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Neurocysticercosis: validity of ELISA after storage of whole blood and cerebrospinal fluid on paper

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Summary Cysticercosis is an infestation of *Cysticercus cellulosae*. When it occurs in the brain, chronic neurological complications can ensue, most commonly seizures. Neurocysticercosis is usually diagnosed by neuroimaging, a technique not available in most endemic countries. Hence immunological tests are valuable for diagnosis and epidemiological surveys. We evaluated the suitability of paper for storing blood and cerebrospinal fluid (CSF) until subsequent testing by enzyme-linked immunosorbent assay (ELISA), by testing whole blood samples on filter paper from 305 patients and CSF samples from 117 patients stored on ordinary white typing paper and on filter paper. Optimal preservation of biological samples is achieved when whole blood is stored on filter paper, CSF on white paper, and when samples are frozen within 1 week after collection. Our results could improve diagnostic capabilities and facilitate epidemiological surveys in endemic countries where immunodiagnostic tests cannot be rapidly performed because of inadequate laboratory infrastructure.

keywords ELISA, Mexico, neurocysticercosis, paper, storage

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Introduction

Cysticercosis is a disease caused by infection with *Cysticercus cellulosae*, the larval stage of *Taenia solium*. This infection is endemic in numerous countries of Africa, Asia and Latin America. In recent years, its incidence in the industrialized north has risen dramatically as a result of increased immigration from endemic areas. Localization of this larva to the central nervous system (neurocysticercosis, NCC) is one of the most frequent neurological disorders in endemic countries, leading to seizures and other complications. NCC is diagnosed by visualization of the cyst using computed tomography or magnetic resonance imaging, or by pathological examination after biopsy. In Mexico, NCC is responsible for 9% of hospital admissions to neurological wards, almost 50% of all convulsions in adults, and 10–13% of the craniotomies performed at the Instituto Nacional de Neurología y Neurocirugía (INNN) of Mexico (Medina *et al.* 1990; Richards & Schantz 1991).

The precise prevalence rate of NCC is difficult to determine because of the pleomorphic clinical presentation, the high number of asymptomatic infections, and the cost of neuroimaging studies which are inaccessible to the majority of the population in endemic countries. Hence immunological tests are a valuable aid in diagnosis. Among these, the enzyme-linked immunosorbent assay (ELISA) is the most frequently used today because of its relative simplicity and low cost. However, it requires a minimal infrastructure (centrifuges, freezer, etc.) which is often unavailable in rural areas. Therefore we evaluated the suitability of paper to store blood and cerebrospinal fluid (CSF) for subsequent ELISA testing. This simple technique facilitates collection, processing and transportation of biological samples and can be extremely helpful in diagnosing the disease in endemic countries.

Patients and methods

Patients

Patients were recruited at the INNN of Mexico between December 1996 and May 1997. Neurocysticercosis was

confirmed by neurological imaging studies (computed tomography or magnetic resonance imaging) and/or by pathological examination. Active form (arachnoiditis, hydrocephalus secondary to meningeal inflammation, cysts, vasculitis) and inactive form (calcifications, meningeal fibrosis) as described by Sotelo *et al.* (1985) were considered separately. Controls were patients for whom the diagnosis of NCC had been excluded by clinical history and neurological imaging studies, and who were hospitalized for seizures, brain cancer, stroke, headache, a psychiatric disorder, or multiple sclerosis.

Samples

The study of blood samples included 305 individuals: 140 cases of NCC (93 active and 47 inactive forms) and 165 controls. Two samples were taken from all individuals: one of venous blood and one of capillary blood by finger prick with a lancet. Venous blood was centrifuged within 2 h of sampling, sera were stored in Nunc® tubes and immediately frozen at –20 °C until testing with ELISA. Capillary blood (four spots of approximately 1 cm diameter) was deposited on one sheet of filter paper (Whatman 311 filter paper, Polylabo, France). The papers were stored at room temperature for 1 week, protected from light and humidity, and then frozen at –20 °C in an airtight container with a desiccant (Silicagel®) until the first ELISA. They were then kept at room temperature in the same condition for 2 weeks until the second ELISA.

For the study of CSF samples, 117 individuals were tested: 48 cases of NCC (42 active forms, 6 inactive forms) and 69 controls. Immediately after collection of the fluid, 1 ml was frozen, 1 ml was placed on filter paper (two 1 cm spots), and 1 ml was placed on white paper (ordinary typing paper, 75 g/m^2) of identical size. The papers were kept at room temperature for 1 week, protected from light and humidity, and then frozen at –20 °C in an airtight container with a desiccant (Silicagel®) until ELISA testing.

Collection of data

The results of neurological imaging studies, CSF analysis and eventual pathological results were collected on a standardized form.

ELISA

This assay was performed in two laboratories: at the INNN in Mexico City, and at the Institut d'Epidémiologie Neurologique et de Neurologie Tropicale (IENT) in Limoges. In Mexico, all samples were assayed, in France only on part of them. The ELISA techniques used were the following: in Mexico, the technique described by Rosas *et al.* (1986), using an antigen prepared from Mexican pig cysticerci; in Limoges, the technique of Chamouillet *et al.* (1997), using a soluble antigen of *C. cellulosae* prepared according to the method of Guerra *et al.* (1982) from African pig cysticerci.

At the INNN, blood sera were used at a 1 : 4096 dilution in PBS; at the IENT, the dilution was 1 : 200. The filter paper impregnated with whole blood was cut in the laboratory into 6-mm diameter discs. These discs were left overnight in 500 µl of PBS at 4 °C. At the INNN, the ELISA was performed using a 1 : 4 dilution of the eluate obtained; at the IENT, directly on the eluate. An optical density (OD) > 0.500 in the Mexican laboratory and > 0.400 in the French laboratory were regarded as positive.

At the INNN, CSF samples were tested at a 1 : 30 dilution in PBS. At the IENT, they were tested without dilution. The papers impregnated with CSF were cut into 0.5 by 1 cm strips. These strips were left overnight in 200 µl of PBS at 4 °C. In both laboratories the ELISA was performed directly on the eluate. An OD > 0.400 was considered as positive.

All ELISAs were realized in duplicate. When there occurred a discordance between the two determinations (18 cases) or when the OD value was located in a ± 0.100 interval of the threshold value (two cases), a third determination was carried out. The final result was established by the two concordant determinations.

Statistical analyses

The database was captured at the INNN of Mexico on Epi Info software (Centers for Disease Control, Atlanta, USA, 1992). The qualitative data were compared using χ^2 test, Pearson's test or Yates' corrected χ^2 test. The Kappa coefficient (κ) and the intraclass correlation coefficient R were calculated to evaluate the qualitative and quantitative concordances. These two coefficients varied between –1 and +1. The closer their values approached 1, the closer the concordance (Fermanian 1984a,b; Bernard & Lapointe 1995). We determined ELISA's sensitivity and specificity considering as cases all the forms of NCC (active and inactive) and separately, active forms only.

Results

The results of the study are presented in Table 1, concordance results in Table 2, and the ELISA's sensitivity and specificity in Table 3.

ELISA of blood samples

At the INNN, significant differences were found in the sensitivity of ELISA between assayed sera and eluates on filter paper ($P = 0.05$ for filter paper kept at room temperature for 1 week and $P < 0.001$ for filter paper kept at room temperature for 3 weeks) although the concordance between ELISA on sera and on eluate of filter paper kept at room temperature for 1 week was good ($\kappa = 0.77$ and $R = 0.88$). At the IENT, no significant differences in sensitivity were discovered between ELISAs of sera and of eluate of filter paper ($P = 0.2$). In both laboratories, ELISA sensitivity was higher for the active forms of the disease; this difference was significant for ELISA of sera ($P = 0.01$ in the INNN, $P = 0.02$ in the IENT) and at the INNN for ELISA of eluate of filter paper kept at room temperature for 1 week ($P < 0.05$). Sensitivity of ELISA of sera did not significantly differ ($P = 0.18$) between laboratories.

ELISA of CSF samples

At the INNN, sensitivities of ELISA on CSF and on eluate of white paper were not significantly different ($P = 0.27$ when all forms of NCC were considered and $P = 0.22$ when only active forms were considered). At the IENT, significant differences did exist ($P < 0.05$). In neither laboratory significant differences in the sensitivities were found when all forms of NCC or when only the active forms of the disease were considered. Sensitivities between the two laboratories did not differ significantly when ELISA was made on CSF ($P = 0.8$ when all forms of NCC were considered and $P = 0.9$ when only active forms of NCC were considered) but there were significant differences when ELISA was made on eluate of white paper ($P = 0.01$ in the two types of samples).

Discussion

The purpose of this study was to evaluate whether paper could be used to store biological samples for immunological testing and diagnosis of NCC. This storage method considerably simplifies sample processing, is inexpensive, and samples can be sent by mail (Farzadegan *et al.* 1987; Lindhart *et al.* 1987; Varnier *et al.* 1988). Our results validate this mode of blood and CSF sample storage. At the INNN, concordance between the two methods (classical and paper) was excellent for blood when papers were frozen 1 week after sampling and for CSF when conserved

Table 1 Number of patients and serological results (enzyme-linked immunosorbent assay) according to the sample, activity of the disease and laboratory

Samples	Population	Nb patients INNN	Nb ELISA + INNN	Nb patients IENT	Nb ELISA + IENT
Sera	NCC all forms	140	75	136	62
Immediate conservation	NCC active forms	93	66	91	56
	Controls	165	17	143	3
Filter paper of whole blood	NCC all forms	140	93	137	53
1 week storage at RT	NCC active forms	93	77	90	47
	Controls	165	21	150	6
Filter paper of whole blood	NCC all forms	134	45	NP	NP
3 weeks storage at RT	NCC active forms	93	41	NP	NP
	Controls	163	4	NP	NP
CSF	NCC all forms	48	35	45	32
Immediate conservation	NCC active forms	42	33	39	30
	Controls	69	0	69	1
CSF on white paper	NCC all forms	48	30	39	14
1 week storage at RT	NCC active forms	42	28	34	13
	Controls	69	0	62	4
CSF on filter paper	NCC all forms	29	15	NP	NP
1 week storage at RT	NCC active forms	26	13	NP	NP
	Controls	54	0	NP	NP

Nb, number; NP, not performed; RT, room temperature; ELISA, enzyme-linked immunosorbent assay; CSF, cerebrospinal fluid; NCC, neurocysticercosis; INNN, Instituto Nacional de Neurología y Neurocirugía; IENT, Institut d'Epidémiologie Neurologique et de Neurologie Tropicale.

Table 2 Concordance of the results according to technique and laboratory

Samples	Storage of the papers by freezing	Laboratories	κ	CI 95%	*R*	CI 95%
Concordance according to technique (blood on paper vs. *sera and CSF on paper* vs. *CSF)*						
Blood on filter paper *vs.* sera	After 1 week at RT	INNN	0.77	(0.69–0.85)	0.88	(0.87–0.89)
Blood on filter paper *vs.* sera	After 1 week at RT	IENT	0.71	(0.59–0.83)	0.81	(0.76–0.85)
Blood on filter paper *vs.* sera	After 3 weeks at RT	INNN	0.58	(0.56–0.60)	0.69	(0.63–0.74)
CSF on filter paper *vs.* CSF	After 1 week at RT	INNN	0.73	(0.58–0.88)	0.51	(0.33–0.65)
CSF on white paper *vs.* CSF	After 1 week at RT	INNN	0.90	(0.70–1.00)	0.81	(0.73–0.86)
CSF on white paper *vs.* CSF	After 1 week at RT	IENT	0.44	(0.25–0.63)	0.57	(0.42–0.69)
Concordance according to laboratory (INNN and IENT)						
Sera	Immediately		0.57	(0.46–0.68)	0.62	(0.54–0.69)
Filter paper of blood	After 1 week at RT		0.51	(0.41–0.61)	0.53	(0.44–0.61)
CSF	Immediately		0.98	(0.94–1.00)	0.89	(0.84–0.92)
White paper of CSF	After 1 week at RT		0.57	(0.38–0.76)	0.70	(0.59–0.79)

κ, Kappa coefficient; *R*, intraclass correlation coefficient; CI, confidence interval; RT, room temperature; CSF, cerebrospinal fluid; INNN, Instituto Nacional de Neurología y Neurocirugía; IENT, Institut d'Epidémiologie Neurologique et de Neurologie Tropicale.

Table 3 Sensitivity and specificity of ELISA according to the laboratory and the activity of the neurocysticercosis and blood or CSF samples

Samples	Patients	Laboratory	Sensitivity (%)	CI 95%	Specificity (%)	CI 95%
ELISA on blood samples						
Sera	NCC all forms	INNN	54	(45–62)	90	(84–94)
		IENT	46	(37–54)	98	(94–99)
	NCC active forms	INNN	71	(61–80)	90	(84–94)
		IENT	62	(51–71)	98	(94–99)
Blood on filter paper*	NCC all forms	INNN	66	(58–74)	87	(81–92)
		IENT	39	(31–47)	96	(92–98)
	NCC active forms	INNN	83	(74–89)	87	(81–92)
		IENT	52	(42–62)	96	(92–98)
Blood on filter paper†	NCC all forms	INNN	34	(26–42)	98	(94–99)
	NCC active forms	INNN	44	(34–55)	98	(94–99)
ELISA on CSF samples						
CSF	NCC all forms	INNN	73	(59–84)	100	
		IENT	71	(57–83)	99	(93–100)
	NCC active forms	INNN	79	(64–89)	100	
		IENT	77	(62–88)	99	(93–100)
CSF on white paper	NCC all forms	INNN	63	(48–75)	100	
		IENT	36	(22–52)	94	(85–98)
	NCC active forms	INNN	67	(51–80)	100	
		IENT	38	(23–55)	94	(85–98)
CSF on filter paper	NCC all forms	INNN	52	(34–69)	100	
	NCC active forms	INNN	50	(31–69)	100	

ELISA, enzyme-linked immunosorbent assay; CSF, cerebrospinal fluid; CI, confidence interval; NCC, neurocysticercosis; INNN, Instituto Nacional de Neurología y Neurocirugía; IENT, Institut d'Epidémiologie Neurologique et de Neurologie Tropicale.
*Freezing after 1 week at room temperature.
†Freezing after 3 weeks at room temperature.

on white paper. These results are in agreement with those of Garcia and Sotelo (1989) and those of Peralta *et al.* (2001). At the IENT, the concordance was weaker, particularly for CSF, probably because of *C. cellulosae* antibody instability on paper. This phenomenon has previously been described with human immunodeficiency

Neurocysticercosis	INNN ($n = 305$)	IENT ($n = 279$)	Rosas *et al.* ($n = 750$)	Ramos-Kuri *et al.* ($n = 678$)
All forms				
Sensitivity (%)	54	46	43	69
Specificity (%)	90	98	69	71
Active forms				
Sensitivity (%)	71	62	50	NP
Specificity (%)	90	98	70	NP

ELISA, enzyme-linked immunosorbent assay; INNN, Instituto Nacional de Neurología y Neurocirugía; IENT, Institut d'Epidémiologie Neurologique et de Neurologie Tropicale; NP, not performed.

Table 4 Neurocysticercosis: sensitivity and specificity of ELISA on sera. Comparison of the results of the present study carried out in two laboratories (INNN and IENT) with those of the literature (Rosas *et al.* 1986; Ramos-Kuri *et al.* 1992)

virus (HIV) antibodies (Lindhart *et al.* 1987; Peckham *et al.* 1990). Samples were analysed in Limoges 4 months after collection and despite conservation by freezing, antibody alteration is possible.

Concordance and thus serum *C. cellulosae* antibody levels clearly decreased when papers were tested three weeks after collection (κ decreased from 0.77 to 0.58 and R from 0.88 to 0.69), despite conservation of filter paper in containers that protected against humidity and light. These results differ from those reported in studies on HIV and hepatitis B virus, where antibodies remained stable for 3–6 weeks after storage of blood on filter paper (Farzadegan *et al.* 1978; Fortes *et al.* 1989; van den Akker *et al.* 1990; Behets *et al.* 1992). Ordinary white typing paper seems better suited for testing CSF than filter paper, undoubtedly because of differences in texture. Filter paper, which is dense and loosely meshed, rapidly absorbs CSF, but elution is more difficult. White paper is very thin and yields better results.

Although comparing the results of the two laboratories was not our principal objective, as differences exist between the two techniques used, some data are interesting. Sera and CSF dilutions used in Mexico and in France were very different (at the INNN, 1 : 4096 for sera and 1 : 30 for CSF; at the IENT, 1 : 200 for sera and without dilution for CSF). Perhaps these differences are related to genetic variety of cysticerci between Mexico and Africa. Such variability could explain better reactivity of Mexican samples with Mexican than with African antigen. Preliminary results (not yet published) comparing African and Latin American cysticerci seem to confirm this diversity. More experiments are necessary to establish it.

Our study confirms that sensitivity of ELISA is limited, particularly with blood products (Tables 3 and 4). Western blot may be the most sensitive test (Tsang *et al.* 1989; Feldman *et al.* 1990), but it is also costlier and difficult to perform. Although Western blot can be used for blood samples stored on filter paper (Jaffri *et al.* 1998), we chose ELISA because of its better availability in endemic countries because of its simplicity and low cost (Rosas *et al.* 1986; Ramos-Kuri *et al.* 1992).

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