

Genetic variation in the *Cytb* gene of human cerebral *Taenia solium* cysticerci recovered from clinically and radiologically heterogeneous patients with neurocysticercosis

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Neurocysticercosis (NC) is a clinically and radiologically heterogeneous parasitic disease caused by the establishment of larval Taenia solium in the human central nervous system. Host and/or parasite variations may be related to this observed heterogeneity. Genetic differences between pig and human-derived T. solium cysticerci have been reported previously. In this study, 28 cysticerci were surgically removed from 12 human NC patients, the mitochondrial gene that encodes cytochrome b was amplified from the cysticerci and genetic variations that may be related to NC heterogeneity were characterised. Nine different haplotypes (Ht), which were clustered in four haplogroups (Hg), were identified. Hg 3 and 4 exhibited a tendency to associate with age and gender, respectively. However, no significant associations were found between NC heterogeneity and the different T. solium cysticerci Ht or Hg. Parasite variants obtained from patients with similar NC clinical or radiological features were genetically closer than those found in groups of patients with a different NC profile when using the Mantel test. Overall, this study establishes the presence of genetic differences in the Cytb gene of T. solium isolated from human cysticerci and suggests that parasite variation could contribute to NC heterogeneity.

Key words: neurocysticercosis - *Taenia solium* - cysticercosis - genetic variability

Taenia solium neurocysticercosis (NC) is a parasitic disease that seriously threatens human health in developing countries (Rodríguez-Canul et al. 1999, Sciotto et al. 2000). This disease is also present in Mexico (Fleury et al. 2010) and Mexico's close proximity to the United States of America (USA) is thought to be one of the main causes of a recent NC emergence in the USA due to worker immigration (Sorvillo et al. 2011).

NC exhibits a heterogeneous clinical manifestation that ranges from asymptomatic cases to severe neurological symptoms (Fleury et al. 2004, 2011, Sotelo 2011). Current evidence indicates that patient genetic background and immune status contribute to the clinical course and outcome of the disease (Del Brutto et al. 1988, Fleury et al. 2004, 2010, Chavarría et al. 2005, Sáenz et al. 2006, Cárdenas et al. 2012). In addition, age and gender have previously been shown to correlate with

clinical differences; some studies have shown a lower prevalence of symptomatic NC in children than in adults and females exhibit a stronger inflammatory response to *T. solium* infection than males do (Fleury et al. 2004, 2011, Chavarría et al. 2005). Less attention has focused on the relevance of parasite variations on NC severity. Studies performed in *T. solium* cysticerci recovered from pigs with different geographical origins have shown that genetic variations of the parasite may be involved in pathogenicity (Maravilla et al. 2003, 2008, Vega et al. 2003, Hinojosa-Juarez et al. 2008, Bobes et al. 2010). Moreover, genetic differences in the mitochondrial *cox1* and ribosomal *ITS1* genes were observed in 13 cysticerci obtained from NC patients; however, this study did not attempt to correlate parasite variation and NC severity (Hinojosa-Juarez et al. 2008).

As no previous study has tested the correlations between polymorphisms in any specific parasite gene and clinical differences among NC patients, the focus of this study was to search for an association between these two factors. The *Cytb* gene is a mitochondrial gene that is highly conserved among species (Hey 1997, Simmons & Weller 2001). In this study, *Cytb* was selected as a molecular marker because polymorphisms in this gene may reflect variations in other genes, some of which could directly participate in disease outcome (Laclette et al. 1992). In addition, *Cytb* was recently used in the phylogenetic analysis of taenids and was one of the genes whose analysis first indicated that *T. solium* may have di-

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verged into two strains. One of these strains was isolated from Asia and the other strain was isolated from Africa and Latin America (Okamoto et al. 2001). The possible reasons for this divergence have been recently explored (Michelet & Dauga 2012). *Cytb* has also been employed for molecular epidemiological surveys of other parasitic diseases (Sakuma et al. 2012) and in the clinical diagnosis of cysticercosis (Kobayashi et al. 2013).

Thus, an analysis of the *Cytb* gene sequences isolated from different clinical patients with NC was conducted.

SUBJECTS, MATERIALS AND METHODS

Samples - Twenty-eight *T. solium* cysticerci were obtained from 12 patients who underwent surgical cysticercus removal after being diagnosed with NC based on clinical assessment and radiologic imaging. Twenty-two cysticerci were excised from patients at the National Institute of Neurology and Neurosurgery Manuel Velasco Suárez, Secretary of Health, Mexico City, and six cysticerci were excised from patients at the National Medical Center Siglo XXI (Hospital Specialties Dr Bernardo Sepúlveda G, Research Unit of Neurological Diseases), Mexico City. Cyst samples were donated by both hospital centres and the DNA was extracted from each sample. The patient demographic data, the number and location of the recovered parasites and the patient inflammatory status are described in Table I. Immediately after the surgical procedure, the parasites were exhaustively washed with phosphate buffered saline, dried and frozen at -70°C until DNA extraction.

Clinical features - The clinical files of 12 NC patients were retrieved to collect demographic data (age and gender), clinical and histological findings (intracranial hypertension and inflammation level, respectively), characteristics of cerebrospinal fluid (cell count and protein level) and radiological features (location, number and status of parasites, arachnoiditis, ependymitis and hydrocephalus) (Table I).

Genomic DNA (gDNA) extraction - gDNA from *T. solium* cysticerci excised from infected human patients was purified using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's recommended protocol for DNA isolation from animal tissue.

Polymerase chain reaction (PCR) amplification and DNA sequencing - The complete 1,068-bp *Cytb* gene was amplified using the primer set described by Nakao et al. (2002) (*Cytb*/F forward 5'-ATAAAGTATAGAT-TGTGGTTC-3' and *Cytb*/R reverse 5'-CATATGACT-GTCTAATGAAGAAA-3'). All PCR reactions were performed in a final volume of 50 µL containing 10 µL of 5X Phusion HF buffer (Finnzymes), 1 µL of 100 mM deoxyribonucleoside triphosphates (Invitrogen), 1 µL of the corresponding forward and reverse primers at a concentration of 20 ng/µL and 0.5 µL of Phusion DNA polymerase (Finnzymes). gDNA (500 ng) was added to each reaction. Briefly, the PCR conditions were as follows: initial denaturation at 72°C for 30 s, followed by 30 denaturation cycles at 72°C for 10 s, annealing at 58°C

for 30 s, extension at 72°C for 1 min and a final cycle at 72°C for 5 min on a Corbett Research thermal cycler CG1-96 (Corbett Life Sciences, San Francisco, USA). Amplification products were harvested and purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. All sequencing reactions were performed in a Big Dye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Life Technologies, Carlsbad, California, USA) and sequenced using a 3130XL Sequencer (Applied Biosystems).

Sequence analysis - The 28 sequences were assembled manually, edited in BioEdit v. 7.09 (Hall 1999) and aligned with CLUSTALX v. 2.0.12 (Thompson et al. 1997). A maximum-likelihood phylogenetic inference analysis of the 28 sequences was performed with approximate likelihood ratio test Maximum-Likelihood Phylogenies (atgc.lirmm.fr/phyml/) (Guindon & Gascuel 2003) using the F81 model (Felsenstein 1981) and the data were analysed with ModelTest software (Posada & Crandall 2001). This model was the most appropriate for our sequence set according to the Bayesian Information Criterion (BIC) implemented in ModelTest v. 3.7 (Posada & Crandall 2001) (BIC = 3288.625, -lnL = 1445.567; nucleotide base frequency: FreqA = 0.231, FreqC = 0.091, FreqG = 0.215, FreqT = 0.461). The results were confirmed with Network 4.6.1.0 (fluxus-engineering.com) (Polzin & Daneschmand 2003) using the median-joining method (Bandelt et al. 1999) with the default parameters of this software.

Haplotypes (Ht) (a statistically defined set of single-nucleotide polymorphisms in a specific gene) present in our sequence sets were defined directly from the alignment. In addition, we defined haplogroups (Hg) (a collection of potential Ht that belong to same group or clade) based on the clustering of 28 sequences into a tree and network. The Ht and Hg were tested for associations with patient clinical manifestations.

Statistical analysis - To evaluate a possible association between 11 clinical manifestations in each patient (Table I) and cyst genetic variation, we used two methods: (i) the relationship between the matrices of parasite pairwise genetic distance and the NC patients' pairwise phenotypic distance using the Mantel test and (ii) the association of different Ht and Hg with clinical manifestations in NC patients (Table I) using Fisher's exact test.

For the first method, genetic distances among cysticerci were estimated using the F81 nucleotide substitution model (Felsenstein 1981). As the phenotypic data were coded as binary data (1, 0) (Table II), a phenotypic distance among NC patients was estimated using the relation 1-single matching index (Romesburg 1990). Finally, F81 and phenotypic distance matrices were compared using the non-parametric Mantel test (Mantel 1967) after 10,000 permutations with NTSYSpc v 2.02j (Rohlf 1997).

For the second method, Hg were coded as binary data to compare one Hg to the remaining three Hg in 2 × 2 contingency tables. Likewise patients, classified as those with a single cyst vs. those with multiple cysts and patients harbouring cysts with a single sequence Ht at the

TABLE I
General, clinical, radiological and inflammatory characteristics of the 12 neurocysticercosis patients

Patient number	Age	Gender	ICH	Parasite location	Number of parasites	Arachnoiditis and/or ependymitis	Parasite status	Hydrocephalus	Histological findings	CSF data		
										Inflammation (cells/mm ³)	Protein level (mg/dL)	Haplotype
1	59	Male	Yes	Ventricle	Multiple	Yes	Colloidal	Yes	ND	56	263	6, 7
2	76	Male	Yes	Ventricle	Multiple	No	Vesicular	Yes	Yes	106	109	1, 2
3	36	Male	Yes	Parenchyma	Single	No	Colloidal	No	Yes	ND	ND	6
4v	39	Male	Yes	Subarachnoid sulci	Multiple	No	Vesicular	Yes	Yes	ND	ND	6
5	75	Female	Yes	Subarachnoid basal cisterns	Single	Yes	Colloidal	Yes	Yes	6	31	8
6	29	Female	No	Spinal cord	Multiple	Yes	Vesicular	No	No	2	15	6
7	27	Male	Yes	Ventricle	Multiple	Yes	Colloidal	Yes	ND	69	94	5
8	48	Female	Yes	Ventricle	Multiple	Yes	Colloidal	Yes	Yes	62	96	3, 4
9	42	Male	Yes	Ventricle	Multiple	Yes	Colloidal	Yes	No	11	88	3, 9
10	46	Female	Yes	Ventricle	Multiple	Yes	Colloidal	Yes	ND	32	71	8
11	43	Female	No	Subarachnoid sulci	Multiple	Yes	Vesicular	No	ND	ND	ND	8
12	27	Male	Yes	Ventricle	Multiple	Yes	Vesicular	No	ND	ND	ND	6

parasites from 12 patients were recovered during medical-indicated surgery. All data were recovered from each patient's clinical and radiological file. Parasite location refers to the position of the parasite recovered during surgery. Data on parasite location, number of parasites, presence of arachnoiditis and/or ependymitis and parasite status were obtained from the radiological studies and interpreted by a neuroradiologist; histological studies were conducted in a section of the parasites extracted from each patient by the Histopathology Service of each hospital. Data on cellularity and protein level in the cerebrospinal fluid (CSF) were obtained by each hospital laboratory. ICH: intracranial hypertension; ND: not determined.

studied *locus* vs. those with more than one sequence Ht were tested for association with general, clinical and radiological features using the same statistical test. Results were considered statistically significant at a p value < 0.1 .

Ethics - Cysticerci were removed during neurosurgery. Surgery was indicated by the neurologist based on the clinical condition of the patient and the feasibility of the procedure. Cysticerci samples were kindly donated by the hospital centres for genetic variation studies and all samples were anonymous. According to hospital policy, patients were required to sign informed consent forms for the surgical procedure. This study conformed to all research regulations regarding the use of human subjects as required by Mexican law and international regulations and the study adhered to the ethical guidelines in the General Rules of Health from Clinical Investigation. The research protocol was approved by the Ethical Committee of XXI Century National Medical Center, Mexican Social Security Institute.

RESULTS

Sequencing results of the *Cytb* gene from all tested cysticerci suggested that all cysticerci were of the American/African genotype (Nakao et al. 2002). The *Cytb* gene sequences from 28 cysticerci from 12 patients were analysed; only one insertion/deletion (nucleotide 761) was detected in the first amplicon obtained from parasite 5, which was excised from patient 2. In addition, 13 polymorphic sites were found, 61.53% of which were informative sites (Fig. 1).

Significant statistical associations were observed between the F81 genetic distance and 11 host variables ($r_{Mantel} = 0.327$, $p = 0.0001$). Furthermore, nine Ht (accesses

JQ973073 H2, JQ973074 H5, JQ973075 H3, JQ973076 H4, JQ973077H6, JQ973078 H7, JQ973079 H8 and JQ973080 H9) were identified among all 28 sequences (Fig. 1), which were subsequently clustered into four Hg in the tree topology (bootstrap values $\geq 80\%$) (Fig. 2).

The first Hg was comprised of seven parasites, which were all excised from the same patient and contained polymorphisms at nucleotides 340, 360 and 651 [guanine (G) was replaced by cytosine (C), adenine (A) by G and C by thymine (T), respectively]. The parasite with a deletion at nucleotide 761 was included in this group. The second Hg included six cysticerci from three different patients. These sequences were grouped based on the variant at nucleotide 340 (G by C); however, three of them exhibited mutations at other positions (Fig. 1). The third Hg included 10 parasites from five patients with variations at positions 543 (G by A) and 546 (A by G). The fourth Hg was comprised of five parasites from three patients who exhibited variations at nucleotides 672 and 914 (C by G and A by G, respectively) (Figs 1, 2).

A tendency towards association between Hg 3 and age was observed ($p = 0.07$): of the five patients harbouring parasites of Hg 3, four were younger than 40, whereas six of the seven patients exhibiting cysts from other Hg were older than 40. The three patients with parasites from Hg 4 were female. In contrast, only two out of seven patients infected with parasites from other Ht groups were female ($p = 0.04$). Younger patients tended to harbour parasites with the same Ht, whereas the four patients presenting with cysticerci containing two Ht were older than 40 ($p = 0.07$). No statistically significant association between Hg and clinical or radiological manifestations was observed.

TABLE II
General, clinical and radiological features of neurocysticercosis patients used in the association analysis and binary transformation

Category	Patient features	Binary transformation
1	Age	> 40 (1), < 40 (0)
2	Gender	Female (1), male (0)
3	Clinical severity (intracranial hypertension symptoms)	Yes (1), no (0)
4	Parasite location	SAb or Sc or Iv (1), SAs or P (0)
5	Number of parasites	Multiple (1), single (0)
6	Arachnoiditis and ependymitis	Yes (1), no (0)
7	Parasite status	Colloidal (1), vesicular(0)
8	Hydrocephalus	Yes (1), no (0)
9	Histological findings	Presence of inflammation (1) ^a , absence of inflammation (0)
10	Cerebral spinal fluid F cell count	Not determined
11	Cerebral spinal fluid protein level	> 10 cells (1), < 10 cells (0) > 90 mg (1), < 90 mg (0)

^a: for histological findings, inflammation was defined as the presence of lymphocytes and macrophages; Iv: intraventricular; P: parenchyma; SAb: subarachnoid basal cisterns; SAs: subarachnoid of sulci; Sc: spinal cord.

Patient number	Cysticercinumber	Nucleotide position												Haplotype	Haplogroup	
		273	292	294	340	360	508	543	546	585	651	672	914			1053
	5	*	*	*	C	G	*	*	*	*	T	*	*	*	1	
	7	*	*	*	C	G	*	*	*	*	T	*	*	*	1	
2	6	*	*	*	C	G	*	*	*	*	T	*	*	*	2	1
	1	*	*	*	C	G	*	*	*	*	T	*	*	*		
	4	*	*	*	C	G	*	*	*	*	T	*	*	*		
	3	*	*	*	C	G	*	*	*	*	T	*	*	*		
	2	*	*	*	C	G	*	*	*	*	T	*	*	*		
9	1	*	*	*	C	*	*	*	*	*	*	*	*	*	3	
8	1	*	*	*	C	*	*	*	*	*	*	*	*	*	3	
	2	*	*	*	C	*	*	*	*	G	*	*	*	*	4	2
7	1	*	*	*	C	*	T	*	*	*	*	*	G	5		
9	3	C	G	A	C	*	*	*	*	*	*	*	*	*	9	
4	1	*	*	*	*	*	*	A	G	*	*	*	*	*		
	2	*	*	*	*	*	*	A	G	*	*	*	*	*		
6	2	*	*	*	*	*	*	A	G	*	*	*	*	*		
	3	*	*	*	*	*	*	A	G	*	*	*	*	*		
12	1	*	*	*	*	*	*	A	G	*	*	*	*	*	6	3
	2	*	*	*	*	*	*	A	G	*	*	*	*	*		
1	2	*	*	*	*	*	*	A	G	*	*	*	*	*		
6	1	*	*	*	*	*	*	A	G	*	*	*	*	*		
3	1	*	*	*	*	*	*	A	G	*	*	*	*	*		
1	1	*	G	*	*	*	*	A	G	*	*	*	*	*	7	
	2	*	*	*	*	*	*	*	*	*	G	G	*	*		
11	3	*	*	*	*	*	*	*	*	*	G	G	*	*		
	1	*	*	*	*	*	*	*	*	*	G	G	*	*	8	4
10	1	*	*	*	*	*	*	*	*	*	G	G	*	*		
5	1	*	*	*	*	*	*	*	*	*	G	G	*	*		

Fig. 1: polymorphic sites in *Cytb* gene sequences of 28 parasites from 12 neurocysticercosis patients. The position and nucleotide changes found haplotypes and haplogroups formed by different haplotypes are shown.

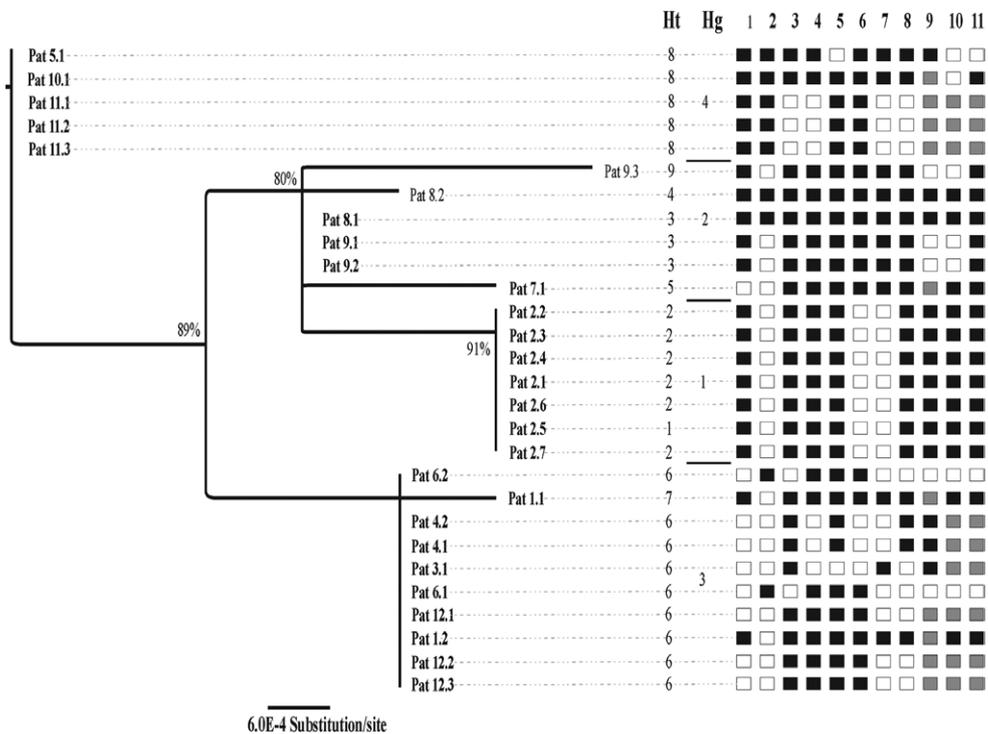


Fig. 2: maximum-likelihood phylogenetic tree based on single nucleotide polymorphism (SNP) differences across the *Cytb* gene of *Taenia solium* cysticerci. A maximum-likelihood phylogenetic tree of the 28 *T. solium* cysticerci from 12 patients based on SNP differences across the 1,068 bp of the complete *Cytb* gene (left) is shown. Haplotypes (Ht), haplogroups (Hg), as well as demographic, clinical, radiological and inflammatory features of each patient-derived cyst are shown (right). The number of each column corresponds to the same clinical category shown in Table II. White and dark squares correspond to the binary transformation according to Table II.

DISCUSSION

In this study, the sequences of the mitochondrial *Cytb* gene were studied in 28 cysticerci surgically excised from NC patients. The genetic variations observed were clustered into nine Ht and four Hg. This finding highlights the genetic heterogeneity among cysticerci isolated from human patients. These genetic variations are consistent with the genetic heterogeneity observed among swine-derived *T. solium* metacestodes isolated from different areas of Mexico and other endemic countries (Maravilla et al. 2003, 2008, Vega et al. 2003, Bobes et al. 2010, Barcelos et al. 2012).

A significant association between the 11 included host features and the genetic distances among parasites was observed (Mantel test). This result indicates that patients with similar features harbour parasites that are more closely related, which suggests that parasite diversity may contribute to the heterogeneous clinical manifestations observed in NC patients (Fleury et al. 2010). Clinical and radiological heterogeneity is a main characteristic of NC. Although previous studies have shown that host genetic background, sex, age and immune status affect the susceptibility to infection and disease (Del Brutto et al. 1988, Chavarria et al. 2005, Sáenz et al. 2006), this is the first study suggesting that parasite heterogeneity may contribute to disease heterogeneity. It is important to note that the observed genetic variation in the mitochondrial *Cytb* gene among the parasites and the association of genetic variation with the observed clinical features could be due to the action of *Cytb* (i.e., *Cytb* encodes a protein that plays an important role in electron transfer, the respiratory chain and oxidative phosphorylation in aerobic organisms) (Fernández et al. 2008, Chen et al. 2009). However, it is possible that this association may reflect an indirect effect of *Cytb* genetic variation due to variation in other genes, which could directly affect disease outcome (Laclette et al. 1992).

The selection of the *Cytb* gene for this molecular study of *T. solium* merits some discussion. First, *T. solium* mitochondrial genes, such as *col*, *Cytb* and *nad*, are more polymorphic than nuclear genes and are more informative for molecular epidemiology purposes (Martínez-Hernández et al. 2009). *Cytb* was also an attractive target for this study because a study of the *col* gene was not feasible: the complete amplification of the *col* gene from human brain cysticerci collected in this study was not possible (data not shown) for reasons that are not yet understood.

In addition, we observed a tendency of association between Hg 3 and 4 with age and gender, respectively. Considering the relevance of gender and age in NC pathogenesis, these tendencies also merit some discussion. It is known that the neuroinflammation accompanying severe NC forms is more intense in women. In addition, the encephalitic form of NC, which is caused by multiple degenerating parasites located in the parenchyma, is an inflammatory form of the disease that is found almost exclusively in female patients (Fleury et al. 2010). With respect to age, most paediatric patients present a benign form of the disease with parasites localised in the parenchyma; however, adult patients present

with an extra-parenchymal form and more severe forms of the disease (Sáenz et al. 2006). Thus, the underlying cause of some of these differences may involve parasite genetic variation.

No other associations among the individual radiological or clinical variables of NC patients and *Cytb* Ht or Hg were observed. These negative results should be interpreted with caution because of our small sample size, which could influence these results.

The influence of genetic variation in infectious organisms on the clinical course of the resulting disease has been described previously (Li et al. 2010). In the case of *T. solium* cysticercosis, genetic variation of the parasite has reportedly been associated with different parasite antibody recognition patterns (Barcelos et al. 2012).

In conclusion, our results suggest that a parasite genetic profile may contribute to NC heterogeneity; however, no individual associations with clinical or radiological characteristics were found.

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