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DIFFERENTIATING VIETNAMESE *TAENIA* SPP. BY MORPHOLOGICAL EXAMINATION AND POLYMERASE CHAIN REACTION - RESTRICTION FRAGMENT LENGTH POLYMORPHISM

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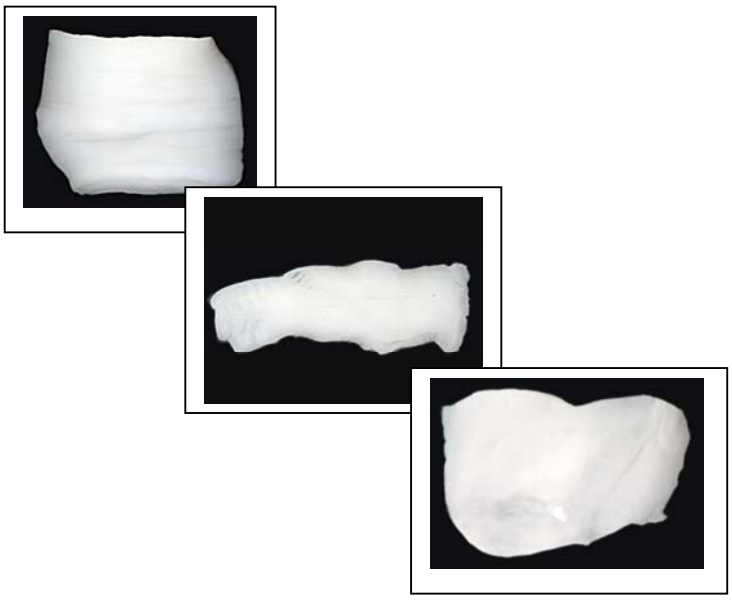

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Dedicated to:

My parents, my husband and my daughter as a promise

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List of the abbreviations

Acidity	pH
Base pairs	bp
Body weight	b.w
Celsius grade	^{0}C
Centimeter	cm
Deoxyribonucleic acid	DNA
Mililiter	ml
Milimeter	mm
Milimol	mM
Mitochondrial genes	mt DNA
Polymerase Chain Reaction	PCR
Retriction Fragment Length Polymorphism	RFLP
Ribosomal genes	rDNA
Species (plural)	spp.

Summary
This study was carried out to evaluate conventional techniques and PCR-RFLP assay for differentiation of *Taenia* spp. in human and to determine which species of *Taenia* are present in certain areas of Northern Vietnam. Thirty-four proglottid samples were collected after treatment with praziquantel from 34 patients at the National Institute of Malariology, Parasitology and Entomology in Hanoi city. Morphological examination enabled differentiation between *Taenia solium* and *Taenia saginata*, however not in all cases. PCR-RFLP assay based on amplification of the 12S rDNA fragment proved to be a reliable tool to distinguish *Taenia s. asiatica*, *T. saginata* and *T. solium* even when proglottids were not available for morphological examination. In addition, this assay allows easy interpretation of the results and does not depend on subjectivity of the observer. This study showed that three *Taenia* spp are present in humans in Vietnam: *T. solium, T. saginata* and *T. s. asiatica*. Fifteen samples were identified as *T. s. asiatica*, 16 samples were *T. saginata* and 3 samples were *T. solium*. The equal distribution of *T. s. asiatica* and *T. saginata* shows that the epidemiological situation on taeniasis/cysticercosis in Northern Vietnam is more complicated than previously described. Community-based surveys and slaughterhouse studies should be carried out to get a better understanding of the epidemiology of cestodes in Vietnam.

Keywords: taeniasis, *T. solium, T. saginata, T. s. asiatica*, morphological examination, PCR-RFLP.

CHAPTER I: INTRODUCTION

Taenia saginata and *Taenia solium* are the two taeniid species of greatest medical and economic importance, causing bovine and porcine cysticercosis and taeniasis in human (WHO, 1983, Yamasaki *et al*., 2002). Man is not only infected by adult tapeworms but also by the metacestodes of *T. solium*. Infection by metacestodes often leads to neurocysticercosis, a major cause of epilepsy associated with considerable morbidity and mortality (García *et al*., 2003). Cysticerci of *T. saginata*, in contrast, are found exclusively in cattle and do not develop in human.

In recent years, there have been numerous reports on a new form of human taeniid, which is known as Asian Taenia because it has been reported in Taiwan, Indonesia, Thailand, Korea, China and the Philippines (Bowles & Mc Manus, 1994). Morphologically, the adult Asian Taenia is similar to *T. saginata* (Eom and Rim, 1993; Fan *et al*., 1995). However, the intermediate host is not cattle but swine (Fan, 1988; Fan *et al*., 1990) and the parasite has a cysticercus more similar to that of *T. solium*. Because of its morphological and genetic resemblances with *T. saginata* it is classified as a subspecies of the latter, *Taenia saginata asiatica*. In practical terms, the important point is that human constitute the definitive host of three taeniids, *T. saginata, T. solium* and *T. s. asiatica* (Galan-Puchades & Fuentes, 2000). For this reason, fast, reliable and specific diagnosis of adult tapeworm infection in human is an important public health priority.

Diagnosis of adult *Taenia* spp. can be done by recovering eggs or proglottids in the faeces of infected hosts or by the copro-antigen test (Allan *et al*., 1990). Infection by the metacestodes can be detected by biopsy of subcutaneous cysts, neuroimaging techniques (computed tomography and magnetic resonance) and immunodiagnosis, and can be suspected in endemic regions on basis of neurological symptoms (epilepsy, headache). In recent years immunodiagnostic techniques were established, including detection methods for specific antibodies and circulating parasite antigen in serum or cerebrospinal fluid. However, until now only few of the current techniques have been standardized and fully validated (Dorny *et al*., 2003).

Differentiating *Taenia* spp. is mostly done based on morphological examination; the presence/absence of hooks on the scolex and the number of uterine branches in gravid proglottids are used as the main criteria. Several authors found that it was difficult to identify the species based on morphology because the scolex is often not available following elimination of a tapeworm and because some of the features overlap between species (Mayta *et al*., 2000). Le Riche & Sewell (1978) differentiated taeniid cestodes by isoenzyme electrophoresis using either fresh or frozen somata. In recent years, polymerase chain reaction (PCR) with primers derived from species-specific sequences has provided a rapid and sensitive method for identification of helminth parasites, including *Taenia* species (González *et al*., 2000). PCR- restiction enzyme analysis (RFLP) (Mayta *et al*., 2000; González *et al*., 2000, 2002; Rodriguez-Hidalgo *et al*., 2002) and multiplex-PCR (Yamasaki *et al*., 2004) permit differential diagnosis of *T. saginata*, *T. s. asiatica* and *T. solium*, even when examination by morphology can not be performed, because these methods do not rely on the availability of intact gravid proglottids.

In Vietnam, information concerning porcine cysticercosis is very limited and based mostly on passive surveillance. In a 1989-1993 study at slaughterhouses in Hanoi on a total of 2,091,000 pigs examined, 0.038% were found infected with *Cysticercus cellulosae*, the larval form of *T.*

solium and 0.047% pigs with *C. tenuicollis*, the larval stage of *Taenia hydatigena*, a tapeworm species of which the dog is the final host. A survey of cattle slaughtered in Hanoi from 1989-1993 (N= 144,390) indicated that 0.027% of cattle was infected with larvae of *T. saginata* (Willingham *et al*., 2003). By using a molecular approach, Doanh *et al* (2002) confirmed that cysticerci isolated from pigs in Vietnam belong to *T. solium*. Surveys on human taeniasis in Central and Northern provinces indicated a prevalence of 0.2-7.2%, however, techniques of low sensitivity were used and the results were inconclusive since it was not known with which species of tapeworms these people were infected (Willingham *et al*., 2003). In a study in the northern provinces of Vietnam, De *et al*. (1998a) identified most tapeworms infecting humans as *T. saginata* (78-80%), *T. solium* accounted for only 20-22% of the tapeworms recovered. One tapeworm infection was found to be caused by *T. s. asiatica* by using molecular means (cited by Willingham *et al*., 2003).

In some parts of Vietnam, the culinary habit of eating raw or undercooked meat may favour transmission of tapeworms. However, eating of raw pork ("nem chua") is more common than eating of raw beef; still adult *T. saginata* seems to be more common than *T. solium*. Based on the current knowledge and because of the puzzling situation regarding the species of *Taenia* found in Vietnam, we carried out this study to determine which *Taenia* species are occurring in certain areas of Vietnam. The results will provide necessary information for further research in order to prevent and control taeniasis/cysticercosis in human and animals.

Objectives:

- To evaluate conventional (morphological) techniques and PCR-RFLP assays for differentiating between *Taenia* species in human.

- To examine which *Taenia* species are present in certain areas of Northern Vietnam

CHAPTER II: LITERATURE REVIEW

2.1 Taxonomy

Kingdom: Animalia
Phylum: Platyhelminthes
Class: Cestoidea
Order: Cyclophyllidea
Family: Taenidae
Genus: Taenia
Species: solium
saginata

In recent years a tapeworm has been described as Asian *Taenia* (Eom & Rim, 1993), which shows morphological similarities with *T. saginata,* but has pig as main intermediate host. According to García (2001) and Ito *et al*. (2002), based on genetic analysis, Asian *Taenia* should be considered as a subspecies of *T. saginata* and designated as *T. s. asiatica*.

2.2 Distribution

T. saginata is cosmopolitan in its distribution, not restricted to traditional cattle breeding but equally occurring in industrialized countries. *T. solium* has a more restricted range, it is associated with poverty, pork consumption and poor pig husbandry practices (Rajshekhar *et al*., 2003) and known to be a serious economic problem and public health risk in Latin America, Asia and Western and Central Africa (Zoli *et al*., 2003). *T. s. asiatica*, a species whose possible existence had been noted in Taiwan since 1935 (Eom and Rim, 2001), has now been found also in Korea, China, Indonesia and Vietnam, and is associated with the eating of uncooked viscera of pigs that are slaughtered at home (Fan, 1988; Willingham *et al*., 2003).

2.3 Life cycle

The life cycle of taeniid tapeworms is complex, involving two hosts and a free-living stage:
The adults live in the small intestine of man, the definitive host. The gravid segments of *T. saginata* are very active and escape through the anus, releasing large numbers of eggs in the peri-anal region or on the ground where they can survive for long periods. The gravid segments of *T. solium* are less active and usually leave the host with the stools; often several attached proglottids may leave at the same time. When ingested by pig or cattle, the eggs hatch, each releasing an oncosphere, which migrates through the intestinal wall and blood vessels to reach striated muscle within which, it encysts, forming cysticerci. When inadequately cooked meat containing the cysts is eaten by man, the oncospheres excyst, settle in the small intestine and develop there into adult cestodes over the next three months or so. In contrast to *T. saginata* the eggs of *T. solium*, when accidentally ingested by man may develop into metacestodes in muscles, subcutaneous tissue or central nervous system. Brain localization of cysticerci of *T. solium* may cause neurocysticercosis. The segments of *T. solium* are somewhat less active than those of the beef tapeworm but its eggs, if released in the upper intestine, can invade the host (auto-infection), setting up the potentially dangerous larval infection known as cysticercosis in muscle or any other site (Peters & Pasvol, 1995).

The third form of tapeworm in man, *T. s. asiatica* is as an adult very similar to *T. saginata* (Eom and Rim, 1993; Fan *et al*., 1995). The intermediate host is mainly the pig, although

cattle can also be infected (but the parasite does not develop well) (Fan, 1988; Fan *et al*., 1990). *T. s. asiatica* metacestodes are viscerotropic; they settle mainly in the liver and visceral organs and not in the muscles of the intermediate host (Eom & Rim, 2001). As a consequence, transmission to man is caused by consumption of raw or undercooked liver or viscera.

2.4 Morphology

*2.4.1 Adult tapeworm**:***

Tapeworms are flat, ribbon shaped and segmented. Adults are hermaphrodite. The body forms three parts:

-The scolex (head): a very small structure, provided with 4 oval suckers that may or may not possess a muscular rostellum at the apex of the scolex. This rostellum is retractable and may be provided with one or more rings of hooks.

- The neck: is not segmented and has no specific morphological features. It is in this cervical region that strobilization takes place.

- The strobila: indicating the beginning of formation of segments. These can be classified in 3 groups: immature proglottids, mature proglottids and gravid proglottids.

T. solium: Adult measures 2 to 10 metres and contains 1000 proglottids. The scolex bears a rostellum with two crowns of hooks (Beaver *et al*., 1984). Mature proglottids have 3 ovary lobes and no vaginal sphincter is present. The gravid segment measures about 12 by 6 mm and the lateral uterine branches, visible by the mass of eggs, are less numerous than in *T. saginata*: 7-13 unilaterally (Bogisth & Cheng, 1998). Gravid segments are less mobile and usually expelled with the stools, separately or in groups of 3 to 6 proglottids (WHO, 1983)

T. saginata: Adult can reach a length of 4 to 6 metres (sometimes up to 25 metres) and contain 1000-2000 proglottids. The scolex is without a rostellum and no hooks are present (Beaver *et al*., 1984). Mature proglottids have 2 ovary lobes and a vaginal sphincter is present. The gravid proglottid is 20-30 mm long, 5-7 mm wide and has in excess of 12 main lateral braches (Bogitsh & Cheng, 1998). Usually, proglottids detach themselves spontaneously from the strobila and are rather motile. About 750,000 eggs may be expelled daily (WHO, 1983).

T. s. asiatica: Adults are large sized tapeworms (mean 341 cm long and 9.5 mm wide) with on average 712 segments. The scolex is spheroidal, with a cuspidal rostellum. The cervical swelling is distinct. Proglottids are rectangular. Anterior proglottids are wide and short; the posterior proglottids are long and narrow. Free proglottids bear a posterior protuberance. Mature proglottids have two ovary lobes that are unequal in size. The vaginal sphincter is round to oval (Eom and Rim, 1993). The uterus has numerous lateral branches, between 16-32. The way of leaving the host is as a single proglottid and spontaneously, independent from defaecation as for *T. saginata* (Fan, 1988; Ito *et al*., 2003).

2.4.2 Cysticercus: A bladder filled of liquid, with a bud on the cyst wall from where a single invaginated scolex will be formed.

The cysticercus of *T. saginata* is named *Cysticercus bovis*. These cysticerci are ovoid, have a milky white structure and measure 7,5-10 by 4-6mm. The invaginated scolex is already the scolex of the future adult stage, thus without hooks (Beaver *et al*., 1984).

The cysticercus of *T. solium* is named *Cysticercus cellulosae*, measures 5 by 8-10mm and has a scolex provided with rostellar hooks (Beaver *et al*., 1984).

The cysticercus of *T. s. asiatica* is named C*ysticercus viscerotropica*. These cysts are ovoid; have a milky white bladder, measuring 2,09 by 2mm. The outer bladder surface is covered by a wartlike formation. This wartlike formation is composed of numerous microtriches. Rudimentary hooklets may be seen in some scoleces, numbering 1 to 37 (Eom and Rim, 1993).

2.5 Clinical signs and symptoms

Adult tapeworms in human caused by *T. saginata*, *T. s. asiatica* or *T. solium* rarely cause serious clinical problems: abdominal pain, diarrhoea, and nausea, except for some cases where the tapeworms may physically block the intestinal tract and cause appendicitis (http://www.biosci.ohio-state.edu/~parasite/taenia.html). In contrast to taeniasis, caused by infection of the adult tapeworm, human cysticercosis is a serious health problem. The pathology associated with *T. solium* cysticercosis depends on which organs are infected and on the number of cysticerci that establish. An infection consisting of a few small cysticerci in the liver or muscles does not cause problem (García *et al*., 2003). On the other hand, even a few cysts, if located in a particularly "sensitive" area of the body, such as the central nervous system or the eye, may result in irreparable damage and affect human health and productivity (Mukaratirwa *et al*., 2003).

2.6 Epidemiology

Prevalence areas of *T. saginata* can be classified into 3 groups (WHO, 1983):

i. Countries or regions that are highly endemic, with prevalence rates in the human population that exceed 10%: e.g. Central and East African countries.
ii. Countries and regions with moderate infection rate (0.1-10%): e.g. South East Asia: Thailand, India, Vietnam and the Philippines.
iii. Countries and regions with prevalence below 0.1%: e.g. Western Europe.

The infection rate of *T. saginata* taeniasis in man is closely related to the frequency of eating raw beef. The infection is most common in the 20 - 40 age groups. The risk of being infected is 5 times greater in members of a carrier's family, 14 times greater in workers that have professional contact with raw meat and 40 times greater in raw beef eaters (WHO, 1983).

Prevalence of *T. solium* infection varies according to the regional level of sanitation, pig husbandry system and eating habit. It is endemic in most developing countries where pigs are raised and pork is consumed. In Asia, reliable prevalence data on *T. solium* taeniasis and cysticercosis for most countries, except Indonesia, are unavailable (India, Philippines), incomplete or are outdated (Taiwan, Korea, China) (Rajshekhar *et al*., 2003).

Prevalence rates of *T. s. asiatica* in humans have not yet been estimated in the countries where it is present. Factors affecting transmission of *T. s. asiatica* relate to the custom of

eating viscera; especially consumption of the liver and blood of freshly killed animals, seem to be contributing to infection (Fan, 1988; Eom and Rim, 2001).

Economic impact: The annual loss due to porcine cysticercosis was roughly estimated at US$ 43 million in Mexico (Acevedo-Hernandez, 1982) and about 25 million Euro in 10 West and Central African countries (Zoli *et al*., 2003). Fan (1997) estimated that the annual economic loss due to taeniasis (including *T. s. asiatica*) in mountainous regions of Taiwan and in Samosir island of Indonesia amounted to US$ 11million and US$ 2.4 million, respectively.

2.7 Diagnosis of taeniasis

Correct identification of *Taenia* spp is important because the consequences of human infection by these parasites are very different.

2.7.1. Parasitological diagnosis of taeniasis:

- *Coprological examination:* Allows the detection of *Taenia* eggs from stool samples, but eggs of *T. solium* are indistinguishable from these of *T. saginata.* Coprological examination has a poor sensitivity (García *et al*., 2003).

- The finding of loose gravid proglottids (usually *T. saginata*) in the underwear or in the faeces provides proof of infection. This finding is less likely to occur in case of *T. solium* infection. A survey of Fan (1988), Ito *et al*. (2003) in patients with *T. s. asiat*ica infection found that over 90% reported passing proglottids.

- *Morphological examination*: Differential diagnosis between the adult stage of *T. solium* and *T. saginata* is based mainly on morphological criteria on fixed and stained proglottids, mostly based on the number of uterine branches present in well preserved gravid proglottids, or on the presence or absence of hooks in the scolex of the tapeworms (Mayta *et al*., 2000). However, overlapping between the numbers of uterine branches of these species has been described; it is also relatively uncommon to obtain a whole adult tapeworm with an intact scolex following treatment. Recently, Jeri *et al* (2004) improved the treatment method to obtain a recognizable tapeworm, making differentiation between *T. solium* and *T. saginata* easier. Proglottids can be stained with the Semichon's acetocarmine stain method to allow morphological differentiation. According to Eom and Rim (1993), the presence of posterior protuberances in gravid proglottids is a useful characteristic for the diagnosis of *T. s. asiatica*.

2.7.2 Immunodiagnosis

Immunological methods have been developed for the diagnosis of *Taenia* spp infection. The best available diagnostic assay for taeniasis is the coproantigen detection ELISA. It has a sensitivity of about 95% and specificity greater than 99% (García *et al*., 2003); however, this test does not provide species-specific diagnosis and there is a potential biohazard of collecting and handling faeces from *Taenia* carriers (Allan *et al*., 2003). The possibility of diagnosing *T. solium* taeniasis by the detection of species-specific circulating antibodies has been demonstrated (Wilkins *et al*., 1999). This test uses excretory-secretory derived antigens from *T. solium* tapeworms in an enzyme-linked immuno-electrotransfer blot (EITB).

2.7.3 Molecular approaches

The Polymerase Chain Reaction (PCR) assay for amplification of DNA sequences was developed by Mullis in 1983. Nowadays PCR has become an important diagnostic tool and a tool to study the phylogeny of infectious agents (Mariaux *et al*., 1996). This technology has

been shown differentiate parasites starting from small amounts of their DNA (Allan *et al*., 2003).

Differentiation of human *Taenia spp*. by molecular assays is normally done on proglottids expelled from carriers after treatment (Eom *et al*., 2002; Rodriguez-Hidalgo *et al*., 2002; González *et al*., 2002). In recent years, PCR tests for species-specific confirmation of *Taenia* spp have been developed base on the detection of the parasite DNA in faecal samples (copro-DNA) (Yamasaki *et al*., 2004), or on cysticerci (Yamasaki *et al*., 2002; Yamasaki *et al*., 2004) or eggs present in the faeces (Yamasaki *et al*., 2004).

Different methods and several loci were used for differentiating *Taenia* species:
Harrison *et al* (1995) and González *et al* (2002) designated primers and applied these in multiplex PCR, giving a differential detection of *T. saginata* and *T. solium*.

Mayta *et al* (2000) used PCR-RFLP to differentiate *T. solium* and *T. saginata*. They amplified the 3' region of the 18S and the 5' region of the 28S ribosomal gene (spancing the 5.8S ribosome gene) and used 3 restriction enzymes (either AluI, DdeI or MboI) for analysis of the PCR amplicons. Each enzyme gave a unique pattern for each species. In this assay, the primers amplified DNA from all cestodes, not only from *Taenia* spp.

Nickisch-Rosenegk *et al* (1999) used the sequence of mitochondria 12S rDNA, a gene that is frequently used for phylogenetic studies. These authors reported sequence differences between 2 *Taenia* species but did not apply this in practice.

Rodriguez-Hidalgo *et al*. (2002) differentiated *Taenia* spp by PCR-RFLP using the 12S rDNA but developing new primers to reduce non-specific amplification experienced when using field samples and DdeI was used as restriction enzyme.

In Asia, where *T. s. asiatica* is present, several methods were developed to distinguish *T. s. asiatica* from *T. solium* and *T. saginata,* such as the internal transcribed spacer (ITS) and intergenic spacer (IGS), as well as several mitochondrial protein coding genes, have been found sufficient polymorphism for elucidating phylogenetic relationships at lower taxonomic level (Hwang and Kim, 1999).

Eom *et al*. (2002) amplified the ITS2 region and the complete 5.8S rDNA, using primers from conserved sequences of the 5' end region of 5.8 S and 28S rDNA. The result showed that there is sufficient polymorphism in the ITS2 sequence to distinguish *T. s. asiatica* from *T. saginata*. In this study, random amplified polymorphic DNA (RAPD) assay was used also to amplify the ITS2 region with success to differentiate human *Taenia* species. But this is cumbersome test and being difficult in getting reproducible results.

Yamasaki *et al* (2002) used a base excision sequence scanning thymine-base (BESS T-base) system for differential diagnosis of *T. sagi*nata, *T. s. asiatica* and the two genotypes of *T. sol*ium. This test is based on sequences of mitochondrial genes, and compares characteristic T-base peak profiles without the need for DNA sequencing. This method can be used when nucleotide data are available, but is a complicated and expensive.

Yamasaki *et al* (2004) used a multiplex PCR to amplify a diagnostic Cox1 fragment to differentiate *T. saginata* and *T. solium* infection on faecal samples and also to differentiate *T. saginata* and *T. s. asiatica* using proglottids samples. Multiplex PCR is a useful diagnostic

test but it appeared that the sensitivity of this test was lower than that of the copro-antigen detection test when applied to faecal samples.

2.8 Prevention and control of taeniasis in man

The prevention and control of taeniasis in man is based on three fundamentals: education, diagnosis and treatment. Health education may play an important role by helping to change eating habits and to promote the self-diagnosis of *T. saginata* infection as well as to improve sanitation, husbandry system and meat inspection in slaughterhouses. Human taeniasis may be decreased by detection and treatment of tapeworm carriers or by treatment of the whole population (WHO, 1983). In regions where taeniasis is only due to *T. saginata* and human cysticercosis does none occur, praziquantel can be used. Mass treatment of the human population may be performed in areas where *T. solium* infection is endemic (Alan *et al*., 1997). For treatment of human taeniasis in *T. solium* endemic areas bithionol, niclosamide or praziquantel may be used, however, niclosamide is preferred because it is highly effective against the intestinal stage of the parasite and has no effect on the cystic stage (WHO, 1983; Miyazaki, 1991; Alan *et al*., 1997). Community cooperation is very important for any intervention to achieve sustainable control.

CHAPTER III: MATERIALS AND METHODS

3.1 Parasite material

Thirty-four samples (proglottids) were collected from 34 patients originating from different areas of northern Vietnam, during the period from June 2002 to October 2003 at the National Institute of Malariology, Parasitology and Entomology (NIMPE) in Hanoi city. The samples were obtained following treatment with praziquantel (15 mg/kg b.w) and purgation with $MgSO_4$ (30g/patient). These patients came to hospital because they had in their stool or had seen actively passing segments. The proglottids were preserved in 70% ethanol and kept at 4^oC until used for differentiating by morphological examination and PCR-RFLP.
A list of samples is shown in Table 1.

Table I: Origin of the samples

			Sex (age)		Clinical signs				Collection	
Code	Date	Origin (province)	M	F	APP	AP	S	E	Scolex	Proglottids
VN 1	9/01/03	Bacninh		34	ni	ni	ni	ni	-	+
VN2	14/1/03	Langson	48		ni	ni	ni	ni	+	+
VN 3	21/1/03	Hanoi	38		ni	-	-	-	-	+
VN 4	22/1/03	Hungyen	35		+	+	-	-	-	+
VN 5	24/1/03	Hatay	26		+	ni	-	-	-	+
VN 6	14/2/03	Hanoi	30		+	+	-	-	-	+
VN 8	21/2/03	Thanhhoa	24		+	+	-	-	-	+
VN 9	22/2/03	Hatay	27		+	-	-	-	-	+
VN11	24/2/03	Hoabinh		24	-	-	-	-	+	+
VN12	5/3/03	Hatay	43		ni	ni	ni	ni	-	+
VN13	6/3/03	Hanoi	62		-	-	-	-	-	+
VN14	6/3/03	Haiphong	28		+	-	-	-	-	+
VN15	18/3/03	Hanoi	27		-	+	-	-	-	+
VN16	19/3/03	Hatay	55		+	+	-	-	-	+
VN17	25/3/03	Hanoi	28		+	-	-	-	+	+
VN18	28/3/03	Haiphong	37		+	+	-	-	-	+
VN19	2/3/03	Hatay	45		-	-	-	-	+	+
VN20	4/4/03	Hatay		30	+	+	-	-	-	+
VN21	8/4/03	Hoabinh	43		+	+	-	-	-	+
VN23	22/4/03	Hatay	43		+	-	-	-	-	+
VN24	5/5/03	Hatay	28		-	+	-	-	-	+
VN25	6/5/03	Hatay	30		+	+	-	-	-	+
VN26	14/5/03	Hanoi		42	-	-	-	-	-	+
VN27	14/5/03	Hatay	3		+	+	-	-	-	+
VN28	16/5/03	Hoabinh	30		+	-	-	-	-	+
VN29	21/5/03	Hatay	27		-	+	-	-	-	+
VN30	21/5/03	Hungyen	17		+	-	-	-	-	+
VN31	22/5/03	Hanoi		20	+	+	-	-	-	+
VN32	26/5/03	Hungyen	30		+	-	-	-	-	+
VN33	26/5/03	Hatay	28		-	+	-	-	-	+
VN01	20/6/02	Baccan	38		-	+	+	-	-	+
VN02	18/4/03	Baccan	42		-	+	+	-	-	+
VN03	24/10/03	Hatinh		17	-	+	-	-	-	+
VN04	24/10/03	Hatinh		35	+	+	-	-	-	+

Note:
ni: no information

M: Male	F: Female
APP: Actively passing of proglottids	AP: Abdominal pain
S: Subcutaneous cysts	E: Epilepsy

3.2 Materials and method for morphological examination

3.2.1 Materials

- Acetic acid-glacial 100% (Merck, ref: K29637063)
- Carmine Red stain (Merck)
- Ethanol 95%
- Formaldehyde 37% v/v (Merck, ref: K27267301)
- Entellan
- Hydrochloric acid (Merck, ref: K21372717)
- Xylen (Merck, ref: K25440681)
- Object slides and cover slips

Solutions:

- AFA (Alcohol, Formaldehyde, Acetic acid):

Formaldehyde 37% v/v	10ml
Ethanol 95%	50ml
Acetic acid glacial	2 ml
Distilled water	40ml

- " Semichon's carmine" * stain:

Acetic acid glacial	50ml
Distilled water	50ml
Carmine's stain	until saturation

To prepare the Semichon's carmine stain the ingredients are mixed in a bottle and left in a warm water bath at 95^0C for 15 minutes. Next the solution is decanted and the supernatant filtered. Before usage the solution is diluted in ethanol 70% in equal parts.

3.2.2 Staining of proglottids of Taenia spp. *(*Morgan and Hawkins 1949)

The proglottids were washed in saline to remove mucus and all other debris from the tegument. Next, relaxation of the specimen was allowed by leaving them in ethanol 10% in a recipient placed in an ice bath for several hours. The specimen was then placed between two slides under gentle pressure and transferred to a petri dish containing "AFA" (Alcohol, Formaldehyde, Acetic acid) and left overnight for the fixation process to continue in this way. Next, the specimen was released and transferred in ethanol 70% for 30 minutes.

In a next step the proglottids were transferred to a new petri dish containing semichon's carmine stain. The proglottids were left between 8-24 hours in the staining solution. The time of staining depended on the concentration of the stain, and the size and thickness of the specimen. Next the excess of staining was eliminated by transferring the specimen into:

Ethanol 20% for 45 minutes,
Ethanol 50% for 45 minutes,
and Ethanol 70% for 45 minutes

The specimen was destained in a solution of hydrochloric acid (0.1 to 0.5% v/v in distilled water) until it showed a pink color, followed by dehydrating in ethanol for 30 minutes and ethanol 100% for 30 minutes. The specimen was then cleared up in xylene for one minute and mounted in Entellan.

The slides were checked under the microscope. The number of main uterine braches in gravid proglottids and/or the ovary lobe in mature proglottids was counted.

Table II: Parameters used for identification of *Taenia* spp. by morphological examination (based on mature proglottids and gravid proglottids):

Taenia spp.	Number of uterine branches/side	Number of ovary lobes
T. solium	7 - 12 [a]	3 [a]
T. saginata	Excess [a]	2 [a]
T. s. asiatica	21 (11-31)[b,c]	2[c]

a- Bogitsh & Cheng, 1998.
b- Fan, 1988.
c- Eom *et al*., 2002.

3.3 Materials and method for PCR

3.3.1 Procedures for DNA extraction

DNA extraction protocol (slightly modified from Boom *et al*., 1990):

The segments were cut into 1-3 mm^3 pieces and transferred into a 1.5 ml tube. 250 µl of the lysis buffer (consist of 60 mM Tris-HCl, pH 7.4, 60 mM EDTA, 10% Tween, 5 mM MgCl2, 1% Triton X-100, and 1.6 M Gu-HCl), 250 µl of milli-Q water (Millipore, Brussels, Belgium) and 50 µl of protein K (20 mg/ml, Roche Diagnostics, Brussels, Belgium) were added into the tube. Samples were incubated in a thermomixer compact (Eppendorf, Koln, Germany) overnight at 60^0C and shaken at 1400 rpm. Then, 40 µl of diatomaceous earth suspension (Sigma-Aldrich, Bornem, Belgium) was added, mixed and incubated at 37^0C for one hour while shaking at 900 rpm. The suspension was centrifuged at 15,300 rpm for 20 seconds and the supernatant discarded. The pellet was washed with 900 µl of 70% ethanol (v/v) at 4^0C and centrifuged at a speed of 15,300 rpm for 20 seconds, next the supernatant was discarded. This washing step was done twice. Then, the pellet was washed with acetone, centrifuged and the supernatant was discarded. Subsequently, the pellet was dried in the thermomixer at 50^0C for 20 minutes and 90 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) was added to the pellet and incubated for 20 minutes at 60^0C while shaking at 1000 rpm in the thermomixer. Finally, 60 µl of the supernatant was transferred to a new PCR tube after centrifuging at 15,300 rpm for 40 seconds, and stored at -20^0C. This tube was used in PCR assays.

3.3.2 Primers

Two pairs of primers were designed by Rodriguez-Hidalgo *et al*. (2002) based on comparison of the conserved areas of the 12S rDNA fragments from *T. saginata*, *T. asiatica* and *T. solium,* that were obtained from the Genbank. The following primers were chosen: ITMF/ITMTnR and TAEnF/ITMTnR because it was found that these would hybridize universally with all *Taenia* species. Specificity of the primers against all DNA sequences in the Genbank was checked by using the nucleotide-nucleotide BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

3.3.3 Polymerase Chain Reaction

PCR was performed using 5µl of the extracted DNA as template and 20 µl of PCR solution containing 12.5 µl of PCR-buffer (20 mM Tris-HCl, pH 8.4, 100 mM KCl, 0.2% Triton X-100, 1.6 mM $MgCl_2$), 4.3 µl of milli-Q water, 2 µl of a mix of the 4 deoxynucleotide triphosphates (dNTP, final concentration 0.2 mM), 0.4 µl of each primer and 0.4 µl of *Taq*-polymerase (50 U/µl, Eurogentec, Searing, Belgium). *T. crassiceps* DNA was used as the positive control and milli-Q water was used as the negative control. The amplification was carried out in a PTC-3 Thermal cycle and programmed for one cycle of denaturation at 94^0C during 4 minutes, then, 40 cycles with the following steps: 94^0C during 45 seconds for

denaturation, 61^0C (in case of using ITMF/ITMTnR primers) or 55^0C (in case of using TAEnF/ITMTnR primers) for 45 seconds for annealing, 72^0C during 45 seconds for elongation and the final cycle was 72^0C for 8 minutes. Next, 5 µl of the amplified DNA was taken after mixing with 3 µl of loading buffer and this was placed into an appropriate slot of 2% agarose w/v (Eurogentec) and TAE buffer (0.04 M Tris-acetate plus 0.002 M EDTA) for electrophoresis, using a Mupid-21 system (UL® Cosmo Bio Co), including a marker of 100 bp (MBI Fermentas, GmbH, St.Leon-Rot, Germany). The electrophoresis was activated at 100 volts for 20 minutes. The gel was taken out and was stained in ethidium bromide (Sigma-Aldrich) for 30 minutes. The result was visualized under ultraviolet light and a picture was taken.

Theoretically, the following molecular sizes of products must be obtained: approximately 370 bp for PCR with ITMF/ITMTnR primers and approximately 890 bp for PCR with TAEnF/ITMTnR primers.

3.4 Restriction Fragment Length Polymorphism (RFLP)

3.4.1 Restriction enzymes

By using the REBASE programme, new digestion enzymes Hpy8I (10 U/µl, Biolabs) and HinfI (10 U/ µl, Bochringer Mannheim GmbH) were chosen to distinguish clearly between *T. s. asiatica* and *T. saginata*. DdeI (10 U/µl, Biolabs) was also used according to the protocol described by Rodriguez-Hidalgo *et al*. (2002)

3.4.2 Procedures

Six microliters of the amplified DNA was placed in a tube containing 9µl of enzymatic solution (consisting of 7.2 µl of milli-Q water plus 1.5 µl TBE buffer and 0.3 µl of restriction enzyme), centrifuged at a speed of 14,000 rpm for 20 seconds and put in a water bath (Memmert type WB-7) at 37^0C overnight for digestion. After mixing with 2 µl of loading buffer, 4 µl of digested solution was placed in the appropriate slot of a 10% polyacrylamide gel in TBE buffer (88 mM Tris, 89 mM Boric acid, 2 mM EDTA). The electrophoresis was performed on the electrophoresis apparatus. A marker of 100 bp (MBI Fermentas, GmbH, St.Leon-Rot, Germany) was also included for size identification of the bands. The electrophoresis mechanism was activated at 100 volts for 2h40. Subsequently, the gel was placed into a 10% v/v acetic acid bath for 30 minutes, followed by washing 3 times for 2 minutes with milli–Q water. Then, the gel was stained for 20 minutes with 2% silver nitrate and was developed for 2 to 8 minutes with development solution after washing for 30 seconds in milli-Q water. The developer reaction was blocked for 10 minutes with EDTA and the gel packed in a plastic sheet after keeping in preservation solution for 30 minutes.

Theoretically, the target sequences obtained with ITMF/ITMTnR primers will be cut in bands of the following length (bp) (by using REBASE program) (table III):

Table III: Theoretical band lengths (bp) of the amplicons obtained with ITMF/ITMTnR primers were digested with restriction enzymes DdeI or Hpy8I:

Taenia species	DdeI	Hpy8I
T. solium	325, 46	339, 32
T. saginata	164, 158, 46, 5	236, 106, 31
T. s. asiatica	165, 161, 46	340, 32

And target sequences from the TAEnF/ITMTnR primers will be cut in bands of the following lengths (bp) (table IV).

Table IV: Theoretical band lengths (bp) of the amplicons obtained with TAEnF/ITMTnR primers were digested with restriction enzymes DdeI or HinfI.

Taenia species	DdeI	HinfI
T. solium	524, 332, 28, 26	613, 290, 7
T. saginata	523, 171, 158, 28, 27, 5	613, 299
T. s. asiatica	525, 172, 161, 28, 27	316, 299, 291, 7

CHAPTER IV: RESULTS

4.1 Morphological examination

The results of the morphological examination of the stained proglottids are shown in table V (in annex).

Twenty-six out of 34 samples were identified as *T. saginata* because the number of uterine branches varied from 16-32 in gravid segments and/or 2 ovary lobes were counted in mature segments (VN2, VN5, VN18 and VN32) (figures I and II). Three samples were determined as *T. solium* (figure III) because 8-12 uterine branches were counted in the gravid proglottids. In the remaining samples (VN9, VN12, VN13, VN31 and VN04) the uterine branches could not be counted.

Figure I: Mature proglottid with 2 ovary lobes from *T. saginata* after staining with semichon-acetocarmine

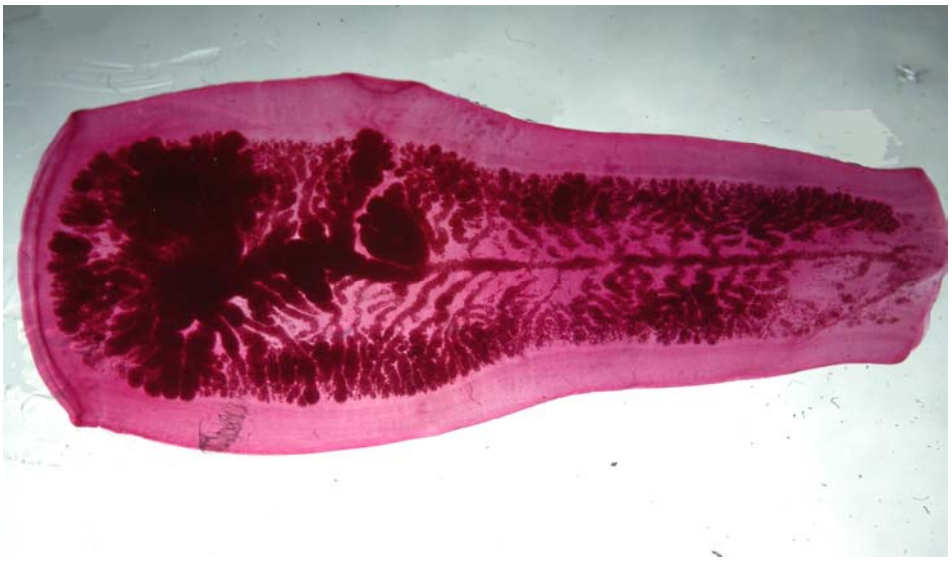

Figure II: Uterine branches in a gravid proglottid of *T. saginata* after staining with semichon-acetocarmine

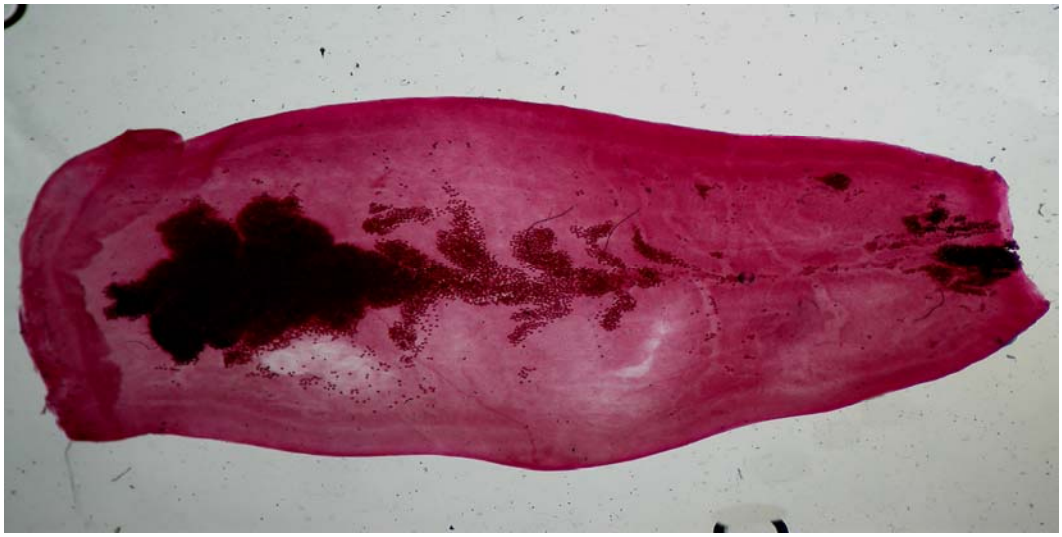

Figure III: Uterine branches in gravid proglottids of *T. solium* after staining with semichon-acetocarmine

4.2 PCR-RFLP

The results of the PCR on DNA extracted from the proglottids are shown in figure IV and fig. V.

With the ITMF/ITMTnR primers DNA from *Taenia* could be detected in 33 of the 34 samples; the molecular size of the amplified product was approximately 370 bp (fig. IV). One sample (VN03) did not give a positive signal.

With the TAEnF/ITMTnR primers DNA from *Taenia* could be detected in all 34 samples; the molecular size of the amplified product was approximately 898 bp (Figure V).

These sizes correlate well with the expected lengths of the products predicted from the Genbank sequences. No non-specific bands were present in both PCRs. This indicated that all the samples belong to *Taenia* species.

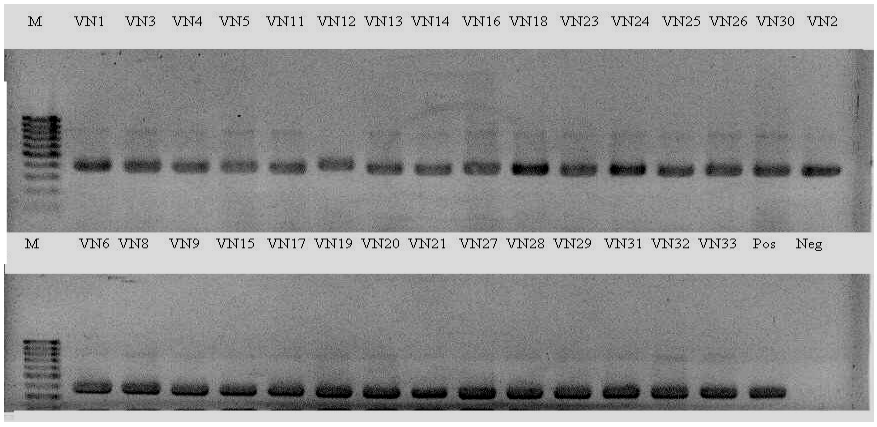

Figure IV: PCR products from the proglottid samples by amplification of ITMF/ITMTnR primers. Analysis is performed on a 2% agarose gel and stained with ethidium bromide. M: 100 bp marker. Pos: positive sample, Neg: negative sample

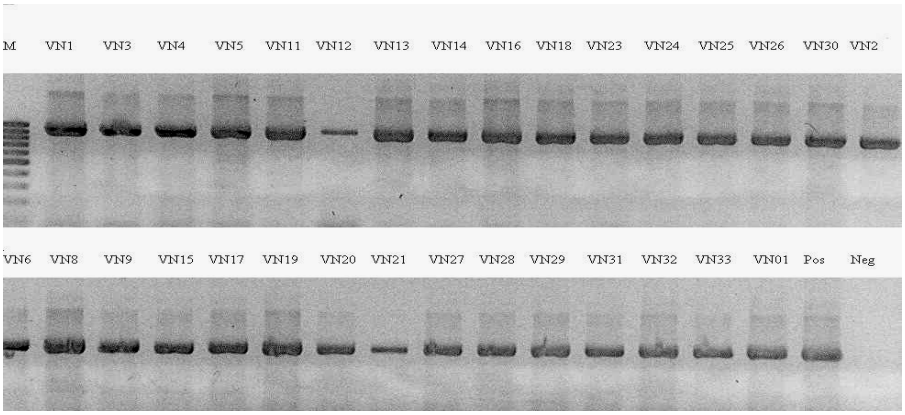

Figure V: PCR products from the proglottid samples by amplification of TAEnF/ITMTnR primers. Analysis is performed on a 2% agarose gel and stained with ethidium bromide. M: 100 bp marker. Pos: positive sample, Neg: negative sample

In order to distinguish the *Taenia* samples at species level, the amplicons from PCR with the ITMF/ITMTnR primers were further analyzed using RFLP with restriction enzyme DdeI. Thirty-one samples showed a band of 164 bp, indicating that these samples were *T. saginata* (samples from VN1 to VN33 and VN04). VN01 and VN02, with a band of 325 bp were *T. solium*.

The second restriction enzyme Hpy8I was used to determine whether there was any *T. s. asiatica* among the *T. saginata* samples. The results showed that 15 of 31 samples were determined as *T. s. asiatica* with a specific band of 339 bp; the other samples were identified as *T. saginata* with a band of 236 bp (figure VI). The pattern of fragments obtained was identical to that predicted.

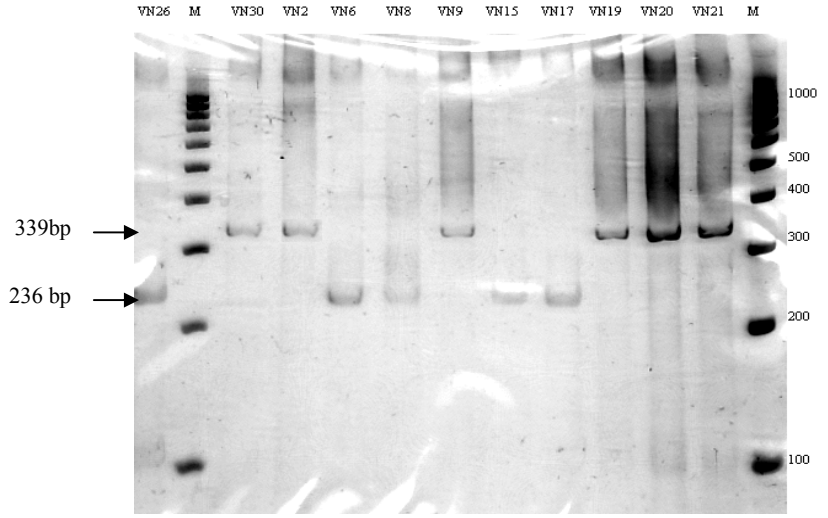

Figure VI: Hpy8I enzymatic restriction of the fragment amplified with ITMF/ITMTnR primers. Bands approximately of 339 bp correspond with the *T. s. asiatica* profile (VN30, VN2, VN9, VN19, VN20 and VN21). Bands approximately of 236 bp are *T. saginata* (VN26, VN6, VN8, VN15 and VN17). Analysis is performed on a 10% polyacrylamide gel and stained with Silver kit. M: 100 bp marker.

Three samples, VN03 together with VN01 and VN02 were amplified with another set of primers: TAEnF/ITMTnR in order to further analyze the negative result. The result was positive and the amplicons were digested with DdeI to determine the *Taenia* species of VN03. Results gave a *T. solium* profile for all three samples (fig.VII).

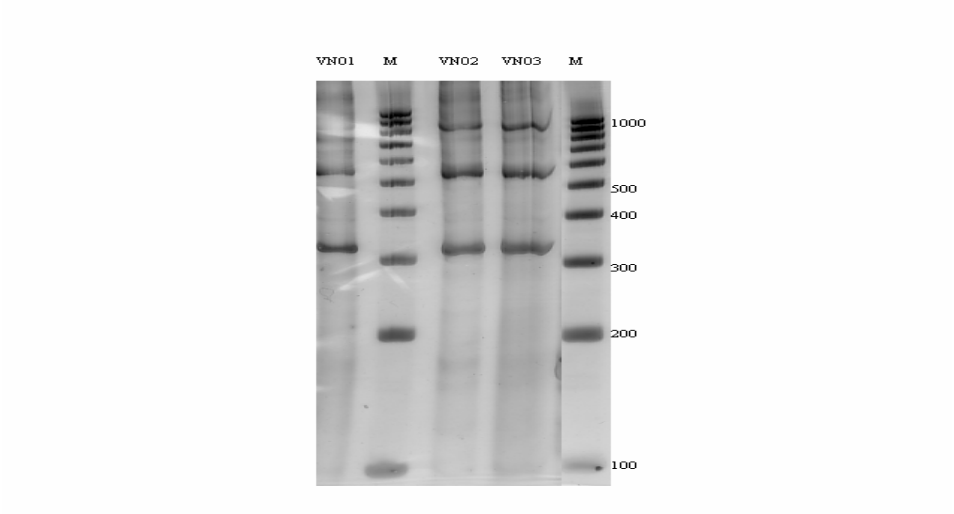

Figure VII: DdeI enzymatic restriction of the fragment amplified with TAEnF/ITMTnR primers. Bands approximately of 520 bp and 330 bp correspond with the *T. solium* profile (VN01, VN02 and VN03). Analysis is performed on a 10% polyacrylamide gel and stained with Silver Kit. M: 100 bp marker**.**

Subsequently, all thirty-four samples were digested with restriction enzyme HinfI following amplification with the TAEnF/ITMTnR primers to compare the results obtained with the ITMF/ITMTnR primers. This PCR-RFLP confirmed the results obtained with the other combinations of primers and restriction enzymes (fig. VIII).

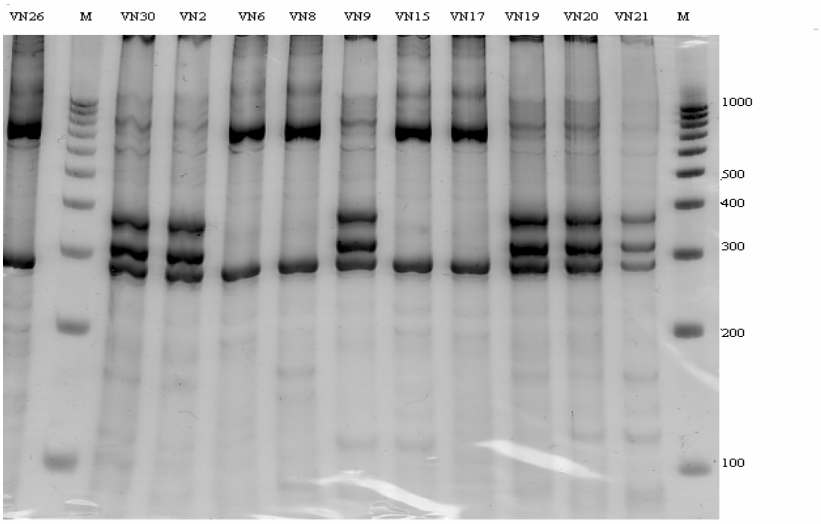

Figure VIII: Hinfl enzymatic restriction of the fragment amplified with TAEnF/ITMTnR primers. Two bands of approximately 600 bp and 300 bp correspond with the *T. saginata* profile (VN26, VN6, VN8, VN15 and VN17). Three bands of approximately 300 bp correspond with the *T. s. asiatica* (VN30, VN2, VN9, VN19, VN20 and VN21). M: 100 bp marker.

Results from morphological examination as well as those from PCR-RFLP assays showed that all the samples gave identical results with the two PCR-RFLP assays and all samples were identified including the 5 samples that could not be identified by morphological examination. Results are summarized in table VI (in annex)

CHAPTER V: DISCUSSION

Determining to which species a human tapeworm belongs is of great importance for diagnostic and epidemiological reasons because *T. saginata* and *T. solium* have a very different impact on public health. *T. saginata* and *T. solium* can be differentiated on the basis of morphological differences, however, the parasitic material, either scolex, or mature or gravid proglottids, naturally expelled or following treatment, may in some cases be too decomposed to allow assessment of the morphological characteristics (Mayta *et al*., 2000). Besides, morphological examination cannot be used to differentiate *T. saginata* from a third tapeworm sub-species, *T. s. asiatica* that is found in some Asian countries, including Vietnam.

PCR methods (Eom *et al*., 2002; Yamasaki *et al*., 2004) offer an interesting alternative for identification of *Taenia* samples because they can be applied on samples that are partly digested and they can also be used for differentiating *T. saginata* and *T. s. asiatica* (Eom *et al*., 2002). In addition, these methods are much more precise and reliable for species identification of tapeworms than morphological methods, easy to interpret the results and not dependent on the subjectivity of the observer.

In this study five of the 34 tapeworm samples could not be identified on morphological characteristics because they were either too decomposed or the uterine branches were not readily distinguishable. From the remaining 29 samples, 26 were identified as *T. saginata* – like and 3 as *T. solium*. On the same samples, PCR and RFLP were applied using different primer sets and restriction enzymes, respectively.

By using the ITMF/ITMTnR primers or TAEnF/ITMTnR primers in PCR, unique products (with 370 bp or 890 bp, respectively) were obtained from the 12S rDNA fragment of *Taenia* spp. This indicated that the primers bound to only one locus. In the subsequent RFLP clearly different bands were obtained by using DdeI restriction enzyme for differentiating *T. saginata* from *T. solium* and Hpy8I or HinfI restriction enzymes for differentiating *T. s. asiatica* from *T. saginata*.

We chose the 12S rDNA fragment in the mitochondrial genes as a target sequence for differentiation because mitochondrial genes (mtDNA) accumulate mutations much faster than other genes and are inherited only through the maternal line (Obwaller A. *et al*., 2004). According to Ito *et al*., (2002), the study of taeniid mtDNA has been useful in differentiation as well as in elucidating similarities and differences between individual species, e.g. a novel taeniid*, T. s. asiatica.* . On the other hand, according to Nickisch-Rosenegk *et al*. (1999), the 12S rDNA was specific for cestodes and primers derived from this sequence allowed the specific amplification of helminth DNA without amplification of host DNA.

Specificity of the primers was checked through alignment of the 12S rDNA sequences from *T. solium*, *T. saginata* and *T. s. asiatica* with the primers. Only one mismatch was found between ITMTnR primer and a template from *T. s. asiatica* but this position is not near the

primer's 3 end. These primers did not show any homology with human DNA sequences when using the BLASTN program.

One sample (VN03) gave a negative PCR product with ITMF/ITMTnR primers but was positive with the TAEnF/ITMTnR primer set. This could be explained by the presence of one or more mismatches in the template. This sample will be screened by cloning and sequencing, to determine where the mutation has occurred.

All other samples gave comparable results in both PCR-RFLP assays. This experiment showed that PCR-RFLP is a reliable tool for discriminating *T. s. asiatica* from *T. saginata* and *T. solium* even when proglottids are not intact or mature proglottids. However, this assay requires that proglottids are preserved in 70% ethanol, not in formalin. To our knowledge this is the first time that the 12S rDNA fragment was used to differentiate *T. s. asiatica* from *T. saginata* and *T. solium* by using universal primers in a PCR-RFLP assay. In addition, this assay is rapid, simple and accurate.

Some other loci have been studied for differentiation purposes. Eom *et al*. (2002) used the RAPD assay to amplify the ITS2 sequence for differentiation of *T. s. asiatica* from *T. saginata*. However, this method uses 3 different arbitrary primers, giving with each primer several bands identical to *T. saginata* or *T. s. asiatica*. This makes the reading of results difficult and cannot be used when samples are mixed with human DNA. Yamasaki *et al*. (2004) used multiplex PCR with a set of primers from the Cox 1 fragment. These authors succeeded in differentiating *T. s. asiatica* infection from *T. saginata* and *T. solium* when using proglottids samples, however, success of this assay depends very much on the ratio of the different primer pairs.

The molecular tests on 34 samples from Vietnam showed that *T. s. asiatica* and *T. saginata* were equally distributed in certain areas of northern Vietnam and that *T. solium* was less common. These results are at least surprising. Firstly, because northern Vietnam is considered to be endemic for *T. solium,* and *T. saginata* is only sporadically observed in bovine carcasses in the abattoirs (Willingham *et al*., 2003). Therefore, more *T. solium* positive would be expected. It must be mentioned that the sampling of the parasitic material was not random, but hospital-based. As a result there were more samples from urban areas (ten samples were from Hanoi, Haiphong, Hoabinh and Hatay) or from villages that have easy access to medical facilities, and less samples from remote villages, where *T. solium* is expected to be more endemic. Secondly, while one report of an adult *T. s. asiatica* case has been published in Vietnam (Cited by Willingham *et al*., 2003), *C. viscerotropica* has until now never been detected at slaughter, neither in pigs nor in cattle. Therefore, the finding of a high proportion of Asian *Taenia* in the samples is puzzling. *T. s. asiatica* is thought to be transmitted only through the consumption of liver and visceral organs, and not through the muscle of pigs (Eom and Rim, 2001). The habit of eating raw meat, either pork or beef is very common in northern Vietnam (Cited by Willingham *et al*., 2003); in contrast, there is no tradition of eating raw liver or viscera from pigs.

Whether *T. s. asiatica* causes human cysticercosis is being debated among scientists. Some authors consider it unlikely because in countries with a high prevalence of *T. s. asiatica* (i.e. Taiwan and Indonesia), the number of patients with human cysticercosis or neuro-cysticercosis is low (Galan-Puchades & Fuentes, 2000; Ito *et al*., 2003). In northern Vietnam, however, cysticercosis is relatively common: Erhart *et al.* (2002) detected circulating cysticercosis antigen in more than 5% of the population of a village in the Bac Ninh province.

A hospital-based study indicated that the most common clinical signs of human cysticercosis in North Vietnam are the presence of subcutaneous nodules and neurological symptoms (Nguyen *et al*., 2003). In addition, taeniasis is also common among cysticercosis patients (Willingham *et al*., 2003). Subcutaneous cysticercosis is uncommon in other parts of Asia (Rajshekhar *et al*., 2003), and is only sporadically reported in Africa and South America. Molecular analysis of subcutaneous cysts should be performed to confirm whether these are caused solely by *T. solium* or if *T. s. asiatica* is also involved. However, in this survey, there was no indication that *T. s. asiatica* can cause cysticercosis since none of the patients that were infected with a *T. s. asiatica* tapeworm were actually showing clinical signs (neurological or subcutaneous cyst).

CHAPTER VI: CONCLUSION AND RECOMMENDATIONS

The present study demonstrated that a PCR-RFLP assay using primers derived from the 12S rDNA fragment is a reliable tool for species identification of *Taenia* in Vietnam. It permits differentiation of *T. solium, T. saginata* and also of *T. s. asiatica*, making this technique superior to morphological examination that cannot differentiate the two latter (sub-) species. However, PCR-RFLP requires a good infrastructure and more expensive equipment than the conventional morphological technique. Moreover, this technique used two different restriction enzymes. To reduce the cost when applying this assay in larger scale surveys, another gene on mtDNA could be identified that allows differentiation with only one restriction enzyme. This PCR-RFLP assay worked well for proglottids samples. It also needs to be tested on cysticerci samples and on faecal extracts for the detection of copro-DNA.

Determining which tapeworm species is occurring provides more understanding in the prevalence and the transmission patterns of cestode infections. This limited study on 34 tapeworm samples collected in hospitals showed that all three *Taenia* spp. of man occurred in Vietnamese people and *T. s. asiatic*a is equally distributed as *T. saginata* in certain areas of northern Vietnam. More samples should be collected in different areas of the country to get a clearer picture of the prevalence of the 3 (sub-) species of *Taenia.* We also recommend that livers and organs from pigs should be thoroughly inspected for the presence of *C. viscerotropica* and that biopsies from subcutaneous human cysticercosis should be analyzed with PCR-RFLP for species identification. This will help to clarify the complex epidemiology of *Taenia* species in Vietnam.

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Annex:

Table V: Results of morphological examination of the proglottids, based on number of main uterine branches and / or ovary lobes.

Samples	Number of uterine branches /side	Ovary lobes	Result
VN1	19/21		*T. saginata*
VN2	16/16	2	*T. saginata*
VN3	15/18		*T. saginata*
VN4	21/19		*T. saginata*
VN5		2	*T. saginata*
VN6	23/22		*T. saginata*
VN8	24/24		*T. saginata*
VN9	ND		
VN11	23/26		*T. saginata*
VN12	ND		
VN13	ND		
VN14	29/31		*T. saginata*
VN15	21/19		*T. saginata*
VN16	20/17		*T. saginata*
VN17	20/23		*T. saginata*
VN18	20/16	2	*T. saginata*
VN19	22/21		*T. saginata*
VN20	24/26		*T. saginata*
VN21	20/22		*T. saginata*
VN23	28/30		*T. saginata*
VN24	28/28		*T. saginata*
VN25	26/29		*T. saginata*
VN26	19/20		*T. saginata*
VN27	23/22		*T. saginata*
VN28	20/20		*T. saginata*
VN29	31/30		*T. saginata*
VN30	20/19		*T. saginata*
VN31	ND		
VN32	17/17	2	*T. saginata*
VN33	27/27		*T. saginata*
VN01	8/9		*T. solium*
VN02	9/9		*T. solium*
VN03	10/12		*T. solium*
VN04	ND		

ND: not done

Table VI: Summary of the results from the morphological examination and the two PCR-RFLP assays

Samples	Morphological examination	PCR with ITMF/ITMTnR primers		PCR with TAEnF/ITMTnR primers	
		DdeI	Hpy8I	DdeI	HinfI
VN1	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN2	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN3	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN4	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN5	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN6	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN8	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN9	nd	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN11	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN12	nd	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN13	nd	*T. saginata*	*T. saginata*		*T. saginata*
VN14	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN15	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN16	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN17	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN18	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN19	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN20	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN21	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN23	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN24	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN25	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN26	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN27	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN28	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN29	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN30	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN31	*nd*	*T. saginata*	*T. saginata*		*T. saginata*
VN32	*T. saginata*	*T. saginata*	*T. s. asiatica*		*T. s. asiatica*
VN33	*T. saginata*	*T. saginata*	*T. saginata*		*T. saginata*
VN01	*T. solium*	*T. solium*		*T. solium*	
VN02	*T. solium*	*T. solium*		*T. solium*	
VN03	*T. solium*	nd		*T. solium*	
VN04	*nd*	*T. saginata*	*T. saginata*		*T. saginata*

nd: not done