern in developing countries including India. Approximately 9 -18.6% of all epilepsy in India is because of NCC [12].

Current diagnostic criteria for NCC are heavily biased towards neuroimaging. However, neuroimaging fails to provide definitive diagnosis of NCC in a substantial number of patients, resulting in both underdiagnoses and overdiagnoses. The common immunodiagnostic methods for NCC include EITB and ELISA, for detection of antibodies to cysticercal antigens in serum and CSF and monoclonal antibody-based detection of cysticercal antigen in serum or CSF [3].

While ELISA based detection of antibodies to cysticercal antigens is no longer recommended as diagnostic criterion, due to high rate of false positives, Detection of cysticercal antigen by monoclonal antibodies mediated methods has not attained widespread acceptance. EITB, though highly sensitive and specific for multiple NCC, lacks sensitivity for solitary NCC [3]. Thus, there is need of alternative serodiagnostic tests for aiding the diagnosis of NCC.

RT- LAMP based detection method of cysticercal DNA, has a future potential to overcome these present obstacles. We have already evaluated "Utility of real time LAMP assay of blood (for detection of *T. solium cox*1 subunit gene) in diagnosis of NCC". The sensitivity and specificity of real time LAMP assay was 74% (solitary NCC- 73.5%; multiple NCC-74.5%) and 90% respectively [11]. Here we have evaluated utility of RT- LAMP for detection of *T. solium cox*1 subunit gene in urine. It is likely that *T. solium* DNA is present in blood during active infection and excreted in urine, detection of which using real time LAMP assay will help in confirming the diagnosis of NCC. Urine based tests have advantage as collection of urine sample does not require any invasive procedure and is thus free from risk of any blood infections such as hepatitis virus etc. In addition, urine samples are easier to collect and urine based diagnostic tests are likely to be more useful in community-based studies. Urine samples are easier to handle and transport to a centralized laboratory compared to blood tests which require expertise for collection and transport.

We found real time LAMP assay of urine to be useful in evaluation of NCC. We could detect trace quantities of cysticercal DNA [1 femto grams) in urine of patients with NCC, confirming its exquisite sensitivity for detection of cysticercal DNA in urine. The overall sensitivity of RT- LAMP assay was 60.3%, while its specificity was 91.7% for various forms of NCC. Notably its sensitivity was 100% in patients with NCC in multiple stages of development with a specificity of 91.7%.

The overall PPV (positive predictive value) of RT-LAMP in diagnosis of NCC was 93-94%. After the patients are found positive for *cox*1 gene of *T. solium* by real time LAMP assay of urine samples, the possibility of confirmation of NCC by detection of lesions in such patients by using neuroimaging techniques such as MRI, CT scan are very high. In addition, it has a very high negative predictive value for patients with vesicular cysts alone, calcified cysts alone, racemose cysts and cysts in multiple stages of evolution.

Real time LAMP assay has several advantages. It has a very low cost compared to conventional polymerase chain reaction (PCR) and EITB, which remains an important factor in developing countries. It is relatively easy to perform and does not require high expertise required for PCR and can yield positive results within matter of hours [13–15]. Given all these advantages, real time LAMP appears to be a useful tool for diagnosis of NCC in developing nations.

Core strong points of our research were - strict adherence with the protocol wherein all enrolled patients underwent regular imaging and clinical follow up. Limiting factors included a relatively smaller sample size. Future studies with a larger sample size will help in describing role of LAMP assay of urine in diagnosis of NCC more easily and in a non-invasive method.

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Declaration of Conflicting Interests: The authors declare that they have no conflict of interest.

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