## REVIEW

# USEFULNESS OF IMMUNOLOGICAL AND MOLECULAR TOOLS: PROGRAMS TOWARDS CONTROL AND ERADICATION OF CYSTICERCOSIS IN ENDEMIC AREAS

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**Abstract.** Neurocysticercosis (NCC) in the human brain is caused by *Taenia solium* and is one of the most serious parasitic zoonoses. Programs for the control of the taeniasis/cysticercosis complex are focused on elimination of the parasite in both humans and animals. The accuracy and the specificity or sensitivity of the detection tests for real cysticercosis and taeniasis cases are important for the control and eradication of taeniasis/cysticercosis in endemic and non-endemic areas. We discuss the advantages and disadvantages of immunological and molecular tools currently available for diagnosis of cysticercosis and taeniasis. ELISA and immunoblot were used with native and recombinant antigens for the detection of human NCC cases and animal cysticercosis cases. Genotyping of *T. solium* and two other human *Taenia, T. saginata,* and *T. asiatica* and copro-DNA tests were used for detection of worm carriers. These tools, available not only for identification of patients or carriers but also for epidemiological studies, are briefly reviewed.

#### INTRODUCTION

Three species of human Taenia are recognized: T. solium, T. saginata, and T. asiatica. It is possible to delimit areas of occurrence and endemicity of each species worldwide (Schantz, 2002). All three species are important public health problems in endemic areas as foodborne zoonoses. Cysticercosis/taeniasis is caused by the "pork tapeworm," T. solium, and is an international emergent and re-emergent zoonosis. It is the major etiological agent of seizures in endemic areas constituting a major public health problem for most of the developing world (Garcia et al, 1991, 2005; Tsang et al, 1995; Schantz, 2006). Neurocysticercosis, caused by T. solium, is the main cause of seizures in endemic areas (Garcia et al, 1991, 2005), where it is a wellknown problem. Now, it is also an important issue in travel medicine programs as the disease

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is recrudescing in non-endemic countries.

The methods used for detection of cysticercosis and taeniasis are performed using direct and indirect techniques used for finding evidence of the parasite from different sources. Recently, we have performed serology using T. solium glycoproteins purified from cyst fluid by preparative isoelectric focusing electrophoresis (IEFE), affinity chromatography and recombinant antigens (Ag1V1/Ag2) using ELISA, and immunoblots for detection of antibodies (Ito et al, 1998; Sako et al, 2000; Sato et al, 2006a). Our routine techniques also include amplification of traces of mitochondrial DNA, searching for direct evidence of the worm by PCR, Multiplex PCR, PCR-RFLP, BESS-T, and DNA sequencing from several material sources making differential diagnosis and clear up any doubt regarding the causative agent of a suspected case (Yamasaki et al, 2002, 2004a, 2005, 2006). These techniques are applied in different situations depending on the purpose of the test, whether it is either clinical differential diagnosis or a part of a control/eradication epidemiological program; in other words, the usefulness of one or other diagnostic test depends on the area (endemic/ non-endemic) and/or the suspected cases.

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Generally, the programs for the control of taeniasis/cysticercosis complex are focused on elimination of the parasite in humans and animals through the use of chemotherapy or elimination of infected hosts in developing and developed countries (Schantz, 2006; Pawlowski *et al*, 2005; Pawlowski, 2006), with the aim of either control and eradication of the parasite or prevention of the parasite entrance in a non-endemic area. This article will review and discuss our experience with different diagnostic methods and their role on taeniasis/cysticercosis control programs.

# Geographical distribution of the human *Taenia*

The presence of the adult worm is reported mainly in under-developed countries, and has a direct relationship with the lack of both sanitary conditions and proper meat inspection for human consumption (Schantz, 2006; Ito et al, 2003a). These two main characteristics of endemic areas maintain the life cycle of the parasite. An important geographical peculiarity is the co-existence of three species of human Taenia in the same area, as we reported in Thailand (Anantaphruti, 2007). In some cases, the endemic areas have natural qualities that can attract migrants, susceptible hosts, who are potentially exposed to the infective agents for taeniasis and/or cysticercosis (Sato et al, 2006b; Wandra et al, 2006) and possibly becoming worm carriers to non-endemic areas, which results in the introduction of an exotic disease. Even if taeniasis is not an important public health problem in a specific area with a well established system of sewage treatment, the lack of both basic hygiene practices and detection of taeniasis patients can put at risk the people living around a worm carrier, because the parasite is releasing viable eggs that can cause cysticercosis (Schantz et al, 1992).

By observing the geographical distribution of *T. solium* it is possible to distinguish two scenarios: 1) developed countries with reported cysticercosis cases in humans, but not in animals, and with no reported taeniasis cases or very rare cases of taeniasis (there are imported exotic disease in humans or a recrudescent disease); and 2) underdeveloped countries with taeniasis (possibility of three species) and cysticercosis cases in humans and animals, with the risk of exporting taeniasis and cysticercosis cases to humans and likelihood to be an endemic area.

# Genotypes of *T. solium*, *T. saginata*, and *T. asiatica*

Full sequencing of the mitochondrial DNA of T. solium from worldwide samples allowed us to observe regional genetic differences in the parasite. Intraspecific pairwise divergences between the two genotypes range from 0.9-1.3% for the Cox1 gene and from 1.6-2.1% for the Cytb gene. Divergences of American and African samples  $(0 \pm 0.4\%$  in Cox1 and  $0 \pm 0.6\%$  in Cytb) were also observed. T. solium African/ American and Asian genotypes were determined (Nakao et al, 2002). Concerning other human taeniid tapeworms, T. saginata and T. asiatica, pairwise divergences between these closely related species were observed, reaching 4.7% for Cox1 gene and 4.0% for Cytb gene (Nakao et al, 2002). Genotypic differences were also observed in our study of the antigens for diagnosis of cysticercosis. Nucleotide substitutions in the first intron of Ag2 allow differentiating African/American and Asian genotypes of T. solium, which constitute an alternative marker for differentiation of T. solium genotypes (Sato et al, 2006a). The use of these genotypic differences as markers allowed us to determine the original area of isolates and subsequent geographic classification. This is remarkable information for the travel medicine specialty.

### DIAGNOSING CYSTICERCOSIS

Human cysticercosis is usually diagnosed after the late onset of neurological symptoms, as seizures. A patient's history of seizures will support the diagnosis of cysticercosis, but it is not reported as a standard clinical sign for cysticercosis, which makes the clinical diagnosis difficult. Seizures and neurological symptoms appear generally five years after the exposure to the infective agent; however, with high variability that depends on the number, location, size, and viability or stage of degeneration of the cysts (Garcia *et al*, 2005; Takayanagui and Odashima, 2006). Clinical diagnosis is usually done after the onset of symptoms, meaning that the evolution of the disease in the host is already completed. In suspected cases, the metacestodes may be visible by neuroimaging exams as computed tomography (CT) or magnetic resonance imaging (MRI). In these cases, perilesional edemas around calcified lesions are usually present in and directly related with seizures and other focal neurological manifestations (Garcia *et al*, 2005).

In pigs, cysticercosis usually has no symptoms; the clinical diagnosis is based on inspection of areas of predilection of the parasite as tongue and eyes. The tongue inspection method only detects heavily infected pigs. Usually this method has low sensitivity and specificity (Sato et al, 2003, 2006b). For this reason, an early detection of human cysticercosis may decrease the number of the clinical form of the disease, making possible a safe, accurate, and supervised treatment of the disease. In the case of swine cysticercosis, its early and accurate detection leading to the proper treatment of truly infected pigs will improve the profit rate for the farmers who at the same time will provide safer pork products for the consumers.

### Antigens and serology

The antigens used in the cysticercosis serodiagnosis are purified glycoproteins (GPs) with molecular weights ranging between 8 kDa and 50 kDa (Tsang et al, 1989). GPs used for cysticercosis diagnosis are a 120 kDa protein complex in T. solium cyst fluid (Lee et al, 2005). It is comprised of six subunits varying in size from 14-38 kDa with 14 or 18 kDa backbone proteins; it generates a highly specific antibody response by the hosts. The synthesis of those GPs using isoelectric focusing electrophoresis (IEFE) is described by Ito et al (1998); resulting in highly specific tests for the detection of antibodies against GPs. IEFE purified antigens could be used with similar specificity and sensitivity in ELISA and immunoblot for the detection of, not only infected persons, but also infected pigs and even infected dogs (Ito et al, 2002b; Sato et al, 2003, 2006a,b; Wandra et al, 2003, 2006). The 8 kDa backbone protein of the GP antigens belongs to a family of proteins that is closely related to that of the antigens for T. solium (Sako et al, 2000, 2006; Hancock et al, 2003). These proteins usually show from 0-3 Nglycosylation sites (Sako et al, 2000; Sato et al, 2006a). Glycoforms with different masses or a different number of N-linked oligosaccharides are the putative cause of the different banding pattern of GPs from Asian, African, or American geographical origin.

A crucial characteristic to point out is that, although there are some antigenic differences from region to region, all the antigens have shown



Fig 1- Immunoblot of *T. solium* glycoproteins purified by monoclonal (4F10) affinity. Panels A and B correspond to 4F10 monoclonal affinity purified glycoproteins before (A) and after treatment with N-Glycosidase (B) using 4F10 monoclonal antibodies as a probe. The lanes are GPs from 1- Africa (Tanzania), 2- America (Brazil), 3- Asia (China) and 4- Asia (Indonesia).

the same immunoblot banding pattern after deglycosylation (Fig 1). This indicates that the GP protein backbone is similar to the antigens from different geographical origins, and the protein backbones, not the carbohydrate components of the antigen, are sufficient for serodiagnosis purposes (Sato et al, 2006b). Sako et al (2000) described four different antigens deriving from a native Asian antigen and synthesized a chimeric recombinant (RecT) antigen for the detection of infected humans and animals by both ELISA and immunoblot (Ito et al, 2002b; Sato et al, 2003, 2006a,b; Wandra, 2003). Serological tests using African, American, or Asian GPs and recombinant antigens showed a correlation higher than 92%, which indicates that both RecTs and GPs (African, American, or Asian) are suitable for serology in humans and swine worldwide. In conclusion, glycosylation causes more bands to show in the immunoblot tests; however, this feature does not affect significantly the sensitivity of the test in immunoblots or ELISAs then RecTs are recommended for diagnosis of cysticercosis.

# Practical examples of the usefulness of the serology

In a study with experimentally infected pigs, we showed the limit of detection for the ELISA tests as 2.5 cysts per animal, at least 1 month after the infection. The field-infected animals are usually carrying more than 2.5 cysts. Thus, it is possible to detect the total number of infected animals (Sato et al, 2003). We also showed that serology methods in swine are more useful than the tongue inspection method, detecting accurately more T. solium cyst infected pigs. Our results found that approximately 70% of PTI (positive at tongue inspection) pigs and 30% NTI (negative at tongue inspection) pigs positives by serology using GPs or RecTs (Ag1V1/Ag2) (Sato et al, 2003). Regarding the detection of cysticercosis in humans, there is a debate about the better source of material for detecting NCC. In spite of the actual recommendation that the cerebrospinal fluid (CSF) should be used for the detection of NCC (Del Brutto et al, 2001), our results with a blind test of serum samples and encephalic fluid showed 100% of correlation (Takayanagui et al, unpublished data), which indicates that serology is suitable for antibody detection for cysticercosis, especially NCC. Another interesting example concerns highly suspected cases by MRI and CT-Scan showing negative results using GPs and Ag1V1/Ag2 ELISA and immunoblot. According to our experience, these cases may be confirmed after surgical excision of the lesion to be a cystic type of neoplasia (Ito et al, 2006a). The serology using either GPs or Ag1V1/Ag2 ELISA was also used for detecting asymptomatic patients in northeast Brazil in a newly described endemic area (Sato et al, 2006b), and had been used for seroepidemiology in Indonesia and China with high accuracy and sensitivity (Wandra et al, 2003; Margono et al, 2003, 2006; Ikejima et al, 2005; Ito et al, 2006b).

# Molecular tools for diagnosis of cysticercosis

The observation of parasitic material is the most important step for diagnosis in parasitology. However, classical identification is sometimes not possible due to the impossibility of getting the whole parasite, or even a morphologic characteristic of the sample (Ito et al, 2002a, c, 2006a; Yamasaki et al, 2004b). Characterization of the full sequence of mitochondrial DNA of T. solium worldwide allowed us to design useful tools for the diagnosis of parasitic material, even if all the morphological characteristics are lost. These techniques do not distinguish between the stages of the parasite; consequently, they can be applied to identify either adults or metacestodes (Ito et al, 2002a,c, 2006a; Yamasaki et al, 2002, 2004a, 2005, 2006). The analysis of the nucleotide sequences of full human Taenia spp mDNA genome makes possible the determination of the genes suitable for use as diagnostic tools. Observing the alignment sequence entropy graphic of the sequence data, some genes (eg, cox1, cob) have substitutions between species conserved within the genotypes. These data were used to design strategies to discriminate T. saginata, T. asiatica, T. solium and also Asian and African/American genotypes of T. solium.

With the use of the obtained sequence information, it was possible to determine restriction enzyme patterns and then design a PCR-RFLP (Yamasaki *et al*, 2002), which in turn made it possible to differentiate the genus Taenia and even genotypes of T. solium (Nakao et al, 2002). In addition, this method is very useful because it does not require sequencing; however, it is time consuming because of the incubation. Despite its usefulness, this technique is not suitable to perform at the field level because of the need for proper storage and preservation of the enzymes, which is essential for getting accurate results. Studying the sequence data of the cytochrome oxidase c subunit 1 (cox I) gene, it was possible to design a set of genotype-specific primers that could be combined to produce a multiplex PCR to distinguish Asian, African, and American genotypes of T. solium, T. saginata, and T. asiatica in only one reaction batch. This tool allowed us to diagnose human Taenia species from the most diverse origins: eggs, fresh worms, worms fixed in alcohol, biopsies, pathology slides, stools, and other sources of DNA (Ito et al, 2002a, c, 2006a; Yamasaki et al, 2004b, 2005, 2006). Diagnosis is made by observing bands with specific molecular sizes in agarose gel, without sequencing. This is an extremely useful tool because sequencing facilities are not present in most part of laboratories in the under-developed world.

# USEFULNESS OF THE TECHNOLOGIES IN ENDEMIC AND NON-ENDEMIC AREAS

The major difference between cysticercosis and taeniasis control programs in endemic and non-endemic areas is the objectives of the programs. While programs in endemic areas are designed to stop or minimize transmission and spreading of the disease complex, in nonendemic areas it is done for differential diagnosis and confirmation of the disease in exotic cases. For both programs, the reliability of the tests is a *sine qua non* for the accomplishment of the proposed objectives. For endemic areas, it is ideal to detect all the potential human and animal parasite-carriers through programs of mass detection involving field surveys.

ELISA performed in serum samples is the most indicated method for the triage and screening of a population from a study area because the sensitivity and specificity of the antigens, GPs or recombinants, are very high



Fig 2- Multiplex PCR technique in a field survey in Indonesia (Bali). Lane 1 is molecular marker 100bp. Lanes 2, 3, 4, and 5 are positive controls for *T. solium*, *T. asiatica*, *T. saginata*, and a negative control, respectively. Lanes 6 and 7 are samples extracted from segments of an expelled worm. Lane 8 copro-PCR with a sample extracted from fecal sample of a suspected taeniasis case.

(Sato *et al*, 2003, 2006b; Margono *et al*, 2003, 2006; Wandra *et al*, 2003), detecting more accurately the true positives. It is a highly useful tool to determine the ways to conduct or establish control programs, to be done in an area under a control program for improving results or when it is the first contact in a new area (Sato *et al*, 2006b). Multiplex PCR technique has already proved it has the ability to be applied in the field in a survey in Indonesia (Bali) (Fig 2), where we could detect worm carriers accurately using DNA extracted from feces and specimen of a suspected taeniasis case with successful results.

### CONCLUSION

The use of serology and molecular techniques in endemic areas is a palpable reality. The reliability of the methods for application in the field or in laboratories in developing countries with limited facilities has already been proved, with successful results. These tools have real contributions for the improvement of the taeniasis/cysticercosis control and eradication programs worldwide.

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