Combined use of an antigen and antibody detection enzyme-linked immunosorbent assay for cysticercosis as tools in an epidemiological study of epilepsy in Burundi


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Summary

OBJECTIVE To evaluate the benefits of the detection of both circulating antibodies (Ab) and antigens (Ag) for the diagnosis of cysticercosis in people with epilepsy. Neurocysticercosis is a cause of neurological diseases world-wide, especially epilepsy. The clinical symptoms of neurocysticercosis are non-specific and diagnosis is often difficult.

METHODS Serum samples were collected from subjects in a matched case–control study for epilepsy in the Kiremba area, Burundi, between March and April 2001 (epileptic cases = 303; controls without epilepsy = 606). The enzyme-linked immunosorbent assay (ELISA) was used for the detection of antibodies (Ab-ELISA) and circulating Ag (Ag-ELISA).

RESULTS The Ab-ELISA revealed 58.7% positivity in epilepsy cases and 31.4% in healthy controls; and Ag-ELISA revealed 38.3% positivity in epilepsy cases and 20.0% in controls. The matched odds ratios were 3.6 (95%CI: 2.5–4.9) for Ab-ELISA, and 2.9 (95%CI: 2.1–4.3) for Ag-ELISA.

CONCLUSION Both Ag- and Ab-ELISA detected a significantly higher number of seropositives among people with epilepsy than among controls. The risk of epilepsy was high in cases with a positive Ag-ELISA, although less important than in cases with positivity for Ab-ELISA. Dead or degenerating cysticerci appear to be more frequently associated with epilepsy than living cysts. The high number of people with circulating Ag of *Taenia solium* suggests that the study area is a focus of active transmission of the parasite.

KEYWORDS Burundi, cysticercosis, diagnosis, enzyme-linked immunosorbent assay, epilepsy, case–control study

Introduction

Although many different causes of epilepsy (such as brain tumours, perinatal disorders, stroke, head trauma’s and various infections) have been described in sub-Saharan Africa (Preux & Druet-Cabanac 2005), neurocysticercosis (NCC) is recognized as a major cause of neurological disease (Preux et al. 1996; World Health Organization 2002). NCC, the infection of the human central nervous system by *Taenia solium* larvae, is widespread in developing countries of Latin America, Africa and Asia and is particularly prevalent in rural areas in association with poverty and poor sanitation where raw or undercooked pork is consumed and pigs have access to human faeces (Pawlowski & Murrell 2001).

Neurocysticercosis is a pleomorphic disease, ranging from asymptomatic infections to severe neurological syndromes and presumably is the major cause of acquired epilepsy with intracranial hypertension or dementia, and a variety of non-specific mild symptoms in-between (Carpio & Hauser 2002; Garcia et al. 2003). Also, the evolution of the disease is highly variable: in some patients, the parasite dies without any complications, in others its death induces an intense inflammatory reaction that is typically associated with onset of symptoms and potentially dangerous complications (Sotelo & Flisser 1997; Pal et al. 2000; Sciutto et al. 2000). Cysticerci may also occur in muscles or in subcutaneous tissues. Contrary to the situation in Asia, where subcutaneous cysticercosis (SCC) is very common, the prevalence of SCC in Africa differs between regions (Iro et al. 2003).

Diagnosis of NCC is not easy at all. A set of criteria (parasitological, clinical, imaging, serological and epidemiological) has been developed whereby various
combinations of these criteria allow reaching different degrees of diagnostic certainty (Del Brutto et al. 2001). MRI (Magnetic Resonance Imaging) and CT-scan (Computer-assisted Tomography) are very powerful tools, but they are very expensive and unfortunately often not available in cysticercosis endemic areas. Therefore, serological tests for the detection of specific circulating antigens (Ag) and antibodies (Ab) are more and more frequently used, particularly in epidemiological studies (Dorny et al. 2004a).

In Burundi, several foci of cysticercosis have been identified but only few papers have evoked the possible relationship between cysticercosis and epilepsy (Nzisabira et al. 1992; Newell et al. 1997b). A matched case–control study in Burundi showed a significant statistical association between seropositivity (Ab against cysticercosis) and epilepsy, and this association persisted after adjusting other factors potentially responsible for epilepsy occurrence (Nsengiyumva et al. 2003).

The aim of the study was to evaluate the benefits of the combined use of enzyme-linked immunosorbent assay (ELISA) for the detection of Ab and Ag for the diagnosis of cysticercosis in Burundese patients with epilepsy.

Materials and methods

Serum samples were collected from subjects of a matched case–control study for epilepsy in the Kiremba area, in the north of Burundi, between March and April 2001 (Nsengiyumva et al. 2003). The cases were subjects with epilepsy and the controls had no epilepsy. The exposure factor studied was cysticercosis seropositivity (Ab and Ag). Clinical and paraclinical examinations were carried out in the people with epilepsy and the controls in the Kiremba hospital. People with epilepsy were defined as having had at least two or more unexplained, unprovoked seizures occurring over a period >24 h (all types of generalized or partial seizures were included) (ILAE 1993). The controls had no neurological illness. Both the cases and controls had been living in the Kiremba area for at least 2 years. The cases and controls were included during the same period and only matched by age. Controls were neighbours of patients with epilepsy or subjects coming to the hospital for vaccination. No census or map was available in this community to allow randomized selection among eligible controls. We used the same set of matched pairs of the first cysticercosis study (Nsengiyumva et al. 2003).

A standardized questionnaire which was specifically designed for use in tropical countries was used to collect information on potential risk factors related to epilepsy (Preux et al. 2000). The study was approved by the Ministry of Public Health, which functions as the National Ethical Committee of Burundi.

Matching criteria

Two controls were matched against one subject with epilepsy for the age (±5 years of age). Cases and controls had no blood relationship. A physician who verified the clinical inclusion and exclusion criteria examined each subject.

Diagnostic criteria of epilepsy

People with epilepsy were diagnosed on the basis of the classification defined by the Commission on Classification and Terminology of the International League Against Epilepsy (ILAE 1989). Motor and sensory problems, cerebellar, pyramidal and extra pyramidal syndromes, as well as cranial nerve palsies were thoroughly evaluated in both the cases and the controls. A physician looked for the dates of onset of seizure disorders and the date of the latest seizure. Furthermore, he checked if the epilepsy was active (at least one seizure in the last 5 years), and listed the anti-epileptic drugs used.

Immunodiagnostic tools for cysticercosis

Samples

A 10 ml blood sample was obtained from each subject using non-anticoagulated Vacutainer® tubes. Each sample was then centrifuged and the sera were transferred into cryotubes (Nunc®) and immediately frozen at −20 °C until use.

Antibody detecting ELISA (Ab-ELISA)

The screening for Ab was done by the Ab-ELISA as described by Guerra et al. (1982). The Ag used for coating the polystyrene ELISA plates was a crude soluble extract of *T. solium* cysticerci. The optical density (OD) was measured at 405 nm with a LP 400 spectrophotometer (Diagnostic Pasteur). The reaction threshold was 0.400. The OD values ≥0.400 were considered to be positive. This method has a sensitivity of 86% and a specificity of 92% (Houinato et al. 1998).

Circulating antigen detecting ELISA (Ag-ELISA)

The Ag-ELISA as described by Dorny et al. (2000) was used in this survey. Briefly, polystyrene ELISA plates were coated with a monoclonal Ab (B158C11A10) and blocked with 1% heat inactivated newborn calf serum in PBS with 0.05% Tween 20 (0.05% PBS-Tw20). Then, trichloroacetic acid pre-treated serum samples were incubated, after which a second biotinylated monoclonal Ab (B60H6A4) was added. A conjugate solution (extravidin-horseradish peroxidase) and a chromogen/substrate solution (OPD (o-Phenylenediamine) in citrate buffer and H₂O₂) followed. All steps, except the application of the substrate,
were done in a shaking incubator for 30 min (coating) or 15 min (other steps) at 37 °C. The substrate step was incubated in the dark for 15 min. Plates were washed in between the steps with 0.05% PBS-Tw 20. The plates were read after stopping the reaction with H2SO4, using a spectrophotometer at 492 nm with a reference of 620 nm. The OD of each serum sample was compared with the mean of negative reference serum samples \( (n = 8) \) at a probability level of \( P < 0.001 \) to determine the result using a modified Student’s \( t \) test (Sokal & Rohlf 1981). The ELISA values were expressed as a ratio by dividing the OD of the test sample by the OD of the cut-off value. An ELISA ratio >1 was considered positive. Preliminary results showed that this method has a sensitivity of 94.4% and a specificity of 100% (Erhart et al. 2002). Recent observations have confirmed the high specificity and sensitivity of the Ag-ELISA. Out of 41 patients with filariasis (three), amoebiasis (three), malaria (seven), schistosomiasis (three), trypanosomiasis (eight), hydatidosis (12) and cerebral tumors (five) only one cross-reaction was observed resulting in a specificity of 97.6%. Out of 84 documented cases of NCC, 74 (88.1%) were detected using the Ag-ELISA (Dorny et al. unpublished observation).

Statistical analysis

Data were analysed using Epi-Info 5.01b software (Centers for Disease Control, French version: National School of Public Health 1992) and Statview 5.0 software (SAS Institute Inc, Cary, USA). Quantitative variables were described using mean and standard deviation (SD). The frequency comparisons were done with the Pearson Chi² test. Exposure proportions of the cases and the controls were calculated, and matched odds ratio (OR) and its 95% confidence interval (CI) were estimated. A multivariate analysis was made using logistic regression with age as matching variable. A significance level of 0.05 was used for all analyses.

Results

Three hundred and three triplets were included, 303 epilepsy cases and 606 controls, giving a total of 909 subjects. Four hundred and forty two (48.6%) were males and 467 (51.4%) were females. The epileptic seizures were generalized in 25.8 years (±15.2) for cases and 26.7 years (±14.6) for controls. No significant difference was found between people with epilepsy and control subjects regarding gender \( (P = 0.07) \).

Immunodiagnosis

Among the 909 subjects examined, 40.5% \( (368/909) \) were positive in the Ab-ELISA \[ 52.2\% \ (192/368) \] were males and 47.8% \( (176/368) \) were females; \( P = 0.07 \). The matched OR between epileptic cases and controls for Ab against cysticercosis was 3.6 \( (95\%\text{CI}: 2.5–4.9) \). Antibodies were detected in 58.7% \( (178/303) \) of the people with epilepsy and in 31.4% \( (190/606) \) of the controls \( P < 0.001 \). Main risk factors significantly associated with a positive Ab-ELISA were increasing age \( (P < 0.001) \) and belonging to a pig-breeding family \( (P = 0.04) \). Table 1 summarizes the relation between various risk factors and a positive Ab-ELISA.

Of 909 people examined, 26.1% \( (237/909) \) were positive in the Ag-ELISA. The matched OR between epileptic cases and controls for circulating Ag of cysticercosis was 2.9 \( (95\%\text{CI}: 2.1–4.3) \). Among the people with epilepsy and the controls 38.3% \( (116/303) \) and 20.0% \( (121/606) \) were positive respectively \( P < 0.001 \). The mean age was

<table>
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<tr>
<th>Table 1 Comparison between some risk factors and results of the Ab-ELISA and Ag-ELISA for cysticercosis</th>
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<tr>
<td>Ab-ELISA</td>
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<tr>
<td>No. of seropositives (%) ( (n = 368) )</td>
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<tr>
<td>Sex (male)</td>
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<td>Water pool nearby</td>
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Ag, antigen; Ab, antibody; ELISA, enzyme-linked immunosorbent assay; OR, odds ratio; CI, confidence interval.
significant higher for people with seropositivity
(\( P < 0.001 \)). The association between various risk factors and a positive Ag-ELISA is shown in Table 1.

Table 2 summarizes the detailed results of the Ag- and Ab-ELISA. In people with epilepsy, 26% (79/303) were only positive in the Ab-ELISA, 5.6% (17/303) were only positive in the Ag-ELISA and 32.7% (99/303) were positive in both the tests. Multivariate analysis using logistic regression showed that positivity for the Ab-ELISA and for the Ag-ELISA was independent factors, but were both significantly related to the occurrence of epilepsy.

**Discussion**

This study is an extension of the matched case–control study for epilepsy carried out by Nsengiyumva et al. (2003), which found a statistically significant association between the presence of antibodies against *T. solium* cysticerci and epilepsy. This association persisted after correcting for other factors potentially responsible for epilepsy. In this study, 303 out of 324 sera from epileptic patients as well as 606 from 648 sera from control persons originally tested by Nsengiyumva et al. (2003) were equally tested by the Ag-ELISA. Significantly more (38.3%) people with epilepsy were positive in the Ag-ELISA than controls (20.0%). Only a few studies on NCC and epilepsy which used both the Ag- and Ab-ELISA have been published (Newell et al. 1997b; Correa et al. 1999; Zoli et al. 2003). The advantage of including both tests is that information can be obtained about the nature of the lesions causing epilepsy (Dorny et al. 2004a). It is well known that seizures may occur at any evolutionary stage of the parasite: living, transitional or degenerating and calcified cysts stage (Carpio & Hauser 2002). However, there is scarce information about the frequency of involvement of the various stages in the aetiology of epilepsy. Unfortunately, neither CT-scan nor MRI were available in the study area so that the serological results could not be confirmed by brain imaging techniques. Although this implies that the results of this study have to be interpreted with caution, the correlation between living cysts as shown by CT-scan and the Ag-ELISA results (Nguekam et al. 2003a) is excellent.

The Ag-ELISA only detects living cysticerci (Garcia et al. 2000; Erhart et al. 2002; Nguekam et al. 2003b) and rapidly becomes negative when the cysts die after treatment (Nguekam et al. 2003a). The Ab-ELISA, on the other hand, detects antibodies against living, degenerating and dead cysticerci and does not allow differentiation between these different kinds of cysts. In this study, 26% of the people with epilepsy had only antibodies against cysticercosis and no circulating Ag, suggesting that only dead or degenerating cysts were causing seizures. Both Ab and circulating Ag were present in 32.7% of the epileptic patients, which suggests that living and dead cysts are simultaneously present. In these cases, it is not possible to deduce whether living or dead cysts are responsible for the seizures. In a small percentage of 5.6% of the people with epilepsy, only circulating Ag could be detected which indicates that in these individuals only living cysticerci are probably responsible for the epilepsy. It also indicates that in these cases the Ag-ELISA is more sensitive than the Ab-ELISA. This confirms previous observations in pigs, where particularly in light infections the sensitivity of the Ag-ELISA was much higher than that of the Ab-ELISA (Dorny et al. 2004b).

Assuming that both living and dead cysts are present in 32.7% of the subjects who are positive in the Ag- and Ab-ELISA, the results of this study indicate that degenerating or dead *T. solium* cysticerci (26 + 32.7 = 58.7%) are more often causing epilepsy than living cysts (5.6 + 32.7 = 38.3%). When these results are compared with those of Zoli et al. (2003), where only 1.2% of the Cameroonian epileptic patients were positive in the Ag-ELISA whereas 44.6% had specific antibodies against *T. solium*, it is evident that epilepsy in Cameroon was even more than in Burundi associated with dying or dead cysts. The very small number of people with epilepsy harbouring living cysts in West Cameroon as compared to the study area in Burundi might also indicate that more active transmission of the parasite is going on in the latter region.

As far as the risk factors are concerned, only increasing age was significantly associated with a higher percentage of positives both for the Ag- and Ab-ELISA. This confirms the observations of Houinato et al. (1998) and Nguekam et al. (2003c)). A significantly higher proportion of Ab-ELISA
positives was found in people belonging to a pig-breeding family, but this was not the case for the Ag-ELISA. On the other hand, being male and regular consumption of pork were risk factors associated with positivity in the Ag-ELISA, whereas the presence of an indoor toilet was negatively correlated with a positive Ag-ELISA result. Contradictory results have been reported about the impact of sex on infection with cysticercosis. The other risk factors for cysticercosis, however, are well known and were reported previously (Garcia et al. 2003; Ngeukam et al. 2003c).

This study has focused on NCC as a cause of epilepsy. It is obvious, however, that many other aetiological agents are able to provoke epilepsy. Among the parasitic infections which might be involved, malaria which is endemic in the study area, must certainly be further investigated for its potential role in causing epilepsy. Cerebral malaria has recently been identified as a potential cause of epilepsy in two studies in Africa using different epidemiological approaches (Ngoungou et al. 2006a,b). Cerebral toxoplasmosis (in association with HIV) and toxocarosis are also able to cause epilepsy (Nicoletti et al. 2002). In a case-control study in Burundi, onchocerciasis was shown to occur significantly more frequently in people with epilepsy than in controls (Newell et al. 1997a). As far as treatment of NCC is concerned, the availability of serological results is not sufficient to start an antiparasitic treatment. Images are necessary to obtain information on the number and localization of the cysts in the brain and to decide whether or not anthelmintic treatment will be used (Garcia et al. 2002).

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**Utilisation combinée d’ELISA pour la détection d’antigène et d’anticorps de la cisticercose comme outil épidémiologique dans l’étude de l’épilepsie au Burundi**

**Donnée de base** La neurocysticercose (NCC), due à l’infection du cerveau par le stade larvaire du *Taenia solium* humain est de plus en plus reconnue dans le monde entier comme cause de maladies neurologiques, en particulier l’épilepsie. Les symptômes cliniques du NCC sont non spécifiques et le diagnostic est souvent difficile.

**Objectif** Évaluer les avantages de la détection des anticorps (Ab) et des antigènes (Ag) circulants pour le diagnostic de la cisticercose chez les personnes avec épilepsie.

**Méthodes** Des échantillons de sérum ont été collectés chez des sujets dans une étude sur l’épilepsie incluant des cas et des témoins dans la région de Kirimba au Burundi entre mars et l’avril 2001 (cas d’épilepsie = 303; témoins sans épilepsie = 606). L’analyse ELISA a été utilisée pour la détection des anticorps (Ab-ELISA) et des antigènes (Ag-ELISA) circulants.

**Résultats** Ab-ELISA a révélé 58,7% de positivité chez les cas d’épilepsie et 31,4% chez les témoins sains. Ag-ELISA a révélé 38,3% de positivité chez les cas d’épilepsie et 20,0% chez les témoins. Les rapports de cotes étaient de 3,6 (CI95%: 2,5–4,9) pour Ab-ELISA et 2,9 (CI95%: 2,1–4,3) pour Ag-ELISA.

**Conclusion** L’Ag-ELISA et l’Ab-ELISA ont détecté un nombre significativement plus élevé de séropositifs chez les personnes avec épilepsie que chez les témoins. Le risque d’épilepsie était élevé chez les cas avec un l’Ag-ELISA positif, bien que de façon moins importante que chez les cas avec une positivité pour Ab-ELISA. Des cisticercoses morts ou dégénérées semblent être plus fréquemment associées à l’épilepsie que les kystes vivants. Le nombre élevé de personnes avec des antigènes circulants de *T. solium* suggère que le domaine d’étude est un foyer de transmission active du parasite.

**Mots clés** Burundi, cisticercose, diagnostic, ELISA, épilepsie, étude cas-témoins.
Uso combinado de la detección de antígenos y anticuerpos por ELISA para cisticercosis, como herramientas en un estudio de epilepsia en Burundi

ANTecedentes La neurocisticercosis (NCC), debida a una infección cerebral con el estadio larval del parásito humano *Taenia solium*, está cada vez más reconocida a nivel mundial como causa de enfermedad neurológica, y en especial de epilepsia. Los síntomas clínicos de la NCC son inespecíficos y su diagnóstico es a menudo difícil.

OBJetivos Evaluar los beneficios de la detección conjunta de anticuerpos (Ac) y antígenos (Ag) circulantes en el diagnóstico de la cisticercosis en personas con epilepsia.

MÉTODOS Se recolectaron muestras de suero de sujetos de un estudio caso control para epilepsia en el área de Kiremba, Burundi, entre Marzo y Abril 2001 (casos epilépticos = 303; controles sin epilepsia = 606). Se utilizó la técnica de ELISA para la detección de anticuerpos (ELISA-Ac) y antígenos circulantes (ELISA-Ag)

RESULTADOS El ELISA-Ac reveló una positividad del 58.7% en casos de epilepsia, y de 31.4% en controles sanos; mientras que el ELISA-Ag reveló un 38.3% de positividad en casos de epilepsia y un 20.0% en controles. El odds ratio pareado fue de 3.6 (95%CI: 2.5–4.9) para el ELISA-Ac, y de 2.9 (95%CI: 2.1–4.3) para el ELISA-Ag.

CONCLUSIÓN Tanto el ELISA-Ac como ELISA-Ag detectaron un número significativamente más alto de seropositivos entre personas con epilepsia que entre controles. El riesgo de epilepsia era mayor en casos con un ELISA-Ag positivo, aunque menos importante que en casos con positividad para ELISA-Ac. Los cisticercos muertos o en degeneración estaban más frecuentemente asociados con epilepsia que los cisticercos vivos. El alto número de personas con antígeno circulante de *T. solium* sugiere que el área de estudio es un foco de transmisión activa del parásito.

palabras clave Burundi, cisticercosis, diagnóstico, ELISA, epilepsia, estudio caso-control