THE EPIDEMIOLOGY OF *TAENIA* SPP. AND CYSTICERCOSIS IN ECUADOR

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This thesis is dedicated to Jef Brandt and Washington Benitez who believed in me and taught enriching experiences to me

"No animal has been responsible for more hypotheses, discussions and errors than the tapeworm"

C. Davine, 1860

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LIST OF ABBREVIATIONS

Ab	Antibody
AFC	Age of first contact
Ag	Antigen
CNS	Central Nervous System
CI	Confidence Interval
Cox1	cytochrome *c* oxidase subunits 1
CT-scan	Computerized Axial Tomography
Cyt*b*	cytochrome *b*
DNA	Deoxyribonucleic acid
EITB	Enzyme-linked immunoelectrotransfer blot
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization
gDNA	Genomic DNA
GP	Glycoproteins
GPI	Glucose phosphate isomerase
MRI	Magnetic Resonance Imaging
mtDNA	Mitochondrial DNA
NC	Neurocysticercosis
OIE	Organización International de Epizootias
OMS	Organización Mundial de la Salud
OPS	Organización Panamericana de la Salud
PCR	Polymerase Chain Reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
WHO	World Health Organization

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GENERAL INTRODUCTION

Taeniasis in man is caused by *Taenia solium*, *Taenia saginata* and *T. saginata asiatica.* The final host of these cestodes is man whilst cattle (*T. saginata)* and pigs (*T. solium* and *T. s. asiatica)* act as intermediate hosts. Contrary to the metacestodes of *T. saginata* and *T. s. asiatica*, metacestodes of *T. solium* can also affect man, where its neurotropism makes it one of the most pathogenic parasitoses, referred to as neurocysticercosis (NC). Therefore, while *T. saginata* and *T. s. asiatica* have mainly an economical importance, *T. solium* is an important public health issue.

Apart from a different degree of specificity for the intermediate hosts, the life cycle of these taeniids is very similar (Fig. 1). Man, the obligatory final host, acquires the infection with the adult parasite by eating raw or undercooked pork or beef contaminated with viable cysticerci.

Fig. 1. Life cycle of *Taenia solium* and *Taenia saginata*
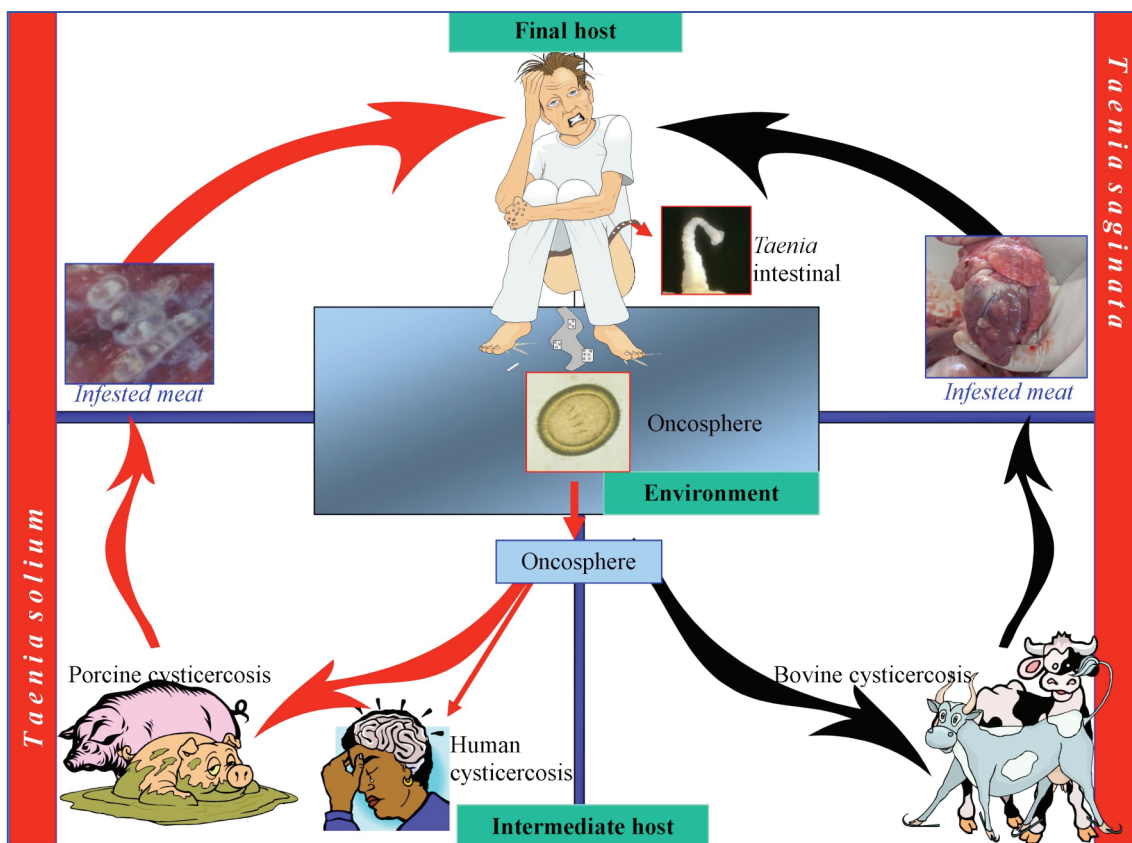
Following ingestion, the cysticercus wall is digested in the stomach; the scolex evaginates and upon arrival in the duodenum attaches itself onto the mucosa. From there the strobila, i.e. a chain of segments or proglottids, grows and two to three months after the infection, gravid segments are formed and detach from the strobila. They are

expelled with the faeces or migrate actively through the anus, as often observed with *T. saginata*. Gravid proglottids contain thousands of eggs, which contain a fully developed larva, capable to infect the intermediate host. Pigs and cattle are the natural intermediate hosts and acquire cysticercosis through ingestion of eggs with faeces or contaminated water or food. The eggs hatch in the stomach and are activated in the duodenum; aided by their vigorously moving hooks and enzymatic secretions, they migrate through the intestinal wall and are distributed all over the body through the circulatory system. In about three months, oncospheres develop to cysticerci that are infective for man.

Ecuador, in the North West of the South American continent, is considered endemic for taeniasis-cysticercosis. *T. solium* in man has been reported since the beginning of the twentieth century. Most of the cases are located in the Andean region (Sierra) with fewer cases in the Costal and Amazonas regions. All factors favouring transmission of *T. solium* are commonly present. Like in most developing countries sanitary conditions are poor and traditional pig husbandry is common. Prevalence in pigs is not well documented, and equally little is known about the impact of cysticercosis on public health and on the economy.

It is commonly accepted that all intestinal *Taenia* are *T. solium* whereas *T. saginata* is considered to be absent or not important. Since 1985, no data on *T. saginata* were listed by Food and Agriculture Organization (FAO), World Health Organization (WHO) and World Organization for Animal Health (OIE).

Fig. 2. Incidence of taeniasis and cysticercosis in man in Ecuador (2004), per 100,000 inhabitants
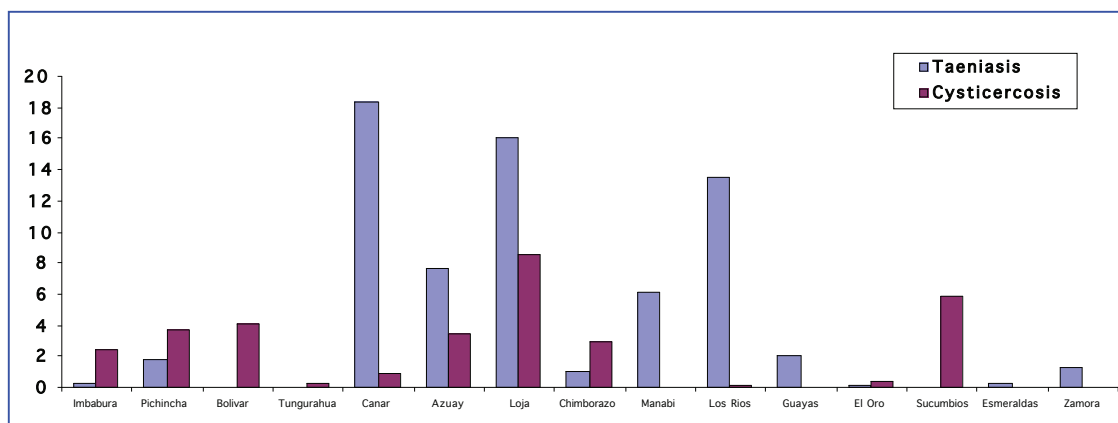
Source: EPI-2, Epidemiological department, Public Health Ministry of Ecuador, (Aguilar, 2005)

Official Ecuadorian health organizations present statistics on a wide variety of diseases, including cysticercosis (Fig. 2). These figures are based on diagnoses and treatments in public hospitals. Most cases of cysticercosis attended in hospital are patients with seizures or epilepsy. The official figures most probably seriously underestimate the real disease burden caused by *T. solium* because people from endemic rural areas have limited access to proper diagnosis and treatment due to financial and logistic constraints.

One of the main problems to control *T. solium*-cysticercosis is the lack of knowledge about the disease among people from endemic areas. Most of them do not know the relation between intestinal *Taenia* known as "solitaria" and cysticercosis neither in pigs nor in man. Consequently, most people see no harm in eating cysticercotic meat and some people even prefer this meat for its special taste.

CHAPTER 1

THE TAENIASIS-CYSTICERCOSIS COMPLEX IN LATIN AMERICA: MORPHOLOGY, CLINICAL IMPORTANCE, DIAGNOSIS, EPIDEMIOLOGY AND CONTROL

1.1   INTRODUCTION

Taenia solium (Linnaeus, 1758), *Taenia saginata* (Goeze, 1782) and *Taenia saginata asiatica* (Eom and Rim, 1993) are medically important tapeworms. Larval stages or metacestodes are *Cysticercus cellulosae* in suids, i.e. domestic pigs and wild boar (*Sus scrofa*), *Cysticercus bovis* in cattle and *Cysticercus viscerotropica*, for *T. solium, T. saginata* and *T. s. asiatica,* respectively (Pawlowski and Schultz, 1972). Contrary to *T. saginata*, *T. solium* has a greater importance in public health because the lesser specificity for its intermediate host can give rise to human cysticercosis, often leading to neurocysticercosis (NC), a major cause of epilepsy associated with considerable morbidity and mortality. Apart from the public health risk, *T. solium*-cysticercosis contributes to high levels of human morbidity and some mortality added to livestock production losses in endemic areas (Carabin et al., 2005). In this context, a preliminary estimate carried out by Carabin et al. (2006) reported that *T. solium*-cysticercosis implies considerable monetary cost to a region that is already economically constrained.

The taxonomy of the *Taenia* spp., according to the "GenBank" database of the NBCI, is shown in Figure 1.1:

Fig. 1.1. Taxonomy of *Taenia* spp.
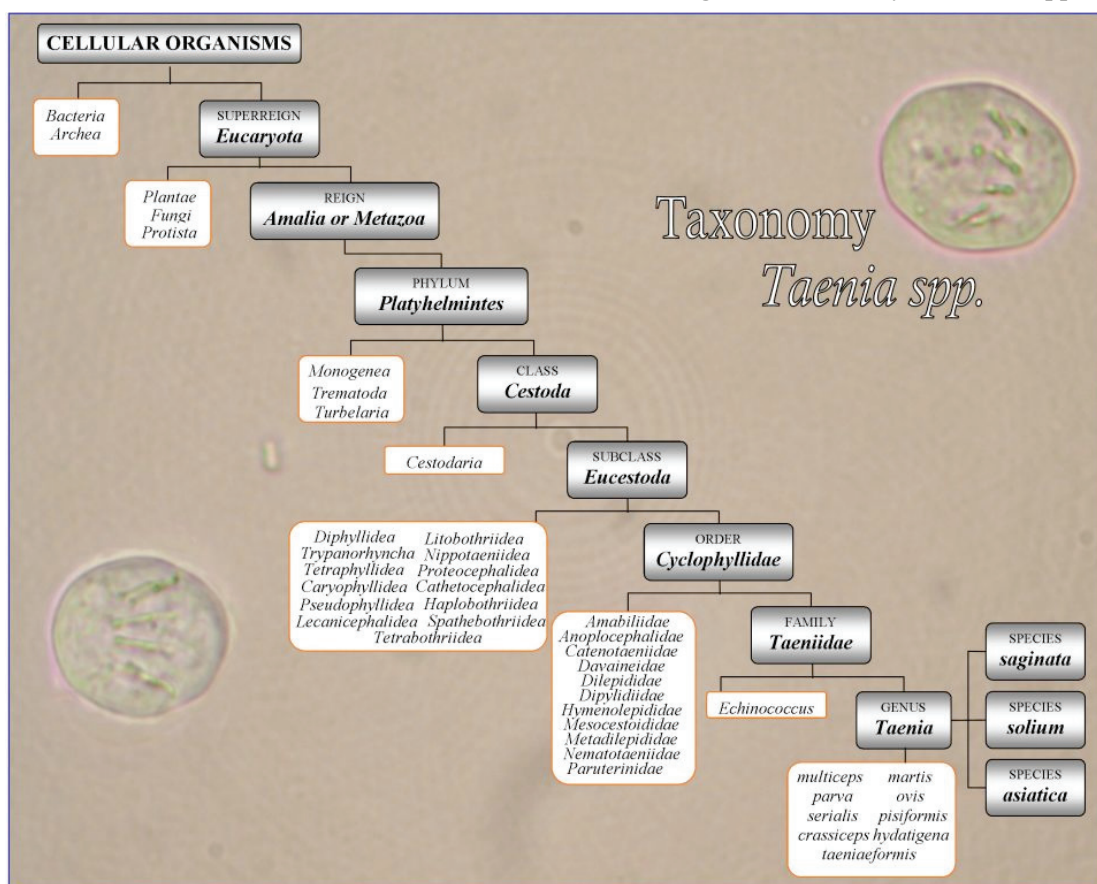
The present review focuses on some general aspects of the taeniasis-cysticercosis complex. It provides updated information on the epidemiology and diagnosis of this zoonotic infection, with special attention to the situation in South America and Ecuador.

1.2   ORIGIN AND HISTORY

Variations in the mitochondrial DNA (mtDNA) or genomic DNA (gDNA) of different species allow to deduce phylogenetic relationship and to study evolutionary sequences among different members of cestodes (De Queiroz and Alkire, 1998; Nickisch-Rosenegk et al., 1999; Hoberg et al., 2001; Ito et al., 2002a). Recently, phylogenetic polymorphism was determined among 13 isolates of *T. solium* from various geographical origins using PCR-amplified sequences of two mitochondrial genes, i.e. cytochrome *c* oxidase subunits 1 (Cox1) and cytochrome *b* (Cyt*b*). The isolates from Asia (China, Thailand, Irian Jaya and India) form a single cluster, whereas the isolates from Latin America (Mexico, Peru, Ecuador, Bolivia and Brazil) and those from Africa (Tanzania, Mozambique and Cameroon) form an additional cluster (Nakao et al., 2002). It was estimated that these two genotypes diverged approximately 4-8 x 10^5 years ago when African hominids in the Pliocene/Pleistocene, scavenging for food or preying on antelope and other herbivores, were exposed to *Taenia hyaenae*, a species that is closely related to *T. solium*. *T. hyaenae* has hyaenids, canids and felids as definitive host and bovids as intermediary host (Hoberg et al., 2001). Hence, it can be assumed that *Taenia* spp. became associated with man, about 10,000 years ago coincidentally with the domestication of intermediary hosts such as cattle and pigs and of carnivorous companion animals (Hoberg et al., 2001). Furthermore, coincidentally with migration of early humans from Africa to Eurasia together with the prototype of *T. solium*, the tapeworm was maintained and diverged in early human colonies through a "human-human" cycle (through cannibalism) and a "human-wild suids" cycle (through hunting) hence, the "human-pig" cycle was established (Hoberg et al., 2001; Nakao et al., 2002). Apparently, with the onset of the colonial era, 500 years ago, the European isolates of *T. solium* were introduced to Latin America and African countries together with pigs and humans (Nakao et al., 2002; Ito et al., 2003a).

Cox (2002) and Wadia and Singh (2002), described the history of human taeniids; apparently, encysted larvae in pigs -cysticerci of *T. solium*- were well known to the ancient Greeks, referred to by Aristotle (384 to 322 BC). In addition, Hippocrates (460

to 377 BC) suggested that the Greek physicians knew that human harboured such cysts in the brain or suffered from any condition associated with them. Edward Tyson (1650 to 1708 AC) was the first person to recognize the "head" (scolex) in the 17th century. These worms continued to be confused long after the work of Tyson, and although Goeze in 1782 had suspected that there were two separate species, the distinction between *T. solium* and *T. saginata* was not obvious until the middle of the 19th century when Küchenmeinster recognized the differences between both tapeworms based on the morphology of the scolex (cited in Cox, 2002). The demonstration of the life cycle of *T. solium,* in 1784, shed new light on the nature of human cysticercosis, and it became apparent that humans could probably become infected with the larval stage of *T. solium* when they ingested the tapeworm eggs.

T. s. asiatica, a species closely resembling *T. saginata*, was first described as a new species in 1993 (Eom and Rim, 1993). However, most authors consider *T. asiatica* to be a subspecies of *T. saginata* rather than a new species, and name it *T. saginata asiatica* (Galan-Puchades and Mas-Coma, 1996; Loos-Frank, 2000).

Before the relationship between *Taenia* and their cysticerci was understood, the larval stages were described with their own scientific name, as if they were separate species/genera. This unfortunate situation still exists today (Acha and Szyfres, 2003). Previously, *T. solium* has also been described as *Taenia cucurbitina* (Pallas, 1766, cit. by Muller, 1975) and *T. saginata* is also known as *Taeniarhynchus saginatum* (Weinland, 1948), *Taenia africana* (Linstow, 1900), *Taenia confusa* (Ward, 1896), *Taenia hominis* (Linstow, 1902 cited by Pawlowski and Schultz, 1972); while in Ecuador and other Latin American countries adult *Taenia* are commonly known as "solitaria", "tallarines" and "Tenia solitaria" (Benitez-Ortiz, 1995; Cruz-Licea et al., 2003). On the other hand, the metacestode of *Taenia* spp. bears names like "granizo", "granillo", "zahuate", "pepa", "tomatillo", "ladilla", "quinua", "coscoja", "grano" and "triquina" in different endemic areas from Mexico, Colombia, Peru, Chile and Ecuador (Camacho et al., 1991; Benitez-Ortiz, 1995; Garcia and Del Brutto, 2000; Garcia and Gonzalez, 2000; Cruz-Licea et al., 2003). It is rather peculiar that "triquina" is a common name for cysticercosis in Latin America (Garcia and Gonzalez, 2000) where *Trichinella spiralis*, another zoonotic helminth associated with pigs, is hardly known.

1.3   MORPHOLOGY OF *TAENIA* SPP.

T. solium and *T. saginata* are flat, segmented and hermaphrodite parasites, measuring between 2 to 10 meter; the adult stages are located in the small intestine. They are composed of a head or scolex (Fig. 1.2) with a diameter of approximately one millimetre bearing four muscular suckers for fixation and allowing some form of locomotion (summarised after Bowman, 1999). Contrary to *T. saginata*, *T. solium* has an armed rostellum, bearing between 22 and 36 hooks ordered in two rows (Fig. 1.3). A thin neck measuring approximately 5 to 10 mm, constitutes the portion with most of the biokinetic activity, (Naquira, 1999), from this part the entire body or strobila is formed.

Fig. 1.2. Scolex of *Taenia solium*

Fig. 1.3. Scolex of *Taenia saginata*

(without staining) 40X
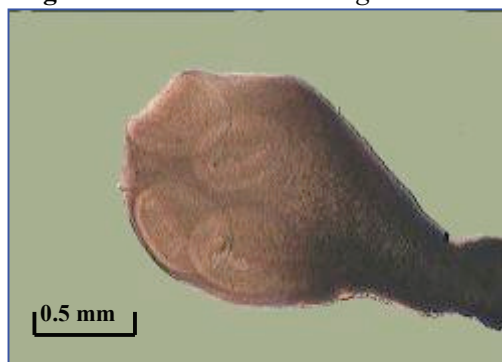
(without staining) 40X

Fig. 1.4. Three-lobed ovarium of *T. solium*
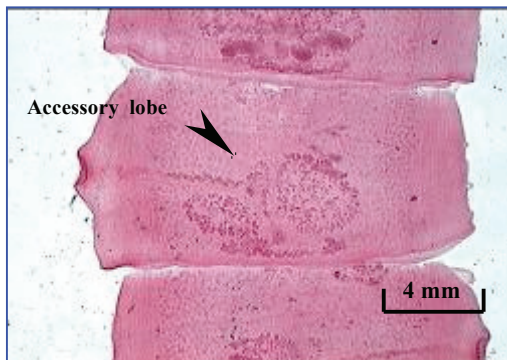
Fig. 1.5. Bilobed ovarium of *T. saginata*
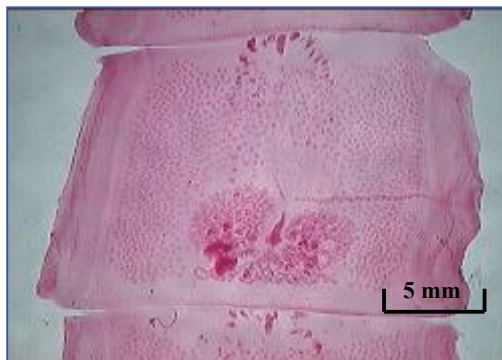
Fig. 1.6. Gravid proglottid of *T. solium*

Fig. 1.7. Gravid proglottid of *T. saginata*
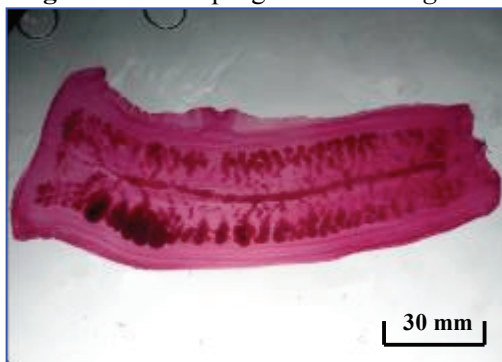
The strobila constitutes of between 800 and 4000 proglottids or segments (1000 on average), which can be grouped as immature, mature and gravid segments. Immature segments are wider transversely than longitudinally; mature segments are square with primary sexual organs completely formed (Fig. 1.4, 1.5); gravid segments have a rectangular shape with longitudinally the longest axis with most of the primary genital organs atrophied but almost entirely filled with a branched uterus packed with oncospheres (Fig. 1.6, 1.7).

Taeniidae possess neither a coelomic cavity nor a digestive system; exchange of nutritients and metabolites occurs mostly through the tegument. An excretory system is formed by longitudinal lateral channels running through each segment with transverse connections. Each segment has an osmo-regulatory system and a nervous system and muscular fibres, allowing rhythmic and co-ordinated movements (Naquira, 1999; Bowman, 1999).

1.3.1 Oncospheres

The uterus contains spherical eggs or oncospheres (Fig. 1.8), measuring between 29 and 77μm for *T. solium* and between 39 and 50μm for *T. saginata*, (Verster, 1967; Muller, 1975). On this basis, differentiation is not possible by standard light microscopy. According to Slais, (1970, in Pawlowski and Schultz, 1972), the eggs of *Taenia* spp. are composed of a chorionic membrane, a thick and grooved embryophore, which is formed by embryophoric blocks (constituted of a keratin-like protein), an embryophoral membrane, two oncospheral membranes surrounding the oncosphere (also named hexacanth embryo) bearing three pairs of hooks (Fig. 1.9). The eggs of *Taenia* spp. are fairly resistant to external

Fig. 1.8. Oncosphere

Fig. 1.9. Hexacanth embryo
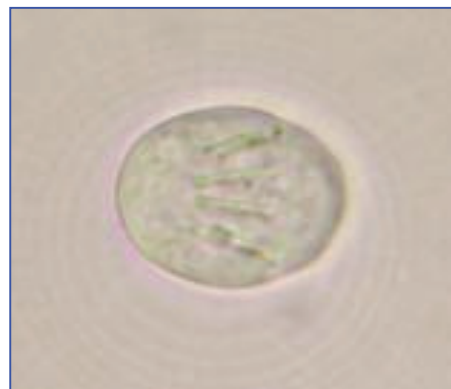
environmental conditions and to common disinfectants. In humid and shady grounds and traditional systems for treating wastewater, eggs can remain viable for weeks or months (OMS/OPS, 1993; Bowman, 1999).

1.3.2 Cysticercus

A cysticercus is an ovoid vesicle of approximately 5-15mm diameter, with a transparent membrane. It contains an invaginated scolex and is filled with in a colourless liquid (Lapage, 1974) (Fig. 1.10). Although it does not constitute an independent entity, the larval stage is known as *Cysticercus* spp. (Rudolphi, 1803); it should be preferably named "metacestode of

Fig. 1.10. *Cysticercus* spp.
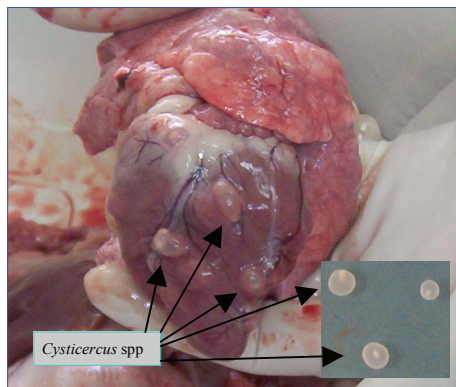
Taenia spp." but traditionally, the old nomenclature remains as it was cited above. A wild growing *T. solium* cysticercus in man is often referred to as "cysticercus racemosus", which is a degenerative form of the metacestode, with the appearance of a cluster of lobes, located in the meninges and the ventricular system of the brain (OMS/OPS, 1993). The old nomenclature has definitely some advantages, specifically with reference to the pathological effects i.e. the cysticercus stage refers to the fully developed infective larvae, whereas a metacestode can be any larval stage. Furthermore, taeniasis refers to the presence of the adult (intestinal) stage, cysticercosis to the consequences of the larval stage housed in the tissues. Taeniasis is usually subclinical or accompanied by mild symptoms, whereas (human) cysticercosis is considered one of the most pathogenic parasitic infections.

The taxonomy revision of the genus *Taenia*, published by Verster (1969), proposed three morphological characteristics to distinguish *T. solium* from *T. saginata*, namely the presence of an armed rostellum, three-lobed ovary and the absence of a vaginal sphincter. Additionally, the number of uterine branches in gravid proglottids is an indicative but not absolute difference between *T. solium* and *T. saginata*. These differences can be observed easily after fixation and staining of proglottids. The differences among *T. solium, T. saginata* and *T. s. asiatica* are summarized in table 1.1.

Table 1.1: Morphological differences between *Taenia solium* and *Taenia saginata* OMS (1983).

Characteristic	Taenia solium	Taenia saginata	Taenia saginata asiatica
STROBILA			
Length (m)	1.5 − 10	4 − 12	1-8
Width (mm)	7 − 10	12 − 14	9-12
Proglottids (quantity)	700 − 1,000	1,000 − 2,000	200-1,200
SCOLEX			
Diameter (mm)	0.6 − 1	1 − 2	0.2-2.0
Suckers	4	4	4
Rostellum	Present	absent	absent or sunken
Hooks (number)	22 − 32	absent	sunken
MATURE PROGLOTTIDS			
Testicles (quantity)	375 − 575	800 − 1200	300-1,200
Ovaria (number of lobes)	3	2	2
Vaginal sphincter	Absent	present	present
GRAVID PROGLOTTIDS			
Uterus (number of unilateral branches)	7 − 20	14 − 35	12-26
Mode of leaving the host	single or grouped (passively)	single or grouped (actively and spontaneously)	single or grouped (90% actively)
Final host	man	man	man
Intermediate host	pig, wild boar, man, dog	bovines	pig
Localisation of adult *Taenia*	duodenum	ileum	small intestine
Common larval name	*Cysticercus cellulosae*	*Cysticercus bovis*	*Cysticercus viscerotropica*
Localisation of the infective metacestode	cardiac and skeletal muscles, brains, eyes, subcutaneous tissues	skeletal and cardiac muscles	visceral organs (liver, omentun serosa, lung)

Source: (OMS, 1983), complemented with observations by Verster (1969); Muller (1975); Acha and Szyfres (2003); Hendrix (1998); Hoberg et al. (2001); Loos-Frank (2000); Eom and Rim (1993) and Flisser et al. (2005)

1.4   CLINICAL IMPORTANCE

1.4.1   Human

1.4.1.1 Taeniasis

The most noticeable sign of taeniasis is the presence of proglottids either in the faeces or actively migrating out of the host. *T. solium* releases with the faeces a few gravid proglottids daily or two or three times a week, while *T. saginata* proglottids are mobile and mostly migrate through the anus spontaneously, single or in chains of up to 7 segments, or they are expelled daily in the faeces (Flisser et al., 2005). Taeniasis is usually asymptomatic, although carriers reported pruritis in the peri-anal region (*T. saginata*) and some unspecific symptoms like gastrointestinal dysfunction, loss of appetite, weight loss and general uneasiness (OMS/OPS, 1993). According to Muller (1975) these symptoms might be due to excretions produced by the parasite.

1.4.1.2 Cysticercosis

Human cysticercosis is characterized mainly by neurological symptoms. Yet, in spite of central nervous system (CNS) localisations, 50 to 70% of the cases are asymptomatic (Palacios et al., 1988). According to Cruz et al. (1999a), Carpio and Hauser (2002) and Del Brutto et al. (2005) epilepsy is the most common, and frequently, the only clinical manifestation of NC among symptomatic patients. Other symptoms like migraine, nausea, nervous hypertension, mental deterioration, endocrinal hypertension and paralysis have also been described (OMS/OPS, 1993). It is commonly accepted, however, that neurological symptoms are mainly immunopathological reactions related to the degeneration of brain cysts (Correa and Medina, 1999; Garcia et al., 2002; Acha and Szyfres, 2003). Additionally, mature cysts may be found in the muscles and subcutaneous tissues where they do not cause any apparent symptoms unless large numbers of cysts are involved, causing muscular pain, cramps and fatigue. In the eyes, retinal and vitreous involvement is most frequently observed and followed by subretinal, conjunctival and anterior segment localization, which may cause uveitis, iritis and retinitis as well as palpebral conjunctivitis, and may affect the eyeball musculature (Acha and Szyfres, 2003; Nash et al., 2005).

1.4.2 Animals

When metacestodes are localised in muscles or surrounding tissues, cysticercosis-related symptoms in animals are usually not manifested. Nevertheless, in experimental infections in pigs, common symptoms were anorexia, fever, bradycardia with increased respiratory rate, nausea, diarrhoea, staggering gait, and in massive infestations, abortion and death have been described (Pawlowsky and Schultz, 1972; OMS/OPS, 1993). Neurological symptoms in pigs are not well documented. Probably they rarely occur, although Acha and Szyfres (2003) described hypersensitivity of the snout, paralysis of the tongue and epileptiform convulsions in infected swine.

1.5   EPIDEMIOLOGY

Human cysticercosis is a disease associated with poverty in areas where people eat raw or undercooked pork and traditional pig husbandry is practised and it is also facilitated by poor hygiene, inadequate sanitation and the use of untreated or partially treated wastewater in agriculture. Although the real prevalence of (neuro-) cysticercosis remains largely unknown, the OMS/OPS (1997); Craig et al. (1996) and Crompton (1999) estimate that the annual incidence of intestinal *Taenia* in man is between 10,000,000 to 50,000,000; more than 50 million pigs and 5 to 6 million humans have cysticercosis, with approximately 400,000 patients having symptomatic disease (Bern et al., 1999) and 50,000 dying each year because of NC (OMS/OPS, 1997). Although, *T. solium*-cysticercosis has been proposed to be declared an international reportable disease (Roman et al., 2000), taeniasis and cysticercosis do not lead to sudden large-scale international outbreaks of disease and therefore would not seem to constitute an appropriate subject for international notification (WHO, 2003; Acha and Szyfres, 2003).

During the last decade data collected on cysticercosis have become considerably more comprehensive. Before that, the only data reported were hospital-based figures on the frequency of NC among hospital patients or among autopsied cadavers (WHO, 1997), and data based on isolated studies of specific sectors of the population, such as schoolchildren and recruits. Furthermore, since many prevalence studies are based on the finding of eggs in faeces, and the eggs of *T. solium, T. saginata* and *T. s. asiatica* cannot be distinguished by conventional methods, the best available data on prevalences in regions where, both species are sympatric, are not up to species level (Acha and Szyfres, 2003). In addition, in the eighties, a region was considered as "hyper-endemic"

when prevalences of taeniasis, porcine and human cysticercosis reached 1%, 5% and 0.001%, respectively measured by conventional methods i.e. coprology, tongue inspection and autopsies (WHO, 1983). However, recent studies demonstrated that in areas with prevalences of taeniasis of around 1%, high to very high prevalences of porcine and human cysticercosis can be measured by antibody detection methods (Gonzalez et al., 1990; Sanchez et al., 1999; Garcia et al., 2003a).

According to OMS/OPS (1993), porcine and bovine cysticercosis are not well documented in Latin America mainly because pigs in traditional husbandry systems are home-slaughtered and meat inspection in slaughterhouses is too superficial. In pigs, meat inspection usually allows easy detection (Benitez-Ortiz, 1995), whereas in bovines, visual meat inspection is hampered by a very low sensitivity (Blazek et al., 1981).

1.5.1 Prevalence of *Taenia solium*-cysticercosis in America and Ecuador

1.5.1.1 General aspects

T. solium infection is widely present in rural areas of developing countries in Central and South America, Asia and Africa, where it is divided in three well-defined areas i.e. high prevalence (endemic) areas, moderate prevalence and low prevalence regions (Fig. 1.11; WHO, 2002). In Central and South America human cysticercosis infection was reported by Schenone et al. (1982) in 18 countries. More recently, a complete classification was made by OMS/OPS (1993) and updated by Roman et al. (2000) (table 1.2).

In America, Welte (1997), Schantz (2002) and OMS/OPS (2005) reported the presence of *T. solium*-cysticercosis (most commonly in Bolivia, Guatemala, El Salvador, Honduras, Ecuador, Colombia, Mexico, Peru, and Brazil). Although, Canada, the Unites States, Argentina, Uruguay, Guyana, Suriname, French Guiana and Paraguay appear to be free of the pig-human cycle, the latter countries are observing an increase in imported and introduced infections related to immigration of persons from endemic countries (Ortega-Herrera et al., 2004), and also because of increasing tourism to endemic areas.

Several Cysticercosis Working Groups from different endemic and non-endemic countries in America have reported prevalences of taeniasis, porcine and human cysticercosis. These data are summarised in tables 1.3; 1.4 and 1.5.

The most recent epidemiological studies reported prevalences of taeniasis between 0% and 12% in America. Several diagnostic methods were used i.e. coprology, coproantigen detection, questionnaires/self-detection and post treatment recovery. Coprology was the most frequently used method to detect taeniasis, followed by coproantigen detection (table 1.3). Apart from a large study in Mexico City by Garcia-Garcia et al. (1999), in which more than 700,000 people were involved, most surveys were conducted in smaller endemic communities. In most reports no differentiation between *T. solium* and *T. saginata* is mentioned. Consequently, few studies report on which species of *Taenia* were involved.

Prevalence data on human cysticercosis in the American continent, reported by the scientific literature are summarized in table 1.4.

Human cysticercosis has been studied through autopsies, immunological and imaging methods. Results of autopsies and imaging techniques are mostly hospital-based; these provide little information on the prevalence. More recently, immunological techniques i.e. ELISA and EITB have been applied in large-scale surveys, using crude or purified antigens and monoclonal antibodies to detect antibodies and circulating antigens, respectively. The use of specific glycoproteins in EITB increased the sensitivity and specificity of antibody detection in human cysticercosis (Tsang et al., 1989). In epidemiological surveys, imaging techniques are seldom used because of their cost and impracticability. Hence, immunological methods are preferred to detect human cysticercosis from endemic communities or hospitals.

Prevalence data on porcine cysticercosis in the American continent, based on literature reports are presented in table 1.5.

Fig. 1.11. Distribution of human cysticercosis

High prevalence (endemic areas)
Moderate prevalence
Low prevalence (imported cases)
Endemic areas within USA
No information available
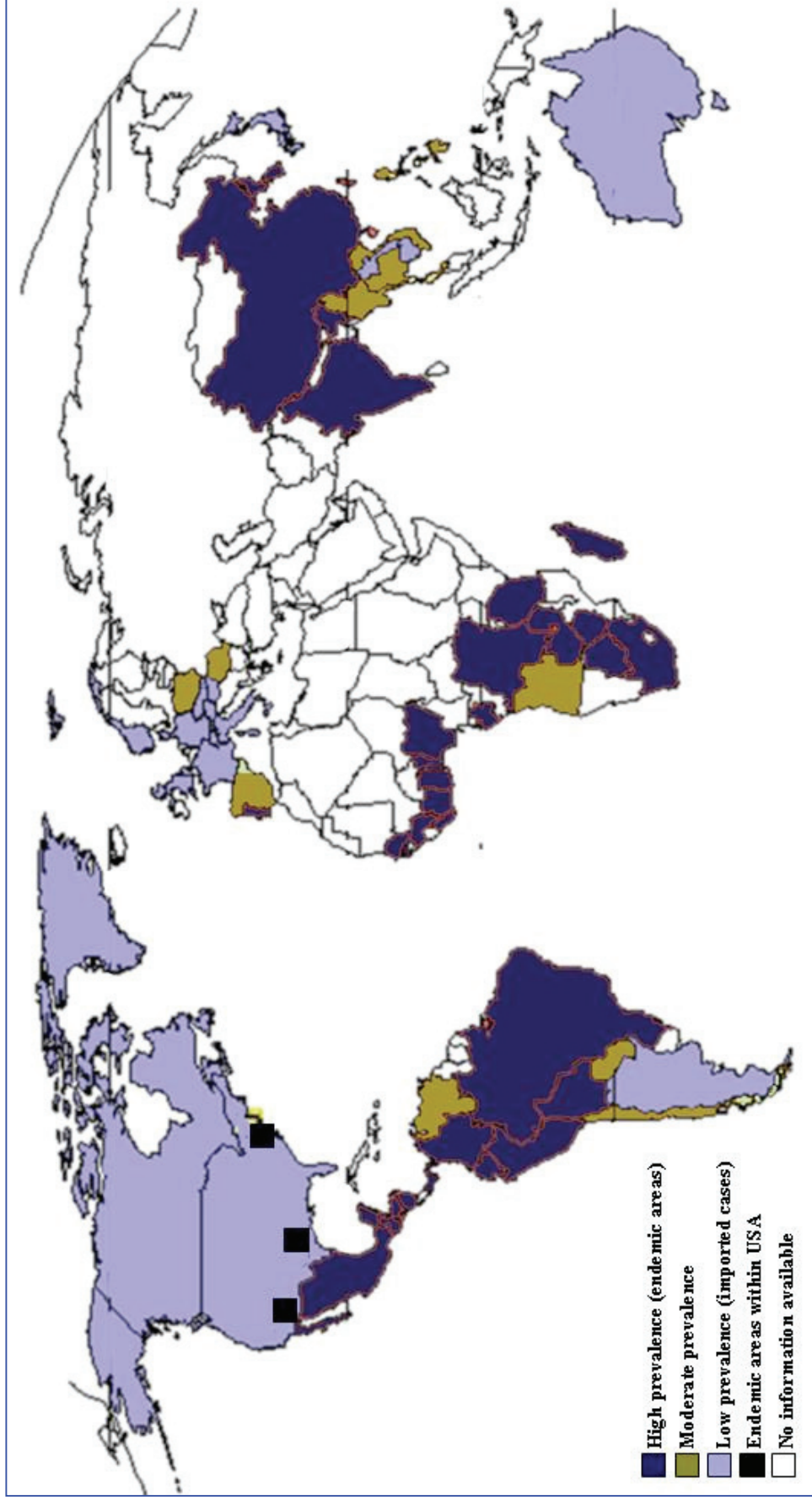
Source: WHO (2002)

Table 1.2. Distribution of taeniasis and human and porcine cysticercosis in America

	Taeniasis and human cysticercosis	Porcine Cysticercosis
Prevalent	Bolivia, Brazil, Colombia, Ecuador, Guatemala, Honduras, Mexico and Peru	Bolivia, Brazil, Colombia, Ecuador, Guatemala, Honduras, Mexico, Nicaragua, Peru Venezuela
The disease exists but is not frequently diagnosed	Argentina, Chile, Costa Rica, Haiti, Panama, Dominican Republic Venezuela, Belice, El Salvador, Nicaragua,	Argentina, Chile, Costa Rica, El Salvador, Haiti, Panama
Imported cases	Canada, Unites States, Cuba, Guyana, French Guyana, Jamaica, Surinam, Uruguay*, Paraguay* and Trinidad and Tobago.	Belice, Dominic Republic, Antigua and Barbuda, Bahamas, Barbados, Bermudas, Canada, Cuba, Dominica, Grenada, Guyana, Jamaica, Paraguay, Suriname, Trinidad and Tobago, Uruguay, United States
No data available or not exist	Uruguay**, Paraguay**, Puerto Rico	

OPS/OMS, 1993; Roman et al., 2000; * imported cases of human cysticercosis; **no data available on taeniasis

Table 1.3. Prevalences of *Taenia* spp. in America

Country	Year	individuals	*Taenia* spp.	*T. solium*	*T. saginata*	Assay	Location/Observations
USA	2001[1]	449		1.1%		EITB	California (1 community)
Mexico	1990[2]	516	1.2%			a	El Salado
	1991[3]	392	1.32%			a	Sinaloa
	1992[4]	1,531	0.3%			a	Morelos
	1994[5]	828	0.2%			a	Michoacan (1 village)
	1996[6]	475	2.1%			b	Yucatan (1 village)
	1998[7]	403	1.2%			a	Gerrero
	1999[8]	713,068		4 cases	37 cases	e	Mexico City
	1999[9]	1,000	0.1%/0.5%			a/b	Soldiers
	2000[10]	115	0%			a	Xochimilco (1 village)
Honduras	1991[11]	536		2%		a	Agua Caliente
	1995[11]	68	1.5%			a	Agua Caliente
	1998[12]	328	0.6%			a	Soldiers
	1999[13]	480	2.5%			a	Salama
Guatemala	1991[14]	1,161/1,204	2.8%/1%	56 cases		b	El Jocote/Quesada
	1991-1994[15]	3,399	2.7%			b	f
	1964[16]	f			1.7%	f	f
El Salvador	1987-1988[16]	f	0.18-1.28%			f	f
Costa Rica	1978-1987[16]	f	0.02-0.09			f	f
Panama	1960[16]	f			0.2%	f	f
Venezuela	1998[17]	1,254	11%			c	f
Colombia	1996[18]	214	0.93%			a	Arboleda
	2000[19]	661	2%			c	Ituango (2 villages)
Peru	1988[20]	30	4 cases			d	Maceda
	2003[21]	2,471/1,620	0.8%/2.8%			f	Quilcas (9 villages)
Bolivia	1995[22]	f	12%			a	Chuquisaca (8 villages)
Argentina	1994-1999[23]	2,447	0.2%			a	Bahia Blanca
Uruguay	1985-1998[24]	f			197 cases	e	Montevideo hospital

a. coprology; b. coproantigens; c. questionnaire; d. recovery; e. self-detection ; f. no information ; 1. De Giorgio et al. (2005); 2. Diaz Camacho et al. (1990); 3. Diaz Camacho et al. (1991); 4. Sarti et al. (1992b); 5. Sarti et al. (1994); 6. Rodriguez Canul et al. (1999); 7. Martinez-Maya et al. (2003); 8. Flisser et al. (2005); 9. Garcia-Garcia et al. (1999); 10. Cruz-Licea et al. (2003); 11. Sanchez et al. (1997); 12. Sanchez et al. (1998); 13. Sanchez et al. (1999); 14. Garcia-Noval et al. (1996); 15. Allan et al. (1996b); 16. OMS/OPS (1993); 17. Ferrer et al. (2003); 18. Sanzon et al. (1999); 19. Agudelo-Florez and Palacio (2003); 20. Diaz et al. (1992a); 21. Garcia et al. (2003a); 22. OMS/OPS (1998); 23. Costamagna et al. (2002); 24. Acuña et al. (2005)

Table 1.4. Prevalence of human cysticercosis in America

Country	Year	individuals	Human cysticercosis	Method	Location/Observations
USA	1989-2000[1]	f	3.9 per million	a	California state
	2001[2]	449	1.8%	d	California (1 community)
Mexico	1990[3]	478	12%	b	El Salado
	1991[4]	392	11%	b	Sinaloa
	1992[5]	1,552	10.8%	d	Morelos
	1994[6]	1,005	4.9%	d	Michoacan (1 village)
	1996[7]	475	3.7%	d	Yucatan (1 village)
	1998[8]	92	3.26%	d	Gerrero
	1999[9]	1,000	0.1/2.8/12.2%	b/c/d	Soldiers
	2000[10]	110	0.9%	d	Xochimilco (1 village)
	2000[11]	2,438	2.17%	e	Mexico (hospital)
Honduras	1991[12]	526	30%	b	Agua Caliente
	1995[12]	110	34%	d	Agua Caliente
	1995[13]	151	30%/62%	d/e	Salama
	1998[14]	404	15.6%	d	Soldiers
	1999[15]	480/148	17%/5%	d/e	Salama
Guatemala	1991[16]	1,161/1,204	17%/10%	d	El Jocote / Quezada
Costa Rica	1976-1988[17]	f	0.006	a	f
Venezuela	1998[18]	1,254	6.7, 27.4%	c/b	f
Colombia	1991[19]	240	23.3	b	Nariño (1 village)
	1995[20]	376/546	9.82/13.92%	d/e	Medellin (hospital)
	2000[21]	661	1.82%	d	Ituango (2 villages)
Peru	1988[22]	371	8%	d	Maceda
	1993[23]	498	15%	d	Lima (hospital)
	1999[24]	108	13%	d	Highland (1 village)
	2003[25]	2,583	13.9% (7.1-26.9%)	d	Quilcas (9 villages)
Bolivia	1990[26]	f	107 cases	e	Cochabamba (hospital)
	1999[27]	447	22%	b	Chaco
Brazil	1987-1990[28]	1,863/459	24.6/8.7%/3.2%	b/d	Cássia dos Coqueiros
	1970-2000[29]	2,639		a	Uberaba (hospital)

a. Autopsy; b. Ab-ELISA; c. Ag-ELISA; d. EITB; e. CT-scan; f. no information; 1. Sorvillo et al. (2004); 2. De Giorgio et al. (2005); 3. Diaz–Camacho et al. (1990); 4. Diaz–Camacho et al. (1991); 5. Sarti et al. (1992b); 6. Sarti et al. (1994); 7. Rodriguez-Canul et al. (1999); 8. Martinez-Maya et al. (2003); 9. Garcia-Garcia et al. (1999); 10. Cruz-Licea et al. (2003); 11. Rodriguez-Leyva et al. (2000); 12. Sanchez et al. (1997); 13. Medina et al. (2005); 14. Sanchez et al. (1998); 15. Sanchez et al. (1999); 16. Garcia-Noval et al. (1996); 17. OMS/OPS (1993); 18. Ferrer et al. (2003); 19. Botero (1999); 20. Palacio et al. (1998); 21. Agudelo-Flores and Palacios (2003); 22. Diaz et al. (1992a); 23. Garcia et al. (1993); 24. Garcia et al. (1999); 25. Garcia et al. (2003a); 26. OMS/OPS (1998); 27. Carrique-Mas et al. (2001); 28. Bragazza et al. (2002); 29. Lino-Junior et al. (2002)

Table 1.5. Prevalence of porcine cysticercosis in America

Country	Year	Population	Porcine cysticercosis	Method	Location
Mexico	1991[1]	72	1.38%	a	Sinaloa
	1991[2]	216	6.5%	a	Michoacan
	1992[3]	571	4%	a	Morelos
	1996[4]	75	23%/35%	a/b	Yucatan
	1999[5]	400,000/year	0.4%	c	Mexico (abattoir)
Honduras	1998[6]	192	27.1%	b	Salama
Guatemala	1991[7]	100 / 25	14% / 4%	a	El Jocote/ Quesada
Nicaragua	2002[8]	8,046	0.34%	c	Regional
Venezuela	2001[9]		23 cases	h	
Colombia	1991[10]	180	13.3%	c	Cordova (2 villages)
	1992[11]	3,300	0.25-22.2%	c	Cordova
	2000[12]	131	5.34%	b	Ituango (2 villages)
Peru	1988[13]	133	33%/43%	a / b	Maceda
	1990[14]	77	23.4/31.2/37.7/51.9%	a/d/f/b	Huancayo
	1999[15]	89	43%	b	Highlands
	2003[16]	534	26%	b	Tumbez (3 villages)
	2003[17]	703	42-75%	b	Quilcas (9 villages)
	2003[18]	1,548	39-76%	b	Highlands (8 villages)
	2004[19]	304	47.3%	b	Apurimac (3 villages)
Bolivia	1995[20]		16.4%	e	Chuquisaca (8 villages)
	1999[21]	273	37%	f	Chaco
Brazil	2005[22]	628	9.71%	g	Belo Horizonte (7 villages)

a. Tongue inspection ; b. EITB ; c. Meat Inspection ; d. autopsy ; e. serology ; f. Ag-ELISA ; g Ac-ELISA ; h. Report; 1. Diaz-Camacho et al. (1991) ; 2. Sarti et al. (1992b); 3. Sarti et al. (1992a); 4. Rodriguez-Canul et al. (1999); 5. Flisser et al. (2005); 6. Sakai et al. (1998); 7. Garcia-Noval et al. (1996); 8. OIRSA (2001); 9. MSDS (2001); 10. Quintero et al. (2000); 11. Reza (2000); 12. Agudelo-Flores and Palacio (2003); 13. Diaz et al. (1992a); 14. Gonzalez et al. (1990); 15. Garcia et al. (1999); 16. Taico et al. (2003); 17. Garcia et al. (2003b); 18. Garcia et al. (2003a); 19. Ayvar et al. (2004); 20. OMS/OPS (1998); 21. Carrique-Mas et al. (2001); 22. De Arruda Pinto et al. (2002)

Some studies were restricted to abattoirs where porcine cysticercosis was diagnosed by meat inspection reporting prevalences between 0.25 to 22.2%. Since immunological tests became available, the number of reported cases of cysticercosis in pigs increased considerably. As such, prevalences between 26% and 76% have been detected in endemic areas. As shown in table 1.5, most studies used only one diagnostic method. Gonzalez et al. (1990) carried out a study using 4 different diagnostic methods in a small group of animals. They concluded that EITB was more sensitive and specific than the other methods i.e. tongue inspection, autopsy and Ag-ELISA.

1.5.1.2 Prevalence of Taenia solium-*cysticercosis in Ecuador*

Ecuador is located in South America bordering Colombia in the north, Peru in the south and east and the Pacific Ocean in the west (Fig. 1.12). The capital is Quito and the country has 22 provinces. The country has 3 well-defined regions i.e. the coastal region (Costa) with a warm humid climate, the Andes region (Sierra) with a temperate to cold climate and the Rain Forest or Amazonia with a hot humid climate. According to

Fig. 1.12. Map of Ecuador

the "IV censo nacional de población y vivienda" and the "III censo nacional agropecuario" carried out in 2001 in Ecuador, 12,156,608 inhabitants and 1,527,115 pigs were recorded, respectively. Among these pigs more than 1,100,000 creole pigs were recorded, that are usually raised in traditional husbandry conditions (INEC, 2006).

The Andean region of Ecuador is considered as hyper-endemic for taeniasis/cysticercosis (Cruz et al., 1989) (Fig. 1.11). Nevertheless, even though improved immunodiagnostic techniques have become available, prevalence data on taeniasis and cysticercosis in man and on porcine cysticercosis in the distinct regions are

still fragmentary. As a result, the impact of cysticercosis on public health and on the economy has not yet been documented.

The first case of NC in Ecuador and Latin America was reported in 1901 (Lasso, 1994). In a clinical survey in hospitals in Quito, carried out between 1954 and 1960, 30 cases of NC were found in 30,000 patients, (Merino, 1961). Between 1947 and 1968, a prevalence of NC of 0.47% was reported in autopsies in Ecuador (Flisser, 2002; Murrell, 2005). In 1968, *T. solium*-cysticercosis was considered for the first time as a public health problem because of an increasing number of NC cases in Ecuador (Rodriguez et al., 1968). The prevalence of human cysticercosis, based on autopsy studies varied from 3% and 6.1% (Gerrero, 1965; Sinche, 1986) and community-based surveys indicated high prevalences: taeniasis between 0.66 and 1.60% (Lopez, 1969; Jimenez, 1976; Cruz et al., 1989) and human cysticercosis between 8.6% to 10% (based on EITB) and 14.4% (based on CT-scan) in the northern Sierra (Cruz et al., 1999a; Del Brutto et al., 2005). All *Taenia* specimens were presumed to be *T. solium* since *T. saginata* was not known to occur in the study areas (Cruz et al., 1989).

Based on official data (Fig. 1.13), it is difficult to get a clear picture on the prevalence of taeniasis/cysticercosis in the country. These figures of taeniasis/cysticercosis were based on hospital records, hence excluding a large part of the rural population. Official reports indicate low and moderate incidences of taeniasis and cysticercosis in humans in some provinces in the Sierra, like Bolivar, Sucumbios and Imbabura (taeniasis/cysticercosis of 0/4.02, 0/5.93 and 0.27/2.41, per 100,000 habitants, respectively).

The overall epilepsy rate in Ecuador was estimated at 12.7/1,000 in 1993 (Cruz et al., 1994) and 25.85/100,000 in 2004 (Aguilar, 2005). Although it is difficult to compare studies that attempt to assess the proportion of epilepsy cases that are due to NC, because of different study designs and techniques used, it appears that in Ecuador this proportion is lower (9.6%) than that found in other endemic countries (between 30-50%) (Carpio and Hauser, 1999, Carpio and Hauser, 2002). In socioeconomically deprived rural areas this rate is higher. In a door-to-door study in rural communities from the Andes (221 participants) and from the coastal region (2,415 participants) Basch et al. (1997) and Del Brutto et al. (2005) found prevalence rates of epilepsy of 22.6/1,000 and 9.9/1,000, respectively. The major causes of epilepsy in Ecuador are

malnutrition, chronic iodine deficiency in the Sierra and NC (Cruz et al., 1994). It was estimated that between 8.6% and 14.4% of neurology cases presented at modern hospitals in Ecuador had evidence of NC, diagnosed by EITB or CT-scan, respectively (Cruz et al., 1994; Del Brutto et al., 2005).

Fig. 1.13. Incidence of taeniasis and cysticercosis in man in Ecuador (1994-2004) per 100,000 inhabitants
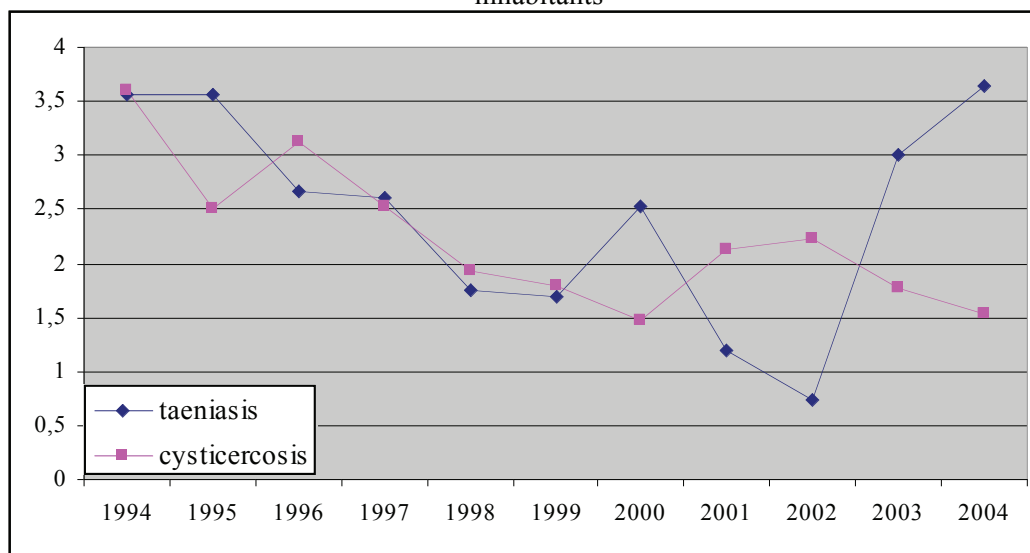
Source: EPI-2, Epidemiological department, Public Health Ministry of Ecuador. (Aguilar, 2005b)

Meat inspection is routinely performed in only 50% of Ecuadorian abattoirs (Ruiz, 1986; Benitez-Ortiz, 1995). Consequently, a similar discrepancy between official reports on porcine cysticercosis and data from surveys was found: slaughterhouses report a prevalence of <0.1% (Erazo et al., 1988), while in surveys in the Southern Sierra between 5.9 and 12% of the pigs were found infected (Jimenez, 1976; Benitez-Ortiz, 1995).

1.5.2   Prevalence of *Taenia saginata*-cysticercosis in America and Ecuador

T. saginata has a cosmopolitan distribution, but the infection is more important in Africa, Asia and Latin America and in some Mediterranean countries (Murrell, 2005). The prevalence of *T. saginata* in humans can be roughly classified into three groups: (i) highly endemic regions with prevalences that exceed 10%; (ii) those with moderate prevalence; and (iii) those with a prevalence below 0.1% or free from *T. saginata*-taeniasis (WHO, 1983).

In America bovine cysticercosis has been reported in Argentina, Brazil, Canada, Chile, Guatemala, Cuba, Mexico and the United States of America (Muller, 1975; Acha and Zsyfres, 2003).

There are no official reports on the presence of *T. saginata* in Ecuador. The only documented evidence can be found in final year dissertations, restricted to slaughterhouse observations in the coastal region of the country. As such, infection levels, observed by post mortem inspections varied from 0.04 to 1.89 % (Briones, 1969; Aragundi, 1969; Intriago, 1976; Sosa, 1981; Aragundi, 1999). According to FAO, OMS and OIE *Cysticercus bovis* was occasionally found in Ecuador from 1973 to 1984; from 1985 to 1995 (last year of publication) no information on this parasite was available (Welte, 1997; Handistatus II, 2005).

According to the same sources in 1995 bovine cysticercosis in South America was only reported in Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay and Venezuela.

Few prevalence data exist on bovine cysticercosis in Latin America. OMS/OPS (1993) showed prevalences between 0.2 to 1.9% in Panama, El Salvador and Chile. Borges et al. (1997) and Camaro et al. (1997) observed bovine cysticercosis in 1.04% and 4.63% of slaughter cattle in Brazil, respectively. In Uruguay 197 cases of bovine cysticercosis were reported between 1985 and 1988 (Acuña et al., 2005).

1.6 DIAGNOSIS

1.6.1 Taeniasis

Several methods have been described to detect intestinal taeniasis. Macroscopic diagnosis is based on the search of proglottids in the faeces or on the recovery of the worm after treatment. As described in sub 1.4 morphological characteristics allow for a distinction between *T. solium* and *T. saginata* based on proglottids or the scolex (table 1.1). Cellulose acetate gel electrophoresis, based on glucose phosphate isomerase (GPI: EC 5.3.1.9.), has been used to differentiate *T. solium* and *T. saginata* (Le Riche and Sewell, 1977; Le Riche and Sewell, 1978; Bâ, 1995). This method can be performed either on fresh or frozen somata, proved to be easy and fast, not requiring large quantities of material and producing consistent results. Molecular methods have been described for differentiation of *Taenia* spp. on parasite material (see 1.7.1.1)

Routine diagnosis is based on the microscopic detection of *Taenia* spp. oncospheres in direct smears or after concentration methods such as the Kato-Katz and formol-ether methods. These methods have a sensitivity of around 38% to 60% (Allan et al., 1996a; Sarti, 1997).

The presence in the faeces of tapeworm metabolic products with antigenic capacity has been used to prepare specific polyclonal antibodies that allow the detection of coproantigens by ELISA (Allan et al., 1990). These methods, however, do not allow differentiation between *T. solium* and *T. saginata*. According to Allan et al. (1990; 1992) coproantigen detection has a specificity and a sensitivity of 100% and 98%, respectively. However, some studies in Guatemala and Peru demonstrated a lower sensitivity (Garcia-Noval et al., 1996; Allan et al., 1996b; Cabrera et al., 2005).

Wilkins et al. (1999) described specific *T. solium* antigens to detect antibodies in serum by Western blot with sensitivity and specificity rates of 95% and 100%, respectively. No cross-reactions were found in serum samples from patients with *T. saginata* or other cestode infections, including *T. solium* cysticercosis. Nevertheless, one sample from a patient with NC without intestinal *T. solium*, tested positive (Allan et al., 2003). Diagnosis of taeniasis by serological examination has obvious advantages over the faeces-based methods, e.g. species-specific diagnosis, avoidance of the potential biohazard of collecting and handling faeces, and also the possibility to combine this test with other immunological assays in the diagnosis of human cysticercosis. However, residual antibodies from past exposure might result in false positives (Allan et al., 2003; Ito and Craig, 2003).

Recently, highly specific PCR-methods have been developed to detect *Taenia* DNA in faeces (Dinkel et al., 1998; Yamasaki et al., 2004; Stefanic et al., 2004; Nunes et al., 2003, 2005, 2006). However, these methods have not yet been properly validated in the field.

1.6.1.1 Molecular methods for diagnosis of taeniasis

Molecular methods such as PCR have been used for amplification and analysis of DNA sequences. Since the development by Mullis in 1983, PCR became an important diagnostic tool.

Several molecular techniques have been described for the detection and differentiation of *Taenia* species using gDNA and/or mtDNA i.e. Dot Blot analysis (Chapman et al., 1995), Multiplex-PCR (Gonzalez et al., 2000; Gonzalez et al., 2002; Nunes et al., 2003; Yamasaki et al., 2004), PCR-Restriction Fragment Length Polymorphism (RFLP) (Mayta et al., 2000; Gonzalez et al., 2002; Nunes et al., 2005; Somers et al., 2006), a based excision sequence scanning thymine-base reader analysis (Yamasaki et al., 2002) and random amplified polymorphic DNA (RAPD) (Eom et al., 2002; Vega et al., 2003).

a   Identification of carriers and differentiation of somata extracts

DNA probes offer the possibility of a sensitive, rapid, and non-invasive diagnostic test which can distinguish *T. solium* and *T. saginata* tapeworms (Chapman et al., 1995). These authors developed highly sensitive, species-specific DNA probes obtained from *T. solium* cysticerci and adult *T. solium* and *T. saginata*. In this study, recombinant clones containing repetitive sequences, which hybridize specifically with gDNA from *T. solium* and *T. saginata* were isolated and characterised. The gDNA's used to make these libraries were [^{32}P] cystidine triphosphate (dCTP) labelled by random primer extension, and the probes thus generated were used to screen the corresponding libraries for high-abundance sequences using a dot blot analysis. Later on, two non-coding DNA probes cloned from a genomic library of *T. saginata* i.e. HDP1 and HDP2 were developed by Gonzalez et al. (2000, 2002) based on the studies of Chapman et al. in 1995 and used in a Multiplex-PCR. These authors designed species-specific oligonucleotides allowing for the differential detection of *T. saginata* and *T. solium*. Their final results showed that HDP1 probe specifically amplified gDNA from *T. saginata* and HDP2 probe allowed the differential amplification of gDNA from *T. solium, T. saginata* and *Echinococcus granulosus*. In 2002, Gonzalez et al. tested 25 *Taenia* samples from different geographical areas with 5 different PCR assays using HDP2, ITS1 and ITS2 gDNA. They concluded that the ribosomal sequence, ITS2-PCR could be an alternative method to the HDP2-PCR in the differential diagnosis between *T. solium* and *T. saginata*. The primers HDP2 were also used to amplify *T. saginata* and *T. s. asiatica* gDNA which could then differentiate between the two by using two different endonucleases. Contrary to *T. saginata*, *T. s. asiatica* did show an aspecific PCR amplification complicating the restriction profile. Hence, a new *T. s. asiatica*

primer on the HDP2 locus was designed to use in a multiplex-PCR resulting in a reproducible pattern for *T. s. asiatica* and allowing a differentiation between *T. saginata* and *T. s. asiatica* (Gonzalez et al., 2004).

Mayta et al. (2000) developed a comparative assay for differentiation of *T. solium* and *T. saginata*, based on the 5.8S ribosomal region amplifying a 1300bp long segment. These authors demonstrated a clear difference between these cestodes species using 3 digestion enzymes (*Alu*I, *Dde*I and *Mbo*I). However, this technique produced poor results when applied on faecal samples spiked with *Taenia* eggs.

The 12s rRNA mitochondrial gene was used to differentiate *T. s. asiatica* from *T. saginata* and *T. solium* in a PCR-RFLP (Somers et al., 2006). This assay proved to be a reliable tool to distinguish among the species and subspecies present in Asia, even when proglottids were in a far state of disintegration (Somers et al., 2006).

b <u>Identification of carriers and species differentiation on faecal extracts</u>

The complex nature of different sources of DNA and the presence in faeces of PCR inhibitors such as haemoglobin, bilirubin, bile salts and chelating agents (Widjojoatmodjo et al., 1992; Shames et al., 1995; Vansnick, 2004) complicate the isolation and purification of *Taenia* DNA in faecal extracts (McOrist et al., 2002). However, novel techniques to detect *Taenia* DNA in faeces have been published. Nunes et al. (2003) described a multiplex-PCR assay on HDP2 gDNA using the primers previously described by Gonzalez et al. (2000) to detect *T. saginata* in faeces spiked with eggs. They established the lower limit of detection at 137 eggs in 1 gram (1096pg of DNA). The Cox1 gene was used as a target gene to develop primers specific for *T. saginata*, *T. s. asiatica* and *T. solium* in a multiplex-PCR (Yamasaki et al., 2004). Only 11 of 23 samples, confirmed to be positive by the coproantigen detection test, were found positive by this test. Possibly, the length of storage (up to 12 years) reduced the test sensitivity. In addition, 5 faecal samples found negative in the multiplex-PCR were detected by a nested PCR (Yamasaki et al., 2004). Nested PCR, by definition, means that two pairs of PCR primers are used for a single locus. The first pair amplifies the locus as seen in any PCR experiment. The second pair of primers (nested primers) binds within the first PCR product and produces a second PCR product that will be shorter than the first one. This reduces the chance to amplify unwanted DNA (non-

specific bands) and increases the number of detectable copies from sources where target DNA is not always evident. Nunes et al. (2005) used the Cox1 gene to develop a PCR-RFLP to differentiate *T. saginata* from *T. solium* in faecal extracts. Out of 12 faecal samples from naturally infected patients, 8 were positive for *Taenia* sp. using copro-PCR, i.e. 3 *T. solium* and 5 *T. saginata*. Furthermore, the lower limit of the DNA detection with PCR-RFLP was 34 eggs in 2g of faeces.

1.6.2   Cysticercosis

1.6.2.1 Visual ante and post mortem diagnosis (animals)

In animals, ante mortem diagnosis is routinely performed in pigs, by visual inspection and palpation of the tongue. Looking for metacestodes in the eyes has been reported but has a low sensitivity (Benitez-Ortiz, 2006, Pers. com.). Contrary to bovines and as a consequence of their coprophageous habits, pigs often show massive infestations with cysticerci in the whole carcass, including in the brain (Borchert, 1975; Perez and Camacho, 1985). The sensitivity and specificity of ante and post mortem inspection are high in massive infections. However, the sensitivity of these techniques is reduced considerably when applied in animals with low cyst burdens. Dorny et al. (2004a) reported a sensitivity of 21% for tongue inspection and 22% for meat inspection whilst, the specificity in both cases was 100%. In bovines ante mortem inspection is pointless, given the localisation of the cysticerci and the low rates of infection (Gonzalez et al., 1990; OMS/OPS, 1993). Meat inspection of bovine carcasses is a standard procedure, and the sole legally compulsory and accepted method. It is performed by incising and inspecting the tongue, masseter muscles, heart, diaphragm, oesophagus and sometimes muscles of the legs, which are considered predilection sites (Blazek et al., 1981). Usually the numbers of cysticerci are low in bovine; hence many cases remain undetected even after conscious post mortem inspection (Blazek et al., 1981; Gonzalez et al., 1990; Gonzalez et al., 1993).

1.6.2.2 Serological diagnosis

Immunodiagnostic methods have been developed to detect either circulating antigens of cysticerci of *Taenia* spp. or antibodies directed against these, in serum and cerebrospinal fluid of man and animals. Recent reviews of these diagnostic methods have been given by Dorny et al. (2004b) and Garcia et al. (2005). For antibody

detection, the enzyme-linked immunoelectrotransfer blot (EITB), using glycoprotein antigens purified by affinity chromatography assay, is considered the method with the highest sensitivity and specificity (Tsang et al., 1989; Garcia et al., 1991; Diaz et al., 1992b; Ito et al., 1998). Because the EITB is laborious and expensive, several researchers developed enzyme-linked immonosorbent assays (ELISA) (Ito et al., 1998; Barcelos et al., 2001; Diaz et al., 1992b; Bueno et al., 2005; Ferrer et al., 2005) using crude or purified antigens of *T. solium* cysticerci or synthetic peptides. These tests have varying degrees of sensitivity and specificity. For antigen detection, monoclonal antibody-based sandwich- ELISA's have been developed by Harrison et al. (1989) and Brandt et al. (1992), modified by Draelants et al. (1995) and Van Kerckhoven et al. (1998). These tests allow detection in serum of excretory/secretory products of *Taenia* spp. cysticerci. Although these assay have a high sensitivity and specificity, detection is restricted to the presence of viable larvae; degenerated or calcified cysts are not detected. Specificity is at the level of the genus, which allows use for the diagnosis of *T. saginata* in bovines and of *T. solium* in humans and pigs.

According to Ito et al. (1998) and Garcia et al. (2005) the EITB has a sensitivity between 94 to 98% and a specificity of 100% for patients with two or more cysts. However, in patients with a single intracranial cysticercus, it shows a lower sensitivity (< 50%) and specificity (65%) (Wilson et al., 1991; Proaño-Narvaez et al., 2002). In addition, false positive serology can result from past infection with *T. solium* (Jain et al., 2005). On the other hand, Antibody-ELISA's were reported to have a similar sensitivity and specificity as EITB for differential serodiagnosis of NC (Ito et al., 2002b). A sensitivity of 85% by antigen detection ELISA was found in selected EITB-seropositive patients (Garcia et al., 2000). Furthermore, the sensitivity in patients with a single viable cyst or only enhancing lesions was 65% (Garcia et al., 2000).

In pigs, EITB was used to detect circulating antibodies with high sensitivity and specificity (Garcia et al., 2003b; Ayvar et al., 2004). The sensitivity and specificity of Ag-ELISA was estimated to be around 85% and 97%, respectively (Nguekam, 1998; Dorny et al., 2004b). Unfortunately, it may be a disadvantage when several *Taenia* species occur in the same host, like in pigs where the Ag-ELISA cannot differentiate between infections with *T. solium*, *T. s. asiatica* and *Taenia hydatigena*.

Antigen detection ELISA in bovines has a sensitivity and specificity of 98.7% and 92.3% respectively in animals with more than 50 living cysticerci (Van Kerckhoven et al., 1998). However, in bovines sensitivity decreases to 12% when the carcass contains less than 20 larvae (Geerts, 1993).

1.6.2.3 Imaging diagnosis

Human neuro-cysticercosis can be diagnosed by Magnetic Resonance Imaging (MRI) or/and Computerized Axial Tomography (CT-scan), which identify vesicular (viable), colloidal (degenerating) and calcified cysticerci or lesions related with cysticercosis in the CNS (Carpio, 1990; OMS/OPS, 1993; Garcia et al., 2005). These neuroimaging techniques are considered as reference diagnostic tools, however, their cost and restricted availability - patients have sometimes to travel long distances - limit their use. Moreover, interpretation of the images demands skilled experts. Finally, only patients with neurological symptoms will undergo a brain scan, meaning that if these symptoms are caused by cysticerci, in most cases the degenerating process has already started, which may be too late to start a causative treatment.

Even with neuroimaging methods and sensitive and specific serological tests, diagnosis of NC remains difficult. Therefore, a panel of experts designed diagnostic criteria for NC (table 1.6), which evaluates the clinical, radiological, immunological and epidemiological data of patients. These proposals are divided in absolute, major, minor and epidemiologic criteria based of their individual diagnostic strength (Del Brutto et al., 2001).

Absolute criteria permit unequivocal diagnosis even if standing alone; *major criteria* strongly suggest the diagnosis but cannot be used alone to confirm or exclude the disease; *minor criteria* are frequent clinical and radiological manifestations of the disease but are relatively non-specific and therefore are unable to significantly differentiate among the diagnostic possibilities; and *epidemiological criteria* include potential exposure that favours the diagnosis of cysticercosis. Proper use of these criteria might help doctors to avoid incorrect diagnosis and to conduct an appropriate treatment, although this assessment has not been prospectively validated (Nash et al., 2005).

Table 1.6. Diagnosis criteria for Neurocysticerosis (Del Brutto et al., 2001)

Categories of criteria		Criteria
Absolute	1.	Histologic demonstration of the parasite from biopsy of a brain or spinal cord lesion
	2.	Cystic lesions showing the scolex on CT-scan or MRI
	3.	Direct visualization of subretinal parasites by fundoscopic examination
Mayor	1.	Lesion highly suggestive of NCC or neuroimaging studies*
	2.	Positive serum EITB for the detection of the anticysticercal antibodies
	3.	Resolution of intracranial cysts lesion after therapy whit albendazole or praziquantel**
Minor	1.	Lesions compatible with NCC or neuroimaging studies§
	2.	Clinical manifestation suggestive of NCC¥
	3.	Positive Cerebro-Spinal Fluid (CSF) ELISA for detection of anticysticercal antibodies or cysticercal antigens
	4.	Cysticercosis outside of NCS¶
Epidemiologic	1.	Evidence of household contact with *T. solium* infection
	2.	Individual coming from or living in an area where cysticercosis is endemic
	3.	History of frequent travel to disease-endemic areas

* CT-scan or MRI showing cyst lesions without scolex, enhancing lesions, or typical parenchymal brain calsifications; ** Solitary ring-enhancing lesion measuring less than 20 mm in diameter in patients presenting with seizures, a normal neurological examination, and no evidence of an active systemic disease; § CT-scan or MRI showing hydrocephalus or abnormal enhancement of the leptomeninges and myelograms showing multiple filling defects in the column of contrast medium; ¥ Seizures, focal neurological signs, intracranial hypertension and dementia; ¶ Histological confirmed subcutaneous or muscular cysticercosis, plain X-ray films showing "cigar-shaped" soft-tissue calcification, or direct visualization of cysticerci in the anterior chamber of the eye.

1.7 TREATMENT OF TAENIASIS IN HUMANS AND CYSTICERCOSIS IN HUMANS AND ANIMALS

Praziquantel is the preferred drug for treatment of human taeniasis at an oral dose of 10mg/kg b.w. (WHO, 2002). According to Herrera et al. (2000) this drug has a low toxicity and minimal secondary effects. Niclosamide followed by a purgative is a good alternative because tapeworms are usually completely recovered, including the scolex, being crucial in the identification and differentiation between species. In addition, this drug is not absorbed in the intestine avoiding the risk of causing neurological symptoms when the carrier also has NC (Jeri et al., 2004). Albendazole has also been used to treat taeniasis although Horton (2000) mentions a low efficacy (85% in 7 studies).

Treatment of cysticercosis in bovines or pigs with albendazole or praziquantel has been restricted to experimental studies. According to Cevallos (2000), the need for repeated dosages, secondary effects, high cost and low efficacy against CNS-cysticerci have limited its use. Oxfendazole administered in a single oral dose of 30mg/kg b.w., was reported to be 100% effective in treating porcine cysticercosis (Gonzalez et al., 1996), although, Gonzalez et al. (1998) and Cevallos (2000) reported lower efficacy against brain cysts.

Since long, anti-parasitic treatment of human (neuro-)cysticercosis has been a debatable issue (Del Brutto et al., 2006). Assuming that symptoms are related to the degeneration process of brain cysticerci, a metacestodicidal treatment may induce or aggravate symptoms. Therefore, many neurologists prefer to alleviate symptoms in CT-scan or MRI positive patients and refrain from the use of anthelmintics. Others take criteria such as the number, localization (parenchymal or extraparenchymal cysticercosis), viability (viable, degenerated or calcified cysts) and the size of the cysts into consideration (Carpio et al., 1998; Chicharro et al., 2000; Garcia et al., 2002; Garcia et al., 2005) and do not advice anthelmintic treatment of a single viable cyst or calcified cysts or asymptomatic NC. Therapeutic measures including anthelmintics, surgical and symptomatic treatment have been reviewed by Garcia et al. in 2002. They agreed that NC is not a single disease for which one therapy can be recommended. Neuro-cysticercosis has been treated using albendazole or praziquantel or a combination of both in addition to corticoids to suppress side effects. Nevertheless, whenever hydrocephalus or intracranial hypertension are present, its management should be the

first priority before using anthelmintics. They also suggest that ventricular NC should be treated surgically.

1.8   CONTROL

Control measures of neuro-cysticercosis have been formulated by the World Health Organization in 2002 (WHO, 2003) based on four facts, i.e. cysticercosis is caused by the larval stage (cysticerci) of the pork tapeworm *T. solium*; cysticercosis is the most important neurological disease of parasitic origin in man; human cysticercosis is associated with poverty in areas where people eat pork and traditional pig husbandry is practiced and cysticercosis of the CNS is an important cause of chronic epilepsy, which places particular demands on health services. Hence, the measures currently available to control cysticercosis i.e. elimination of infected pigs/carcasses (meat inspection), improved sanitation, hygiene and pig husbandry, health education, treatment of intestinal taeniasis, chemotherapy of infected swine and mass treatment in humans and pigs have been proposed by WHO (2002), Allan et al. (2002) and Gonzalez (2002). In this context, the main strategies available are directed towards elimination of infected pig carcasses (meat inspection), underpinned by improved sanitation, hygiene, pig husbandry and most importantly, proper health education. Other measures, summarized by Engels et al. (2003) include treatment of intestinal taeniasis, vaccination of pigs and chemotherapy of infected animals. Agro-industrialisation has led indirectly, even unintentionally, to the eradication of the parasite in industrialised countries. However, as long as traditional pig husbandry is practised in developing countries, the life cycle of *T. solium* is likely to persist (Gonzalez et al., 2003).

In 1993, according to the WHO (2003), the International Task Force for Disease Eradication declared *T. solium* a potentially eradicable disease, for the following reasons: (i) the life cycle requires humans as definitive hosts; (ii) tapeworm infection in humans are the only source of infection for pigs, the natural intermediate host; (iii) the transmission of infection from pigs to man is controllable; (iv) no reservoir for infection exists in wildlife. Furthermore, Schantz (2002) includes two more reasons: (v) there are practical methods for surveillance in pigs (visual inspection of the tongue), in humans (EITB and copro-antigens) and, (vi) safe and effective drugs for mass chemotherapy of taeniasis in humans and cysticercosis in pigs do exist. It is, therefore, expected that the strategic use of anthelminthics against the adult parasite in people and the larval parasite

in swine, combined with health education and regulation of pigs slaughter, should be adequate to interrupt transmission, but this approach has yet to be proven in practice. The lack of a national strategy to eradicate the *T. solium*-cysticercosis complex in some countries like Ecuador, poses a serious constraint. This is understandable because of several limitations i.a. lack of information available to the official sanitary services, economic obstacles, and difficulties to sustain control programmes, and finally, the persistence of bad hygienic habits. Because of inadequate knowledge on the true prevalence and impact of taeniasis and human-, porcine- and bovine cysticercosis, and a lack of adequate infrastructure and technical skills to obtain the required information, it is very unlikely that national control measures will be undertaken.

1.9 CONCLUSIONS

The taeniasis-cysticercosis complex has been widely studied mainly focussing on the epidemiology, risk factors and diagnostic tools. However, new aspects of the epidemiology, such as endemic stability and secondary transmission (Gonzalez et al., 2005b, 2006) complicate the understanding of this parasitic infection. While most epidemiological studies were based on the detection of the antibody response, the value of this tool for measuring infection is hampered by the lack of a gold standard for human cysticercosis, which complicates test validation, and by the fact that exposure to the oncospheres not necessarily leads to establishment of cysticerci (Garcia et al., 2001). In pigs, dissection of the entire carcass, the gold standard for porcine cysticercosis, was used to validate multiple diagnostic tools using a Bayesian analysis (Dorny et al., 2004a). This study showed that, although the use of multiple diagnostic tools is not always feasible in field conditions, it may contribute to a more accurate understanding of the taeniasis cysticercosis complex in endemic areas. These tools included meat inspection, tongue examination, and serological detection of antibodies and antigens. The combined use of these serological methods has also potential for a better understanding of the infection status and transmission dynamics in the human population (Dorny et al., 2004b)

Assessing taeniasis prevalence is mostly done based on microscopic examination or antigen detection on stool samples. These methods do not allow differentiation of infection with *T. solium*, *T. saginata* and *T. s. asiatica*, while these cestodes often co-exist in certain areas. A better understanding of the taeniasis-cysticercosis complex

would greatly benefit from a species-specific diagnosis of tapeworm carriers. Finally, while prevalence data are available and NC has been linked to epilepsy and other neurological disorders in endemic areas, the burden of disease, the impact on human health and the economic impact of this parasitic infection are poorly documented (Engels and Savioli, 2006).

In Ecuador, data provided by governmental sources indicate that this complex is endemic. Nevertheless, the taeniasis-cysticercosis complex has been poorly studied. Moreover, although some prevalence data are available, which have been collected from studies, either on human or porcine cysticercosis, no data have been collected on the impact of the infection (Carabin et al., 2005). More information is needed, not only to improve our understanding of the epidemiology, but most of all to define priority areas and to develop appropriate methods for control.

OBJECTIVES

The main objectives of the thesis were to improve the diagnostic tests and to update the epidemiological knowledge of the taeniasis-cysticercosis complex in Ecuador.

A first specific objective was to improve the detection and differentiation of *Taenia* spp., starting with the development of a PCR to differentiate between *T. solium* and *T. saginata* somata, aiming to extend this technique to specific diagnosis on faecal samples. For this purpose, a new set of highly sensitive primers for amplification of *Taenia* DNA had to be developed for use in a PCR-RFLP assay (Chapter 2). To improve the sensitivity of this technique when applied on faecal samples a new set of primers had to be developed for use in a semi-nested PCR. In addition, DNA extraction techniques on faeces had to be optimalised (Chapter 3).

The second specific objective was to study and compare the epidemiological situation in two climatologically and geographically different regions of Ecuador by using multiple diagnostic tools. A comprehensive survey in a highland area in the northern Andes of Ecuador was done to study intestinal *Taenia* in humans and cysticercosis in humans, pigs and bovines (Chapter 4). A second survey on taeniasis/cysticercosis was conducted in an arid region in the Sierra of southern Ecuador (Chapter 5).

In the general discussion (Chapter 6), the results of the different chapters will be discussed, highlighting the most important findings and implications for the understanding of the taeniasis-cysticercosis complex.

CHAPTER 2

COMPARISON OF CONVENTIONAL TECHNIQUES TO DIFFERENTIATE BETWEEN *TAENIA SOLIUM* AND *TAENIA SAGINATA* AND AN IMPROVED PCR-RFLP ASSAY USING A MITOCHONDRIAL 12S RRNA FRAGMENT

2.1 INTRODUCTION

Humans are the final hosts of *Taenia saginata* and *Taenia solium*. Usually, gravid proglottids of *T. saginata* leave the intestine actively, whereas those of *T. solium* are passively eliminated with the faeces. Schantz (1996) reported a yearly economic loss of about 164 million dollars in Latin America due to the condemnation of the carcasses of pigs and cows, intermediate hosts for these parasites. In addition, *Cysticercus cellulosae*, metacestodes of *T. solium*, can also occur in humans, often leading to neurocysticercosis, a major cause of epilepsy and associated with considerable morbidity and mortality (Cruz et al., 1989, 1999b).

According to Welte (1997), *T. solium* is endemic in most of Latin America, except for Paraguay, Belize and Guyana, whereas *T. saginata* has not been reported from Ecuador and Colombia. Several methods have been described to differentiate between proglottids of the 2 taeniid species. In addition to the absence (*T. saginata*) or presence (*T. solium*) of rostellar hooks on the scolex, Morgan and Hawkins (1949) described a differential method based on the number of uterine branches in gravid segments. They reported that *T. solium* had between 8 and 14 unilateral uterine branches, whereas *T. saginata* had 15 to 24 branches (table 2.1). However, several authors found overlapping numbers, which impeded proper specification (Gemmell et al., 1983; Deluol, 1998; Salfelder et al., 1992; Mayta et al., 2000). Furthermore, mature segments of *T. solium* possess a third accessory ovarian lobe and no vaginal sphincter (Verster, 1969). Le Riche and Sewell (1978) differentiated taeniid cestodes by iso-enzyme electrophoresis based on glucose phosphate isomerase (GPI: EC 5.3.1.9.), using either fresh or frozen somata. Kocher et al. (1989) reported that the mitochondrial 12s rRNA is well conserved among *Echinococcus multilocularis*, *Taenia taeniaeformis*, and *Taenia martis*, giving a PCR fragment of about 440bp when using cestode-specific primers. Gonzalez et al. (2000) developed a diagnostic technique based on multiplex-PCR with species-specific primers from non coding DNA fragments, i.e., HDP1 and HDP2 for *T. saginata* and *T. solium*, respectively. Mayta et al. (2000) reported a PCR assay, targeted at the 5.8S ribosomal gene sequence, with primers annealing, respectively, to the 3' end of the 18S and 5' end of the 28S ribosomal regions. A phylogenetic comparison of 13 taeniid species, based on a 12s rRNA fragment of 300bp, was done by Nickisch-Rosenegk et al. (1999). They reported 50% sequence homology and 30% sequence conservation among the species. This degree of variability was considered

useful for interspecies comparisons. These reports formed the basis for the development of a diagnostic tool to differentiate between proglottids of *T. saginata* and those of *T. solium*, as all methods were found unsatisfactory when used on field samples due to the presence of non-specific, possibly human, amplicons. The development of a diagnostic tool based on these findings is reported herein.

2.2 MATERIALS AND METHODS

2.2.1 Parasites

Proglottids and oncospheres were obtained from 25 patients from different regions of Ecuador (highlands: Quito, Imbabura, and Carchi; coastal region: Manabí). These patients were positive by coprology analysis and parasite material was collected after treatment. In addition, the following reference samples were used: *T. saginata* from a Senegalese patient and *C. bovis* from Ecuador, collected during the present survey. In the absence of adult reference somata of *T. solium*, metacestodes were used, collected from pigs from Cameroon and from Ecuador, and a specimen from a patient from India (table 2.1). Parasites were identified by GPI iso-enzyme electrophoresis (Le Riche and Sewell, 1978) and morphological criteria, i.e., the number of uterine branches in gravid segments, the presence of an accessory ovarian lobe and a vaginal sphincter in mature

Table 2.1. Origin of samples used.

Code	Origin	Date	Host	Code	Origin	Date	Host
E1	Quito/E	17/05/00	Human	E19	Imbabura/E	27/03/01	human
E2	Imbabura/E	14/05/00	Human	E20	Imbabura/E	27/03/01	human
E3	Imbabura/E	18/05/00	Human	E21	Quito/E	27/03/01	human
E4	Quito/E	30/03/00	Human	E22	Quito/E	27/03/01	human
E5	Quito/E	11/04/00	Human	E23	Quito/E	08/12/98	human
E7	Quito/E	17/05/00	Human	E24	Quito/E	07/04/99	human
E10	Imbabura/E	26/06/00	Human	E25	Quito/E	21/04/99	human
E11	Imbabura/E	19/10/00	Human	E26	Quito/E	30/06/99	human
E12	Imbabura/E	16/10/00	Human	E27	Quito/E	26/10/99	human
E13	Manabí/E	14/02/01	Human	E28	Quito/E	26/04/01	human
E14	Imbabura/E	15/02/01	Human	References samples			
E15	Imbabura/E	20/02/01	Human	T.sag	Senegal	16/03/93	human
E16	Imbabura/E	15/03/01	Human	CcE	Ecuador	08/07/83	pig
E17	Carchi/E	15/03/01	Human	CcC	Cameroun	06/12/85	pig
E18	Quito/E	15/03/01	Human	CcH	India	15/12/81	human
				Ccb	Ecuador	11/02/01	bovine

T.sag=*T. saginata*; E=Ecuador; Cc=Metacestode of *T. solium*; Cb=Metacestode of *T. saginata*; H=human; C=Cameroon.

segments after staining (Morgan and Hawkins, 1949), and the presence of rostellar hooks on the scolices. Finally, two pigs were orally infected with oncospheres from a *Taenia* sharing morphological characteristics of both *T. solium* and *T. saginata*.

2.2.2 DNA extraction protocol

The DNA extraction protocol (slightly modified from Boom et al., 1990) was based on guanidinium (Gu-HCl: 6.0M Guanidinium-HCl pH7.5 and 25mM EDTA; Life Technologies, Merelbeke, Belgium), followed by complexing on a suspension of diatomaceous earth (Sigma-Aldrich, Bornem, Belgium). Lysis buffer consisted of 60mM Tris-HCl pH7.4, 60mM EDTA, 10% Tween, 5mM $MgCl_2$, 1% Triton X-100 and 1.6M Gu-HCl 2x concentrated; 250µl of lysis buffer, plus 250µl of milli-Q water (Millipore, Brussels, Belgium) and 50µl of Proteinase K (20mg/ml, Roche Diagnostics, Brussels, Belgium), were added to $3mm^3$ of each sample, i.e., *C. cellulosae* or *Taenia* spp. somata. Samples were incubated in a Thermomixer Compact (Eppendorf, Koln, Germany) overnight at 60ºC, shaking at 1400rpm. Then, 40µl of diatomaceous earth suspension was added, mixed vigorously for 2 seconds and incubated for 1 hour at 37ºC. The suspension was centrifuged for 20 seconds at 12000*g* and the pellet resuspended in 900µl 70% v/v ethanol at 4ºC. This was centrifuged again for 20sec and the supernatant discarded. This process was repeated once in ethanol and again in 900µl acetone. Finally, the pellet was dried in a Thermoblock Dri-bath (Barnstead/Thermolyne, Dubuque, Iowa) at 50ºC for 20 minutes; then, 90µl TE pH8 (10mM Tris and 1mM EDTA) was added, followed by incubation for 20 minutes at 60ºC while shaking at 1000rpm in the Thermomixer. After centrifugation, 50µl of the supernatant was transferred to a new Eppendorf tube. This was kept at 4ºC or stored at −20ºC until used for PCR amplification.

2.2.3 PCR

PCR was performed in a total volume of 25µl, containing 5µl of *Taenia* sp. DNA as template and 20µl of PCR mix containing 4.3µl of milli-Q water; 12.5µl buffer (20mM Tris-HCl, pH8.4; 100mM KCl; 0.2% triton X-100, 3.3mM $MgCl_2$); 2µl of a mix of the 4 deoxynucleotide triphosphates (dNTP, final concentration 0.2mM); 0.4µl of each primers and 0.4 units of Taq-polymerase Silverstar, 50U/µl (Eurogentec, Seraing, Belgium). The amplification was performed in a PHC-3 Thermal cycler (Techne,

Cambridge, U.K.) with an initial denaturation step at 94ºC for 4 minutes, followed by 40 cycles consisting of 94ºC for 1 minute, 58ºC for 1.5 minutes and 72ºC for 2 minutes. Five µl of the amplified products together with Gene Ruler™ (MBI Fermentas, GmbH, St.Leon-Rot, Germany) marker of 100bp was separated by electrophoresis using a Mupid-21 system (Eurogentec) in 2% agarose w/v and TAE buffer (0.04M Tris-acetate, 0.002M EDTA) for 20min at 100volts. The gel was stained with ethidium bromide (Sigma-Aldrich) for 30 minutes and the DNA products visualised using ultraviolet light.

2.2.4   Primers

The primers T60F and T375R, described by Nickisch-Rosenegk et al. (1999), and primers TM12SR and ITMTR2, developed during this study, were used to amplify a region from the 12s rRNA gene (Fig. 2.1). Primers were designed with the aid of the following programs: Amplify (B. Engels 1992) and Right Primer, version M1.25 (R. Isaac, Biodesk, 1994). The Blast program (Genbank http://www.ncbi.nlm.gov/) was used to check the specificity of the primers against all DNA sequences present in the Genbank.

DNA band purification: When a non-specific band was observed, the 300bp band was purified by cutting the DNA segment out of the gel and using the Wizard® DNA Clean-up kit (Promega, Leiden, The Netherlands) following the manufacturer's protocol.

2.2.5   RFLP

RFLP digestion was performed according to manufacturer's specifications (Life Technologies, Merelbeke, Belgium) using 10 units *Dde*I µg-1 DNA, plus 6µl of amplified DNA in a total volume of 15µl. Tubes containing the reaction mixture were incubated for 4hr at 37ºC. Six µl of the digested product was mixed with 2µl of the loading buffer and transferred onto a 10% polyacrylamide gel. A marker of 100bp was included for size identification of the bands. DNA was separated by a Mighty Small horizontal electrophoresis apparatus (Amercham Pharmacia Biotech, Roosendaal, The Netherlands) in TBE (88mM Tris, 89mM boric acid, 2mM EDTA).

The gel was stained using a commercial kit "Plus one" (Amercham Pharmacia Biotech, Roosendaal, The Netherlands) and preserved under plastic foil.

2.3 RESULTS

In total, 25 specimens were analyzed. Primers T60F and T375R amplified a fragment of about 360-bp of the mitochondrial 12s rRNA gene and a second, non-specific 650-bp fragment. The latter was present in most of the analyzed taeniid specimens. In Fig. 2.1, sizes of PCR fragments with these primers are presented. The optimal temperature for annealing was determined to be 58ºC using a Robocycler gradient 40 (Stratagene, Amsterdam, The Netherlands).

Fig. 2.1. Two samples (E2 is field sample E2 and T.sag is *T. saginata*) were used to determine the optimal temperature for annealing using the T60F and T375R primer pair. At 58°C, DNA fragments were clearly observed. Sample E2 shows 2 fragments, the larger is probably non-specific. Analysis is done on a 2% agarose gel and stained with ethidium bromide. Marker is a 100bp DNA ladder.
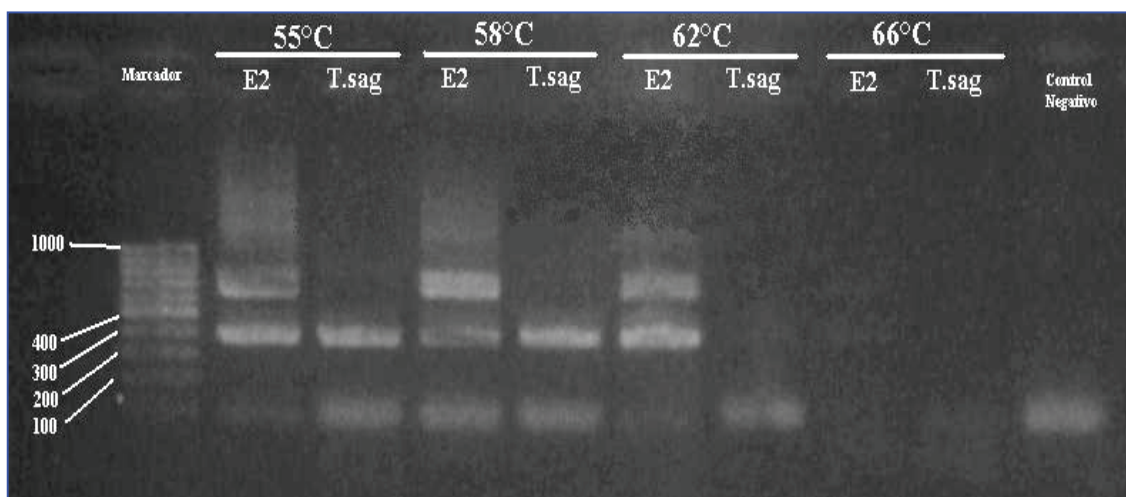
Figure 2.2 shows the alignment of the fragment described by Nickisch-Rosenegk et al. (1999) and Nakao et al. (2000) falling within the 440-bp mitochondrial fragment for cestodes as described by Kocher et al. (1989). On the other hand, primer T375R is part of Nakao's fragment (Nakao et al., 2000). A comparison of the T375R primer sequence as described by Kocher at al. (1989) and Nakao et al. (2000) revealed a nucleotide difference in the third and 27th bp.

Fig. 2.2. Sizes of the mitochondrial 12S rDNA fragments with the location of the T60F and T375R primers shown as published.
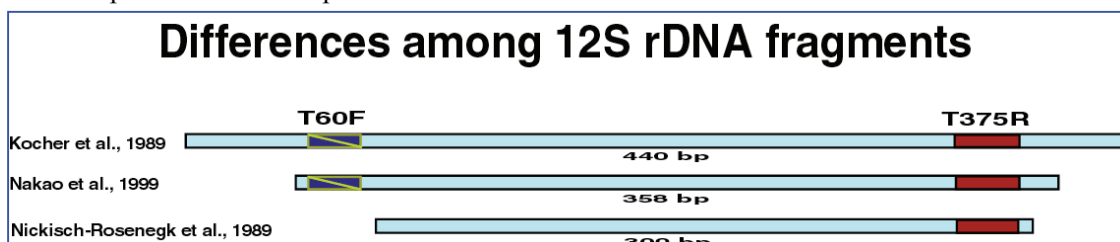
A new primer, ITM12SR (derived from T375R) was synthesized with the following sequence: 5'-**AATCGAGGGTGACGGGCGGTGTGTACA**-3'. The new combination of primers (T60F and ITM12SR) yields the same results as the original combination (T60F and T375R) retaining the second fragment, which is probably non-specific for all taeniids (results not shown).

When unpurified amplicons are used for RFLP, a very complex pattern emerges. Although it is possible to distinguish the taeniid species by their patterns (not shown), the process is unsatisfactory for routine use. Therefore, an investigation was undertaken to determine whether it would be possible to redesign 1 or both, of the PCR primers to eliminate the non-specific amplicons. A new primer, ITMTR2 (5'-**TGACGGGCGGTGTCTACATGAGTTA**-3') was developed using part of the ITM12SR primer sequence. Analysis of this primer by the Genbank program BLAST did not reveal homology to sequences of human or intestinal parasite origin. The amplification result of the mitochondrial 12s rRNA segment gave a clean single fragment of 360-bp (Fig. 2.3).

Fig. 2.3. PCR results corresponding to the different field samples (E1-22) as described in Table I. Amplification of the mitochondrial 12s rDNA segment from taeniids, using primers T60F and ITMTR2. Analysis is done on a 2% agarose gel and stained with ethidium bromide. Marker is a 100bp DNA ladder.
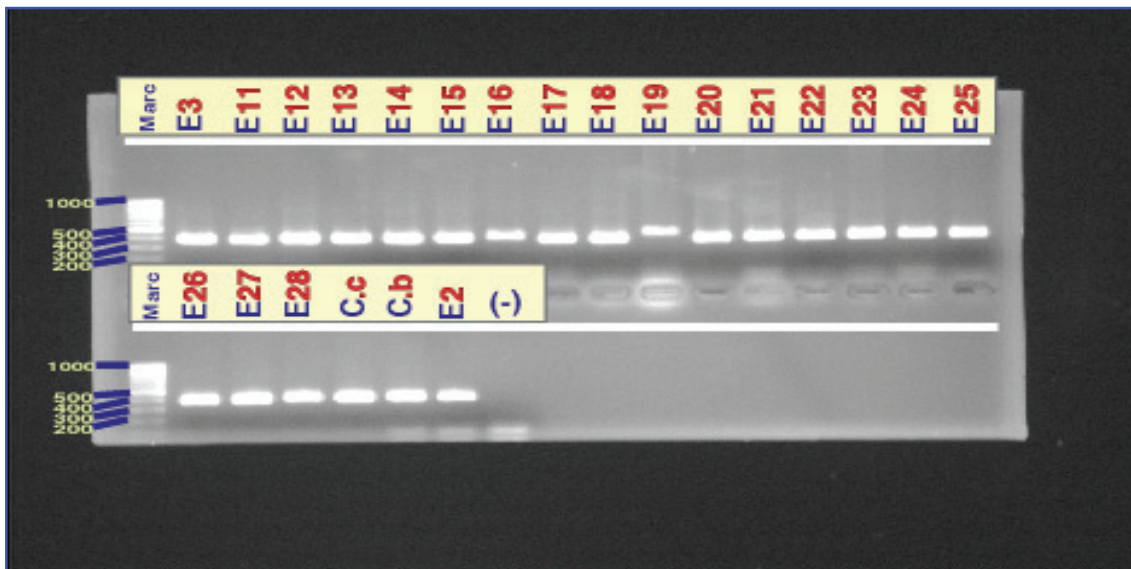
The RFLP results (Fig. 2.4) of this amplicon showed a clear arrangement of the fragments without confounding bands caused by the presence of non-specific segments. Nine samples (E3, E11, E12, E14, E16, E17, E18, E20 and CcE) corresponded to the

profile of *T. solium*, whereas the other 17 specimens plus the reference samples showed a profile typical for *T. saginata*. Minor differences in the low intensity band profiles around the 200bp mark were seen in samples E21 and E19 as compared with samples E13 and E15 (Fig. 2.4).

Fig. 2.4. Restriction results corresponding to the different field samples (E3; E11-21 and E2, E22-28 and ECc and ECb) as described in Table 1.1. *Dd*eI enzymatic restriction of the 12s rDNA segment amplified with the primers T60F and ITMTR2. High single bands (± 300 bp) correspond with *Taenia solium* profiles while the low double bands (± 150 bp are *Taenia saginata* profiles. Non-specific bands are absent. Analysis is done on a 10% poly-acrylamide gel and stained with a silverkit. Marker is a 100bp DNA ladder.
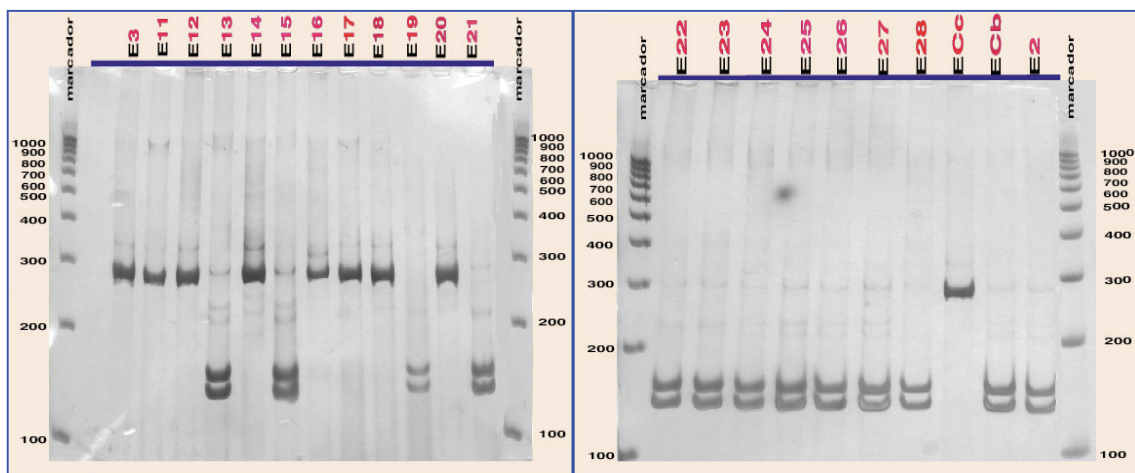
These results corresponded entirely with the GPI zymograms of the 25 specimens and with all the morphological criteria for all but specimen E18. GPI patterns showed the proximal band of *T. saginata* extracts in a more anodal position than that of *T. solium* extracts, as described by Le Riche and Sewell (1978).

As presented in table 2.2, sample E18 showed all the characteristics of *T. solium*, except for having a *T. saginata*-like unarmed scolex and an active migration (observation of at least 1 active migration of a gravid segment through the anal sphincter). Oncospheres of this tapeworm were orally administrated to two pigs, which proved to be infective, yielding cysticerci with armed scolices. GPI pattern of E18 corresponded with *T. solium*. As such, it was concluded that of the 25 specimens analyzed, 17 were identified as *T. saginata* and 8 samples were *T. solium* on the basis of their overall morphology, GPI-zymograms and PCR.

Table 2.2. Speciation of 25 specimens of *Taenia* spp. based on morphological features, i.e., absence or presence of rostellar hooks, maximum number of unilateral uterine branches for putative *T. solium*, minimum number for putative *T. saginata*, number of ovarian lobes and presence, or absence of a vaginal sphincter, and mode of migration of gravid segments.

Specification	Scolex	Uterine branches	Ovarian lobes	Vaginal sphincter	Migration	Code
T. saginata	NO*	22	NO	present	active	E1
T. saginata	NO	22	NO	NO	active	E2
T. saginata	NO	19	NO	NO	active	E4
T. saginata	NO	18	NO	NO	active	E5
T. saginata	NO	18	NO	NO	active	E7
T. saginata	NO	17	NO	present	active	E10
T. saginata	NO	17	2	present	active	E13
T. saginata	NO	18	NO	NO	active	E15
T. saginata	NO	17	2	present	active	E19
T. saginata	Unarmed	17	2	NO	active	E21
T. saginata	Unarmed	17	NO	NO	active	E22
T. saginata	NO	17	NO	NO	active	E23
T. saginata	NO	18	NO	NO	active	E24
T. saginata	NO	17	NO	NO	active	E25
T. saginata	NO	16	NO	NO	active	E26
T. saginata	NO	16	NO	NO	active	E27
T. saginata	NO	17	NO	NO	active	E28
T. sol/T. sag	Unarmed	10	NO	absent	active	E18
T. solium	NO	9	NO	NO	passive	E3
T. solium	NO	9	NO	NO	passive	E11
T. solium	NO	10	NO	NO	passive	E12
T. solium	Armed	9	3	absent	passive	E14
T. solium	NO	8	3	NO	passive	E16
T. solium	NO	9	3	NO	passive	E17
T. solium	Armed	10	2	absent	passive	E20

*NO = no observation possible (disintegration of material or scolices not recuperated).

2.4 DISCUSSION

In phylogenetic studies on several cestode species, Nickisch-Rosenegk et al. (1999) used universal primers T60F and T375R to analyze a 12s rRNA segment. These authors reported sequence differences between these 2 species, but did not aim at a practical application for differentiating at species level. In the present study, the same primers with the same PCR protocol were used and a differential diagnosis between the 2 taeniids could be made through RFLP. Amplification of this segment with T60F and T375R produced a fragment of about 360bp together with a weaker non-specific band of about 650bp (Fig. 2.1). A "Blast" search of the primers ITM12SR and T375R against all sequences in the Genbank database revealed the possibility of amplification of taeniid mitochondrial fragments and DNA from human mitochondria. Since adult

Taenia spp. live in the human intestine, host DNA contamination is a real possibility and could explain the non-specific amplification. Subsequent Blast searches permitted the design of primer ITMTR2, which has no homology with human DNA or any other mtDNA other than that of taeniid mitochondrial origin. The new primer pair T60F/ITMTR2 amplified a 360bp fragment without the occurrence of the non-specific band (Fig. 2.3). When analyzed using RFLP with D*de*I digestion, the pattern of fragments obtained was identical to that predicted by using GeneJockey II. PCR results correlated well with the morphological characteristics. One of the samples (E18) had morphological characteristics of both taeniids, i.e., an unarmed scolex but a typical *T. solium* reproductive system. However, the PCR result, GPI iso-enzyme pattern, and the experimental infection of a pig, were all conclusive for *T. solium*. Apparent size differences in the amplicons from samples E16 (*T. solium*) and E19 (*T. saginata*) and minor profile characteristics in *T. saginata* samples E21 and E19 could be due to strain differences. This possibility requires a considerable amount of further investigation and will be explored in future studies. The specificity of the primers described in this study enabled easy differentiation of adult *Taenia* spp. This test is faster, less complex and easier to interpret than the test described by Gonzalez et al. (2000), which is a method based on 3 primers in a multiplex-PCR. On the other hand, Mayta et al. (2000), using 3 restriction enzymes (A*lu*I, D*de*I, and M*bo*I), could clearly distinguish *T. solium* from *T. saginata*. These authors used primers to amplify a region of the 5.8s rDNA, corresponding to a fragment of approximately 1300bp. In the present study, the 5.8s rDNA segment was also amplified, using the same protocol and primers as Mayta et al. (2000). However, the lack of specificity of the primers, the low sensitivity of the test (about 80% of the samples showed positive amplification), the complexity of fragments in the RFLP, and the size of the segment amplified, are the main disadvantages of Mayta et al. (2000) in comparison with the method proposed in the present study.

In conclusion, the present work contradicts the general assumption that *T. saginata* is no longer present in Ecuador. In spite of being a notifiable disease in Ecuador, there are no official reports of bovine cysticercosis except for the documented evidence by Briones (1969). Consequently, due to the major importance of *T. solium*, all cases of taeniasis are assumed to be caused by the latter. The improved PCR-RFLP assay, amplifying a fragment of the mitochondrial 12s rRNA, clearly differentiated between *T. solium* and *T. saginata* and will be able to confirm in future studies the presence of

strains or new species of *Taenia* in Ecuador or Latin America. This is of major importance in epidemiological studies of human cysticercosis and will be invaluable in evaluating possible zoonotic implications. Differentiation based on morphological criteria may be the least expensive method, but is hindered by difficulties in the acquisition of suitable somata for clear diagnosis. As shown in table 2.2, the difficulty of recovering mature segments and scolices or the advanced disintegration did not often allow proper identification. As for the gravid proglottids, the number of uterine branches in *T. saginata* and *T. solium* seems not to overlap in Ecuador, a conclusion which should to be further substantiated by a higher number of samples. However, relying on a single morphological feature, e.g., the observation of a *Taenia* sp. without rostellar hooks, may lead to a false conclusion. This is evidenced by the fact that the carrier of E18, in spite of the *T. saginata*-like scolex, was nevertheless subjected to cysticercosis screening, a routine procedure for *T. solium*-carriers, resulting in a positive CT-scan for NC.

PCR-based differentiation is not difficult and has a high sensitivity and specificity. However, the high technological demands i.e. expensive infrastructure, and the price per sample is yet an obstacle preventing the general use of this method.

CHAPTER 3

IMPROVED PCR-RFLP ASSAY FOR THE DETECTION AND DIFFERENTIATION OF *TAENIA* SPP. IN FAECAL SAMPLES (PRELIMINARY OBSERVATIONS)

3.1 INTRODUCTION

Taenia saginata and *Taenia solium* are medically and economically important tapeworms, causing bovine and porcine cysticercosis respectively and taeniasis in man (WHO, 2002; Yamasaki et al., 2002). Early detection and identification of an adult *T. solium* infection is important in the control of neurocysticercosis, a major cause of epilepsy associated with considerable morbidity and mortality (Cruz et al., 1989; García et al., 2003c). *T. solium*-taeniasis remains often unnoticed, contrary to infection with *T. saginata*, where the active migration of gravid proglottids is more likely to alert the carrier (Acha and Szyfres, 2003).

Several methods for detection of *Taenia* spp. in faecal samples are available. Detection of copro-antigens by ELISA (Allan et al., 1990, 1996a) has a higher sensitivity (98%) than the visual detection of proglottids and oncosphere detection by coproscopic examination (Flisser et al., 1999). Although easy to perform, these methods do not allow differentiation between *T. solium* and *T. saginata*, unless followed by treatment, recovery and identification of the somata.

Besides morphological differentiation, several molecular techniques have been described for the differentiation of *Taenia* species on the somata, such as, multiplex-PCR and PCR-RFLP methods on gDNA or mtDNA (Chapman et al., 1995; Mayta et al., 2000; Gonzalez et al., 2000, 2002, 2004; Somers et al., 2006; Rodriguez-Hidalgo et al., 2002). These methods were shown to be highly sensitive and specific.

Recently, an immunodiagnostic technique was developed (Wilkins et al., 1999) to detect antibodies against adult *T. solium* in serum samples. This test, using a Western blot format, was reported to have a sensitivity of 95% and a specificity of 100%. No cross-reactions were found in samples from patients with *T. saginata* or other cestode infections. This method has great potential for assessing the epidemiological status in an endemic area, but needs validation under field conditions.

Molecular-based techniques on faecal extracts present another alternative for species-specific diagnosis of *Taenia* spp. (Nunes et al., 2003; Yamasaki et al., 2004; Nunes et al., 2005). These authors sampled different genomic loci in multiplex-PCR or PCR-RFLP assays and used commercial kits, i.e. DNAzol reagent and QIAgen

mini stool kit for DNA extraction. However, sensitivity of these assays was reported to be only 47.8% and 66.6% for the multiplex-PCR and the PCR-RFLP, respectively.

The objective of the present study was to develop a specific and highly sensitive molecular-based test for the diagnosis and differentiation of *Taenia* spp. in faecal samples. The primers described in chapter 2 were highly specific for use on *Taenia* spp. somata, however, when used on faecal extracts these gave rise to non-specific bands, complicating the interpretation of the PCR results. Therefore, a new primer pair had to be developed. In addition, alternative cheaper DNA extraction protocols were tested and compared with the QIAgen commercial kit.

3.2 MATERIALS AND METHODS

3.2.1 Development of a new *Taenia* sp. primer pair on the 12s rRNA gene

Sequencing of the total 12s rRNA from a *T. saginata* somata sample from Ecuador was done using TaenF primer (5'-**GTTTGCCACCTCGATGTTGACT**-3') on the mitochondrial LsRNA partial segment and ITMTnR primer (5'-**CTCAATAATAATCGAGGGTGACGG**-3') on the mitochondrial 12s rRNA partial segment, giving a 901bp fragment.

The complete 12s rRNA sequence of *T. saginata* was then aligned using the Clustal program and compared with the *T. solium* and *Taenia saginata asiatica* complete mitochondrial genome described by Nakao et al. (2003) (Genbank: AB086256) and Jeon et al. (2005) (Genbank: NC004826), respectively (Fig 3.1).

3.2.2 Primers

A new semi-nested primer set (nTAE, ITMF and ITMTnR) was designed by means of the following programs: Amplify (W.R. Engels, 1993) and Right Primer, version M1.25 (R. Isaac, Biodesk, 1994). The specificity of the primers was verified by blasting (Genbank http://www.ncbi.nlm.gov/) against all the DNA sequences present in the Genbank.

The primer pair, nTAE/ITMTnR was used in the first round and ITMF/ITMTnR or T60F/ITMTr2 in the semi-nested PCR. Sensitivity and specificity was compared with the primer pair T60F and ITMTr2 (see Chapter 2). Primers nTAE and ITMTnR

were biotinylated for use in the capture sequence method on faeces as described by Mangiapan et al. (1996).

3.2.3   DNA extraction

Three different DNA extraction methods for faeces were used in a comparative study; a commercial kit (QIAamp$^®$ DNA stool minikit, QIAgen-Germany), the extraction method developed by Boom et al. (1990), adapted by McOrist et al. (2002) and further modified in the present study and the technique described by Mangiapan et al. (1996), modified by Vansnick (2004). A brief description of the extraction protocols is given below:

a. QIAgen Kit

Approximately 180-220mg faeces were suspended in 360-440µl PBS plus 2% v/v polyvinyl polypyrrolidone (PvPP) for 10 minutes at 100°C and then centrifuged at 12000g for 3 minutes. The supernatant was discarded and the pellet re-suspended in 1ml Milli-Q water (Millipore, Belgium) then centrifuged at 12000g for 3 minutes. The pellet was finally lysed with a buffer provided in the kit (QIAamp$^®$ DNA stool minikit, QIAgen, Germany) according to the manufacturer's instructions.

b. Modified extraction method (Boom et al., 1990)

Between 100 and 300mg faeces were dissolved in 200-600µl PBS plus 2% v/v PvPP and boiled for 10 minutes at 100°C. Samples were centrifuged at 12000g for 3 minutes, the pellet was re-suspended in 500µl lysis buffer (60mM Tris-HCl, pH7.4, 60mM EDTA, 10% v/v Tween, 1% v/v Triton X-100, and 1.6M Gu-HCl), and 40µl proteinase K. Samples were incubated overnight in a Thermomixer compact (Eppendorf, Koln, Germany) at 50°C and shaken at 1400rpm. Then, 500µl zirconium beads (Merlin Diagnostics, cat#. 11079105z; diameter 0.5mm) were added and incubated for one hour at ambient temperature. The pellet was washed three times in 5.5M guanidinium thyocyanate in 50mM Tris buffer (pH6.4); twice in 70% ethanol and once in acetone and thereafter dried at 60°C for 15 minutes. DNA was then eluted from the zirconium beads by incubating the beads in 100µl TE buffer at 48°C for 20 minutes.

c. Technique of Mangiapan et al. (1996), modified by Vansnick (2004)

This technique is based on the specific capture of *Taenia* spp. DNA in faeces samples by the sequential addition of biotinylated oligonucleotides and avidin-coupled magnetic beads.

One gram of faeces was homogenised in 2ml PBS plus 2% v/v PvPP in a 60 ml Falcon tube. Samples were heated at 100°C for 10 minutes then centrifuged at 1200g for 30 minutes. The pellet was re-suspended in 2% v/v Tween20 solution in Milli-Q water (1ml per 100mg faeces) and approximately 8 glass beads (diameter 2mm) were added. Thereafter the samples were shaken on a vortex in a 1.5ml Eppendorf tube and the homogenised suspension left to settle for 30 minutes. The supernatant was then centrifuged again for 30 minutes at 1500g. The pellet was re-suspended in 1ml Milli-Q water and transferred to a new 1.5ml Eppendorf and centrifuged for 5 minutes at 12000g. The samples digestion process and capture of the sequences were performed according to Mangiapan at al. (1996), slightly modified by Vansnick (2004) in which biotinylated oligonucleotides were hybridised to *Taenia* DNA and then, were caught with magnetic beads. In this study additional modifications to the protocol consisted in the application of different oligonucleotides, different hybridisation temperature and a transfer to a clean tube during the washing process to solve the problem of faeces debris adhering to the tube wall.

3.2.4 Polymerase Chain Reaction

PCR was performed in a total volume of 25μl containing 5μl faecal extract as template and 20μl PCR mix containing 1μl Yellow sub™ (GENEO Bioproducts, Germany); 3.3μl Milli-Q water; 12.5μl buffer (20mM Tris-HCl pH8.4; 100mM KCl; 0.2% v/v triton X-100; 1.5mM MgCl$_2$); 100pmol/μl of a mix of the 4 deoxynucleotide triphosphates (dNTP, final concentration: 0.2mM); 25pmol/μl of each primer and 0.4U *Taq*-polymerase Silverstar (Eurogentec, Seraing, Belgium). For the semi-nested PCR, a final volume of 25μl was used i.e. 0.5μl from the first PCR round plus 24.5μl PCR mix, as described above yet with (0.3U) *Taq*-polymerase. E2 DNA extracted from somata was used a positive control.

The first PCR round and the semi-nested PCR were performed in a PTC-100 Tetrad Thermal cycler (MJ Research, U.S.) The first PCR round (nTAE/ITMTnR

primers) consisted of an initial denaturation step at 94°C for 4 minutes, followed by 40 cycles consisting of 94°C for 45 seconds, 56°C for 4 seconds and 72°C for 45 seconds. The semi-nested PCR (with ITMF/ITMTnR primers) consisted of an initial denaturation step at 94°C for 4 minutes, followed by 25 cycles of 94°C for 45 seconds, 56°C for 45 seconds and 72°C for 45 seconds.

Five microlitres of each amplified product together with a gene Ruler™ marker of 100bp (MBI Fermentas, GmbH, St. Leon-Rot, Germany) was separated by electrophoresis, using a Mupid-21 system (Eurogentec, Seraing, Belgium) in 2% w/v agarose and 0.04M Tris-acetate plus 0.002 M EDTA buffer for 20min at 100volt. The gel was stained with ethidium bromide (Sigma-Aldrich) for 30minutes and the DNA products visualised by ultraviolet light.

3.2.5 Restricted Fragment Length Polymorphism

RFLP was carried out as described in chapter 2 using the restriction enzyme *Dde*I.

3.2.6 Experimental design

In a first experiment, faecal samples from 35 *Taenia* carriers (including two carriers with a combined infection of *Taenia* spp. and *Hymenolepis nana*) and five negative controls were used for developing a PCR on faecal extracts. These tapeworm carriers had been identified by microscopic examination of stool samples after concentration by the Ritchie's formaldehyde-ether method (Ritchie, 1948). They originated from different regions in Ecuador (Highlands: Quito, Imbabura, Carchi, Tungurahua and Loja; Costal region: Manabí and Guayas) (table 3.1). The five negative control faecal samples were collected from non-infected healthy individuals from Belgium. The samples from Ecuador were collected after administration of a dose of laxative [bisacodyl 2.5mg (Dulcolax®, Boehringer Ingelheim-Germany)], the controls did not receive a laxative treatment before collecting the stools. All faecal samples were immediately stored at 4ºC either as such or in an equal volume of ethanol 70%. The samples were transferred in a freezer upon arrival in Quito. A commercial kit (QIAamp® DNA Stool Mini Kit) was used as the standard protocol for DNA extraction. All tapeworm carriers, as detected by microscopy, were treated with a single dose of praziquantel (10mg/kg b.w.), followed by the administration of a

laxative. Where possible, the expelled adult parasites were collected and species-identified using morphology and PCR-RFLP as described in chapter 2. DNA-extracts from *Taenia* somata (*T. saginata* and *T. solium*), identified by morphology and GPI pattern, collected in Ecuador were used as positive controls in the PCRs.

Table 3.1. Origin and dates of collection of faecal samples from Ecuadorian *Taenia* spp. carriers (detected by microscopic examination)

Code	Origin	Taenia Collection	Code	Origin	Taenia Collection
E13	Manabi	14/02/2001	E47	Loja	6/08/2002
E14	Imbabura	15/02/2001	E49	Loja	18/11/2002
E16	Imbabura	15/03/2001	E55	Pichincha	17/10/2002
E29	Pichincha	27/11/ 2001	E56	Loja	21/12/2002
E30	Loja	22/10/2001	E57	Loja	21/12/2002
E32	Loja	16/02/2002	E59	Loja	16/02/2003
E33	Loja	17/02/2002	E61	Loja	31/03/2003
E34	Loja	17/02/2002	E66	Loja	27/04/2003
E35	Loja	17/02/2002	E68	Loja	27/04/2003
E36	Loja	17/02/2002	E71	Loja	29/06/2003
E37	Loja	17/02/2002	E72	Loja	1/07/2003
E38	Loja	17/02/ 2002	E73	Loja	1/07/2003
E40	Guayas	20/04/2002	E77	Loja	1/07/2003
E41	Guayas	23/05/2002	E78	Loja	1/07/2003
E42	Tungurahua	13/06/2002	E81	Loja	2/07/2003
E44	Loja	06/08/ 2002	E83	Pichincha	26/11/2003
E45	Loja	06/08/ 2002	E84	Loja	13/12/2003
E46	Loja	06/08/2002			

In a second experiment, the same positive Ecuadorian faecal samples and the five negative faecal samples were subjected to two other DNA extraction methods before being analysed by the newly developed PCR (see 3.2.3). The results were compared with those obtained using the commercial DNA extraction kit (QIAgen).

In a third experiment, 53 faecal samples, negative for *Taenia* eggs on microscopic examination, were analysed to assess the specificity of the PCR-RFLP on Mangiapan DNA extractions. These samples were collected from healthy non-infected individuals in Ecuador (N=14) and Belgium (N=39). Only the samples from Ecuador were taken following laxative treatment. While the Ecuadorian negative samples were stored in a similar way as the positive samples (first experiment), for the samples from Belgium no specific protocol for storage was followed as these samples were sent to us by a commercial clinical laboratory for routine parasitological diagnosis.

Fig. 3.1. Multiple sequence alignment of *T. solium*, *T. s. asiatica* and *T. saginata* mDNA fragments. *T. saginata* specific-901bp was amplified using TaenF/ITMTnR primers. The primers used in this study are marked in bold and the end of the 12s rRNA fragments are indicating by arrows respectively. nTAE and ITMF primers are developed during this study. The 3rd base (▼) of ITMTr2 described in chapter 2 shows a difference. T60F (TTAAGATATATGTGGGTACAGGATTAGATACC) and IIMF primers are conserved in the last 13bp, shown by the bp in bold. Stars indicate consensus sequences, gaps indicate differences, - are deletions.

```
CLUSTAL W (1.82) multiple sequence alignment


                                    TaenF                                                        nTAE
T.saginata    GTTTGCCACCTCGATGTTGACTTAGATTAAAGCTTGGGTGCGTAGTCTGAGTTGAGTTGAGTTGAGCCAGGTCGGGTTC    120
T.s.asiatica  GTTTGCCACCTCGATGTTGACTTAGATTAAAGCTTGGGTGCGTAGTCTGAGTTGAGTTGAGTTGAGCCAGGTGGGTTC     120
T.solium      GTTTGCCACCTCGATGTTGACTTAGTTAGATTAAAGCTTGGGTGCGTAGTCTGAGTTGAGTTGTGAGCCAGGTCGGGTTC   120
              ************************ *** ** ***************************       ** ******* ****

                                                                                 LsrRNA
T.saginata    TTATCTATTGTAGAAGTTTATCAGTAACGAAAAGAGATAAGCTTTTATCAGTAGAAGTTTATCAATAATTCCATGTTTTTATTAG   240
T.s.asiatica  TTATCTATTGTAGAAGTTTATCAGTAACGAAAAGAGATAAGCTTTTATCAGTAGAAGTTTATCAATAATTCCATGTTTTTA-ATTAG  240
T.solium      TTATCTATTGTAGAAGTTTATCAGTACGAAAAGGATAAGCTTTTATCAGTAGAAGTTTATCAATAATTCCATGTTTTTTA-ATTAG   239
              ********************************  *********

T.saginata    TTGTTTAACTGTGGCAAAAGAAGAAGTTTGTGTTTCATTAACTGCATAAAAAATTAGTTTTTTTAATTTATTGTGTTTAAAACTAGA   359
T.s.asiatica  TTGTTTAACTGTGGCAAAAGAAGAAGTTTGTGTTTCATTAACTGCATAAAAAATTAGTTTTTTTAATTTATTGTGTTTAAAACTAGA   360
T.solium      TTGTTTAACTGTGGCAAAAGAAGAAGTTTGTGTTTCATTAACTGCATAAAAAATTAGTTTTTTTAATTTATTGTGTTTAAAATCTAGA  357
              ************************** ***********

T.saginata    GTTTTAAAAGGGTGAAACATTAGAAGGGGGATAGGACACAGAGCCAGCAGCAATCTGCGGTTAAACTCTGTGTTTTTCTTTG...     479
T.s.asiatica  GTTTTAAAAGGG-GAAACATTAAGAGGGGGATAGGACACAGAGCCAGCAGCAATCTGCGGTTAAACTCTGTGTTTTTCTTTG...     479
T.solium      TTTTTAGAAGAATGAGACATTAGAAGGGGGATAGGACACACTGCCAGCAATCTGCGGTTAAACTCTGTTTTTCTTTG...          477
              **  *** ***           ** ***************

                                                              T60F              ITMF
T.saginata    TTTTTTTATTTAAGTTTTTCATTTTTTTATAAATTTATATTGTGTGACAGGGATTAGATACCCCATTA...TGTTAGTAATAATAG   599
T.s.asiatica  TTTTTTTATTTAAGTTTTTCATTTTTTTATAAATTTATATTGTGTGACAGGGATTAGATACCCCATTA...TGTTAGTAATAATAG   599
T.solium      TTTTTTATTTAAGTTTTTCACTTTTTTTATGTTTTGTGTGTGACAGGGATTAGATACCCCATTAATG...                    597
              ******  *********** ********

                                                                              ITMTr2
T.saginata    GTAGGTTTTACTAAGCTAAGTCTATGTGTCGCTATTATAAAAGTATACACACGCCCGTCACCCTCGATTATTAATTCGAG          837
T.s.asiatica  GTAGGTTTTAGTTAGTTAAGTCTATGTGTCGCTATTATAAAAGTATACACACGCCCGTCACCCTCGATTATTAATTCGAG          835
T.solium      GTAG-TGTTAGTTAAGCCAAGTCTATGTGCTTGCTACTTTGATAAAGTTTATTAAGGACTTAAAAGTAAAGTTAAAT...           832
              ****

                                                      ▼   ITMTnR
T.saginata    TTTTGTTAAATGTGAAGTAGTAGTTTAGTCATGTTACACACGCCCGTCACCCTCGATTATTAATTCGAG                     901
T.s.asiatica  TTTTGTTAAATGTGAAATAAGTTTAGTCATGTTACACACGCCCGTCACCCTCGATTATTAATTCGAG                       899
T.solium      TTTGTTGGGTGTGAAATAAGTTTAGTCATGTTACACACGCCCGTCACCCTCGATTATTAATTCGAG                        896
              ******  *** ********
```

3.2.7   Ethical clearance

The protocol of this survey was approved by the Ethical Committee of the Central University of Ecuador. Samples from Ecuador and Belgium were obtained after informed consent. The Belgian samples used in the third experiment were obtained from a commercial clinical laboratory, upon request for parasitological examination.

3.3 RESULTS

3.3.1   Primers

Alignment of the newly 901bp sequenced fragment of *T. saginata* somata with the somata of *T. solium* (896bp) and *T. s. asiatica* (899bp), described by Nakao et al. (2003), and Jeon et al. (2005) was used to design new specific primers for use on faecal samples (Fig. 3.1).

Previous primers (T60F and ITMTr2) described in chapter 2 produced non-specific bands on QIAgen extracted faecal samples (data not shown) hence new primers were developed. The new primers (Fig. 3.2) were analysed using the BLAST program (NCBI) and did not reveal any homology to sequences of human or parasite origin, showing a good specificity potential.

Fig. 3.2. Primer sequences developed in this study to amplify *Taenia* spp. DNA present in human faeces

ITMTnR	5'-**CTCAATAATAATCGAGGGTGACGG**-3'
ITMF	5'-**TGTGACAGGGATTAGATACCCCATT**-3'
nTAE	5'-**CGTGAGCCAGGTCGGTTCTTAT** -3'

The amplification results of QIAgen extracted faecal samples with these new primers, compared to the previous primers described in chapter 2 showed a reduction of the non-specific bands in both positive and negative DNA extracts (data not shown) due to improved homology in the primers' sequences. The primers amplified equally well DNA extracted from *T. saginata* and metacestodes of *T. solium*, used as controls.

3.3.2   PCR on faecal extracts

The modifications in the hybridisation temperatures in the Mangiapan technique are shown in Fig 3.3. The best results were observed when both digestion and hybridisation temperatures were set at 50°C

Fig. 3.3. Positive (upper row) and negative faecal samples (lower row) extracted with the Mangiapan protocol. Digestion was compared at temperatures of 50°C and 60°C while, hybridisation was compared at temperatures of 50, 55 and 60°C.
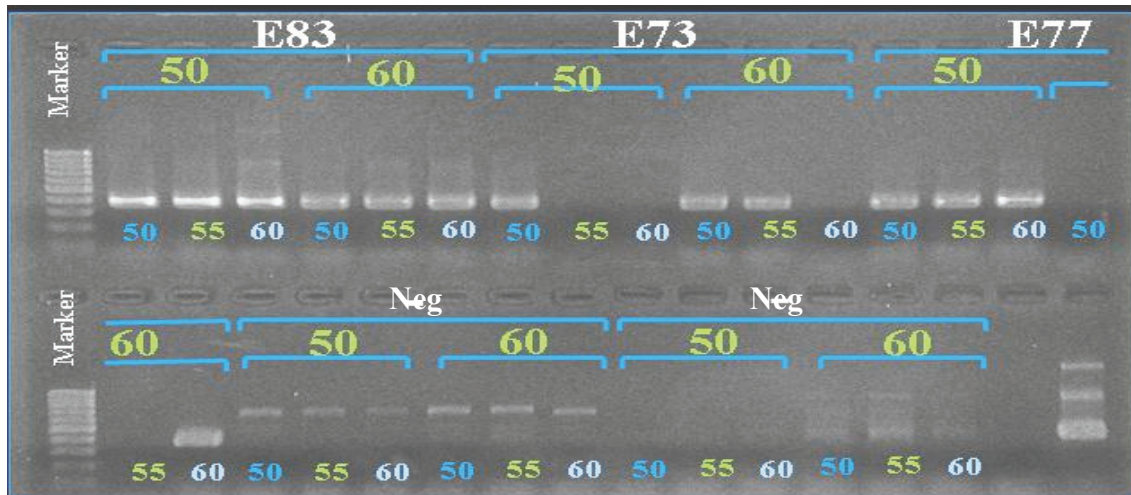
Using the newly developed primer pairs in the semi-nested PCR on faecal extracts of 35 *Taenia*-carriers, extracted with the QIAgen method, a clear single band of around

Fig. 3.4. Experiment 1: PCR results on faecal samples from a selection of Ecuadorian *Taenia* carriers following DNA extraction with the QIAgen kit. The mitochondrial 12s rRNA segment from taeniids in faeces was amplified, using primers ITMTnR/nTAE (first round) and ITMTnR/ITMF (semi-nested). Analysis is performed in a 2% agarose gel and stained with ethidium bromide. The marker is a 100-bp DNA ladder. Positive samples show bands at ± 370bp.
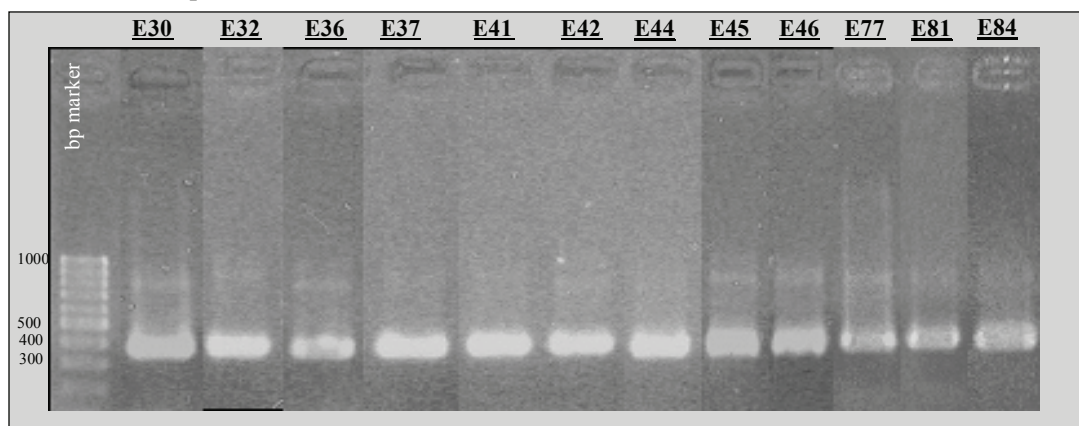
370bp (371bp for *T. solium* and 374bp for *T. saginata*) was found in all samples (experiment 1). No non-specific bands were observed on this group of samples (Fig.3.4). None of the 5 negative control samples gave a band of around 370bp.

Results of the amplification of the DNA extracts from a selection of the 35 cestode-positive samples and of the 5 negative samples, obtained by the 3 different DNA extraction methods are shown in Fig. 3.5 (experiment 2).

Fig. 3.5. Experiment 2: Copro- PCR results on a selection of Ecuadorian faecal samples from *Taenia* carriers and on a Belgian negative control, following different DNA extraction methods i.e. (A) QIAgen, (B) Boom and (C) Mangiapan assay. The primers ITMTnR/nTAE (first round) and ITMTnR/ITMF (semi-nested) were used to amplify the mitochondrial 12s rRNA segment from taeniids in faeces, using. Analysis is performed in a 2% agarose gel and stained with ethidium bromide. The marker is a 100-bp DNA ladder. Positive samples show bands at ± 370bp.
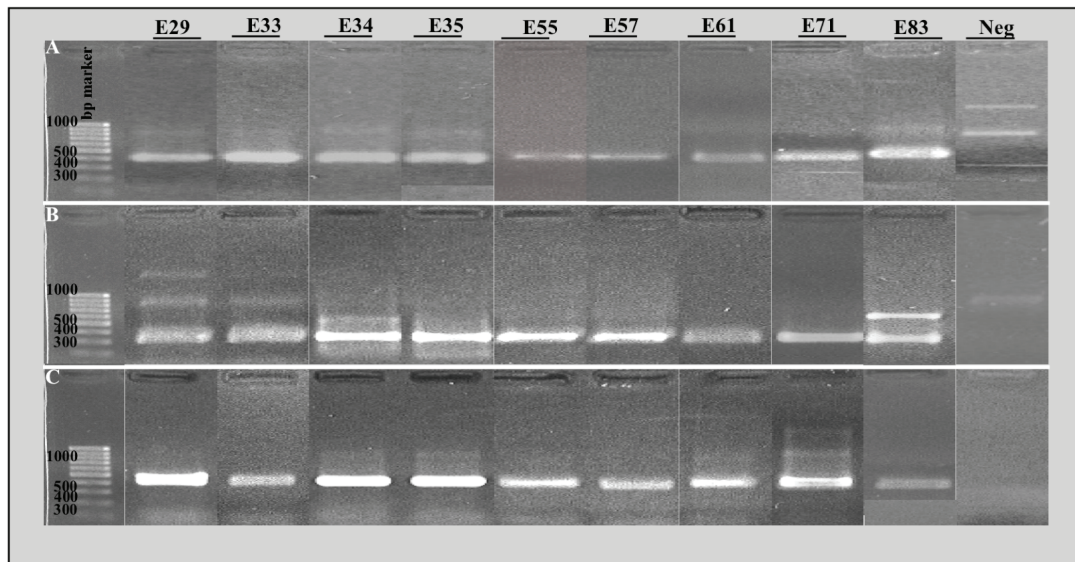
All samples from *Taenia* carriers showed a band of 370bp, irrespective of the extraction method. However, contrary to the Mangiapan extraction, non-specific bands were seen in the PCR processed on QIAgen and Boom extracts (Fig 3.5: samples E29 and E83). Non-specific bands were also observed on the QIAgen and Boom extracts of the negative samples, yet the absence of the *Taenia*-specific band of 370bp in these samples allowed to classify them as negative results (Fig 3.5).

The RFLP results of QIAgen extracted faecal samples (Fig. 3.6) showed a clear distinction between *T. solium, T. saginata* and *H. nana* profiles. The D*de*I enzyme profile of *T. solium* DNA (371bp) consisted of bands of 325bp and 46bp; restriction of *T. saginata* DNA (374bp) with D*de*I gave 4 bands: two bands of 165bp and 163bp and

two bands of 25bp and 21bp. The small band differences could not be differentiated with the resolution of a 10% acrylamide gel, appearing as two singles bands following silver staining. *H. nana* DNA did not give a restriction profile as it was not cut by the enzyme (Fig. 3.6). The *H. nana* profile was confirmed by digestion and DNA extraction of somata and subsequent PCR-RFLP analysis. Cloning and sequencing was obtained and 100% homology with a partial *H. nana* 12s rRNA fragment (Genbank: AB031361.1) was found by blasting the sequence against all Genbank sequences.

Fig. 3.6. D*de*I enzymatic restriction of the 12s rRNA segment. Restriction results positive for *T. solium* (E14, E71, E84, E56, E57 and E59) and *T. saginata* (E13, E16 and E30) and the carriers with both *T. solium* and *H. nana* (E61 and E36) as described in table 1. High single bands (±325bp) correspond with *T. solium* profiles, whereas the lower two bands (±160bp) are *T. saginata* profiles. *H. nana* is not restricted (±370bp). Analysis is performed using 10% polyacrylamide gel and stained with a Silver staining kit. The marker is a 100-bp DNA ladder.
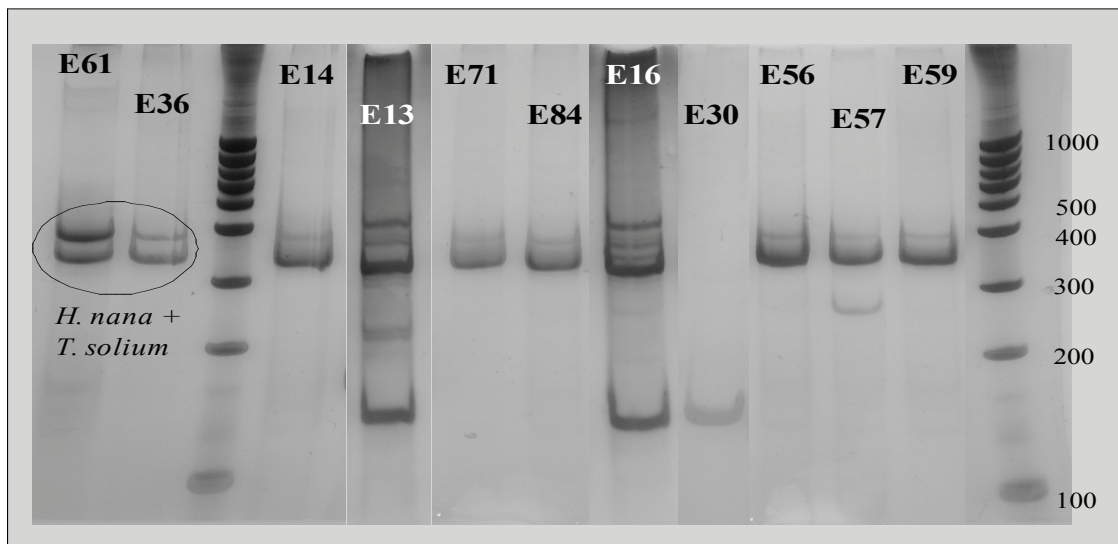
Following praziquantel treatment, taeniid cestodes were recovered from all tapeworm carriers (N=35). *H. nana* (E36 and E61) could not be recovered after praziquantel treatment. The PCR-RFLP results on these 35 somata showed total agreement with the copro-PCR results (data not shown)

Among the 35 faecal samples collected from tapeworm carriers, 27 samples gave a *T. solium* pattern in the RFLP, and 8 were positive for *T. saginata*. Two samples (E36 and E61) showed bands at 370bp, 325bp and 46bp (Fig 3.6, E36 and E61), suggesting a mixed *T. solium*, *H. nana* infection. In these samples oncospheres of *Taenia* spp and *H. nana* were found on coprological examination.

The 53 faecal samples that were negative for *Taenia* eggs on microscopic examination were used to determine the specificity of the new primer pairs on Mangiapan DNA extraction (third experiment). None of the samples gave a *Taenia* positive band of approximately 370bp in the PCR using primers ITMTnR/nTAE (first round) and ITMTnR/ITMF (semi-nested). However, while no non-specific bands were found in the 14 negative samples originating from Ecuador (data not shown), non-specific bands appeared in the 39 negative samples from Belgium, complicating the interpretation of the PCR results (Fig. 3.7).

Fig. 3.7. PCR on *Taenia* negative samples from Belgium extracted with the Mangiapan method. Samples showed many non-specific bands, which complicated the interpretation. On microscopic examination, no *Taenia* eggs were found in these samples.
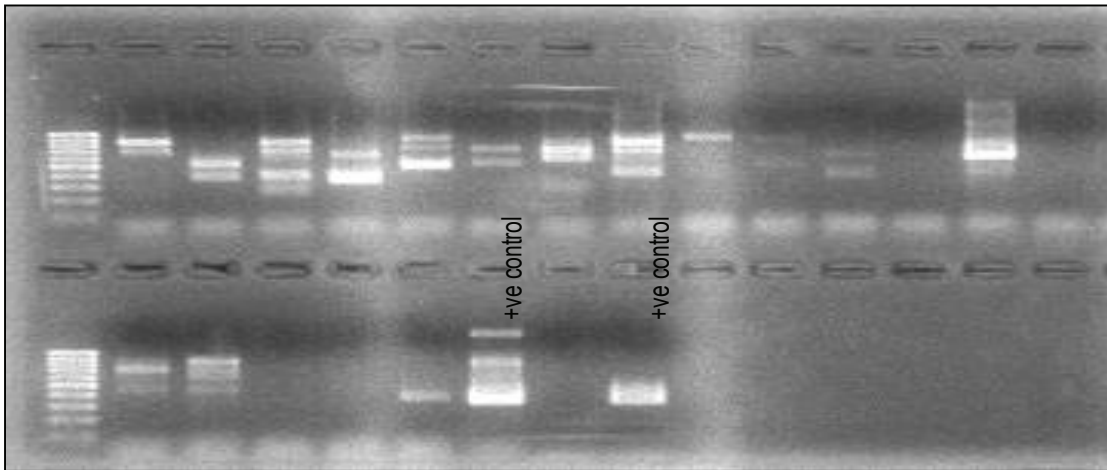
3.4 DISCUSSION

The development of highly specific and sensitive primers was based on the results of a clustal alignment of the newly obtained sequence of the 12s rRNA *T. saginata* fragment with the complete mtDNA of *T. solium* and *T. s. asiatica*. The primers successfully amplified DNA of most important cestode species of which man is the natural host i.e. *T. solium, T. saginata, T. s. asiatica* and *H. nana*. According to the *Hymenolepis diminuta* sequences of the Genbank, our primers will not amplify this sequence. In contrast to *H. nana*, *H. diminuta* is a very rare parasite of man.

The results of the newly developed PCR-RFLP method applied on the faecal extracts totally agreed with that of the extracts of the expelled somata, recovered after treatment. The copro-PCR followed by RFLP allowed a species-specific diagnosis on all stool samples taken from the 35 tapeworm carriers. In addition, this technique

demonstrated that two carriers (E36 and E61) had mixed infections with *T. solium* and *H. nana.* The presence of *H. nana* infections, a common parasite in Ecuador, is not a confounding factor in the detection of *T. solium* or *T. saginata* since the 370bp PCR fragment of *H. nana* is not restricted by the D*de*I enzyme in this PCR-RFLP assay.

Several methods have been developed for the extraction of bacterial, viral or protozoon DNA from faeces (Walsh et al., 1991; Boom et al., 1990; Dinkel et al., 1998; McOrist et al., 2002; Nunes et al., 2005). McOrist et al. (2002) compared five faecal extraction methods for the detection of bacterial DNA and concluded that the QIAamp$^{®}$ DNA stool mini kit (QIAgen, Germany) and the method described by Boom et al. (1990), gave the best results. Therefore, these two methods together with the Mangiapan technique (Vansnick, 2004) were compared in this study. Although the QIAgen kit is an excellent, easy and fast assay to extract DNA from faeces, it is far more expensive than the Boom and Mangiapan methods, The modified Mangiapan test has proven to be a valuable alternative to the QIAgen method with the added advantage of being about 50 times cheaper (Vansnick, 2004). On the other hand it is much more time consuming: i.e. extraction of a sample requires 24 hours for Mangiapan and 15 hours for Boom, whereas QIAgen extraction takes only around 50 minutes.

DNA extraction and amplification from faecal material often give rise to non-specific bands and this was also observed in negative and some positive samples in our study. Among the causes for these non-specific reactions, the complex nature of the different sources of DNA and the presence of PCR inhibitors in the faeces such as, haemoglobin, bilirubin, bile salts, chelating agents and organic components of soil were mentioned (Widjojoatmodjo et al., 1992; Shames et al., 1995; Vansnick, 2004). Moreover, the success rate of faecal DNA amplification varies considerably among species and individuals (Goossens et al., 2000), although the reasons for such differences are unclear (Murphy et al., 2003). In our study, the DNA extraction methods and the origin of the stool samples apparently also played a role.

A few non-specific bands were observed in the PCR's on faecal extracts prepared by the QIAgen and Boom methods from tapeworm carriers, while no non-specific bands were shown following Mangiapan extraction. More problems were encountered when processing faeces from negative controls, especially on those samples collected in Belgium. Here, non-specific bands were observed following the three extraction

methods. The reason for this is not clear but differences in the sampling method – all faecal samples in Ecuador were taken following laxative administration while this was not so for Belgian samples – and/or in sample storage methods may have caused these differences (Murphy et al., 2003; Nsubuga et al., 2004). While in Ecuador samples were immediately cooled following collection, this may not have been the case in Belgium. Although preservation methods were reported to have little effect on the success or failure of PCR of mtDNA (Frantzen et al., 1998), the incubation, refrigeration or freezing of stool samples can affect the DNA recovery (Paglia and Vizca, 2004). Unpublished results of copro-PCR on stool samples from Africa showed the presence of many non-specific bands in positive as well as in negative samples, processed with QIAgen or Mangiapan methods, complicating the interpretation after PCR (D. Geysen, Pers com., 2006). It cannot be excluded that the lower hybridisation temperature used in the Mangiapan extraction method in our study affected the specificity of the PCR.

In previous molecular studies on faecal extracts, description of non-specific bands was not always a recurring feature (Yamasaki et al., 2004; Nunes et al., 2003, 2005, 2006). This may reflect the unstable nature of such non-specific bands.

We can conclude that the new PCR-RFLP assay has potential for use as a research tool in the analysis of faecal samples for specific diagnosis of *Taenia* spp. However, the appearance of non-specific bands in some samples has shown that the protocol needs to be improved. Therefore, a step-wise analysis of the protocol should be performed, thereby assessing the sample storage methods, optimizing the times and temperatures of the extractions methods and PCR protocols; alternatively, the development of more specific oligonucleotides should be envisaged. Finally, performing PCR-RFLP for routine diagnosis in developing countries remains difficult, because of high demands on laboratory equipment and infrastructure, and high costs; though, it can be used to differentiate *Taenia* spp. from samples found to be positive by conventional methods.

CHAPTER 4

Taeniasis-cysticercosis in man and animals in the Sierra of Northern Ecuador

4.1 INTRODUCTION

Taenia saginata is one of the tapeworms of the small intestine of man, its metacestode, commonly known as *Cysticercus bovis*, is specific for cattle. Distribution is almost cosmopolitan but where *Taenia solium* is equally present, the importance of *T. saginata* is often overshadowed by the latter, which is of greater public health importance.

Reports on adult *Taenia* in Ecuador are given by Lopez (1969) i.e. 0.66% without indication of the species but later interpreted as *T. saginata* (Pawlowski and Schultz, 1972). The prevalence of *T. solium* was reported by Jimenez (1976) and Cruz et al. (1989) respectively, 1.02% and 1.6%.

T. solium cysticercosis has been reported both in humans i.e. 14.4% in the northern Sierra (Cruz, 1996) and in pigs in the southern Sierra of Ecuador i.e. 5.9 % by Jiménez (1976) and 12% by Benitez-Ortiz (1995).

There are no published reports on the presence of *T. saginata* metacestodes in Ecuador, the only documented evidence can be found in final year dissertations and is limited to slaughterhouse observations in the coastal region of the country. As such, infection levels, observed by post mortem inspection varied from 1.89% to 0.04% (Briones, 1969; Aragundi, 1969; Intriago, 1976; Sosa, 1979; Aragundi, 1999). There is no evidence of studies on bovine cysticercosis in the Andean Sierra or in the Amazon region of the country.

The objective of the present study is to evaluate whether the presence of *T. saginata* should be taken into account in epidemiological studies of *T. solium*-cysticercosis. Therefore, studies on cysticercosis in humans, pigs and bovines and on intestinal *Taenia* in the same region of the northern Andes of Ecuador have been conducted.

4.2 MATERIALS AND METHODS

The observations were made in two provinces of the Andean Sierra: Pichincha province, with the capital Quito and the neighbouring province to the north, Imbabura province with Ibarra as the capital.

4.2.1 Bovines

Surveys were organised in two slaughterhouses: in the "Camal Municipal de Rastro de Ibarra", Imbabura Province and in the "Camal Metropolitano de Quito" Pichincha Province. The slaughterhouse of Ibarra was visited 20 times between December 2000 and January 2001; a total of 374 carcasses were inspected and 397 blood samples collected from bovines of different age, sex and breed. The limited number of animals slaughtered per day allowed for a close inspection of the so-called predilection sites: masseters, myocard, oesophagus and diaphragm, which are usually not subjected to regular inspection in this slaughterhouse. When an animal was found positive, the complete carcass and all the organs were submitted to a more thorough inspection.

The slaughterhouse of Quito was visited four times during February and March 2001. A total of 472 blood samples were collected and 432 carcasses inspected. In this slaughterhouse the workload is heavier, yet inspection of the predilection sites is supposed to be part of the routine. Here again, positive cases were subjected to a thorough inspection.

4.2.2 Pigs

During 1998, on a total of 8,154 carcasses, no infected carcasses were officially reported in the metropolitan slaughterhouse in Quito. This is the main slaughterhouse for pigs in the Sierra, animals from various regions are presented. For this apparent lack of detection, it was decided to concentrate the efforts on Imbabura province.

Two surveys were held in a slaughterhouse in Ibarra, where only local pigs are slaughtered. During the first survey, between February and April, 1998, 1,101 porcine carcasses were inspected by means of a single incision in the *Musculus masseter, Musculus biceps brachii, Musculus trapezius cervicis, Musculus glutaeus superficialis,* and by superficial inspection of the tongue, heart and diaphragm muscles. In addition, blood samples were randomly taken from 591 of these pigs. In a second survey from February until April 1999, 1,795 pigs were sampled. Every carcass that was found positive for cysticercosis by routine meat inspection was subjected to a more profound examination, as agreed by the local authorities, which consisted in the inspection of the brains and a total larval count of the tongue, heart and diafragma. It was calculated that the remaining muscle mass represented on average 47% of the carcass weight. The

larval burden of that mass was estimated by a larval count in 100g of each of the following muscle-groups: *M. masseter, M. biceps brachii, Musculus longissimus dorsi, Musculus psoas minor and M. glutaeus superficialis*. Thus, on the basis of the larval counts in the 500g muscle samples (lg), the carcass weight (w) plus the sum of the larvae in the brains (lb), tongue, heart and diaphragm (lc), the total larval load could be estimated or total larval load = lg*2*(w/100*47)+lb+lc. Organs, viscera and the inner surface of the carcass were macroscopically inspected for other platyhelminthes.

4.2.3 Humans

4.2.3.1 Human cysticercosis

Between October 1997 and May 2002, 2,368 blood samples were collected from inhabitants of Pichincha province, visiting a health centre and 1,938 blood samples from inhabitants of Imbabura province, consisting of 1,292 samples from volunteers and 646 samples from patients sent by general practitioners because of neurological complaints. The last group of patients volunteered, after informed consent, to have their sera analysed by the Ag-ELISA as described below, when positive, to undergo a CT-scan of the brains. These scans were made by a CTMAX 640 "General Electric" and analysed by experienced neurologists. Samples were collected from both genders between 12 and 80 years old.

4.2.3.2 Taeniasis

From January 2000 until May 2002, a total of 1,935 faecal samples from human volunteers were collected: 695 samples came from inhabitants of Pichincha Province and 1,240 from various rural communities of Imbabura province. *Taenia* carriers were offered anthelmintic treatment i.e. a single oral dose of praziquantel at 10mg/kg b.w. and stools were collected for species identification of the collected tapeworms. In this study, both genders between 1 and 80 years were included.

4.2.4 Techniques

4.2.4.1 Stool examination

Stool samples were examined by the Ritchie technique (Ritchie, 1948).

4.2.4.2 Identification of cestodes

Metacestodes encountered in the muscles were dissected out and transferred to 90ml saline plus 10ml bovine bile at 37ºC to allow evagination and visualisation of the

scolex. Tapeworms collected from human stools following praziquantel treatment were identified according to the methods described in chapter 2.

4.2.4.3 Detection of circulating antigens (Ag-ELISA)

Blood samples were kept at 4°C, centrifuged at 2500×g for 5 minutes and serum stored at -20ºC until use. Sera from bovines, pigs and humans were tested in an Ag-ELISA (Brandt et al., 1992) with the modifications, including pre-treatment of the sera by trichloroacetic acid, as described by Dorny et al. (2000) and Erhart et al. (2002). To facilitate comparison between different plates, all results were expressed as a ratio, calculated by dividing the optical density of each sample by the cut-off value. This cut-off was calculated using a t-test based on the optical densities of eight negative samples of Ecuadorian origin, as such, any value above 1 is considered as being positive.

4.3 RESULTS

An overview of the diagnosis of cysticercosis in the respective hosts, carcass inspection in bovines and pigs and the coprological survey is presented in table 4.1.

4.3.1   Bovine cysticercosis

Inspection of 374 carcasses in the slaughterhouse of Ibarra revealed one positive animal (0.27%). From the 397 sera collected, 23 were seropositive (5.79%; ratio range: 1.01–128.32) in the Ag-ELISA. This parasitologically positive animal had an Ag-ELISA ratio of 128.32. It was a 1-year-old steer of 350 kg from Carchi, in the northern part of the country. It was heavily infected with over 4,000 metacestodes, mainly viable. Meat inspection yielded two positives out of the 432 carcasses (0.46%) inspected in the Metropolitan slaughterhouse of Quito; out of the 472 bovine sera, 12 (2.54%) were positive in Ag-ELISA. In spite of a thorough search of the viscera and the carcass in both positive animals slaughtered, only one living metacestode was found, each time located in the oesophagus muscle. One of the 2 animals positive at inspection was seronegative in the Ag-ELISA whereas the other had a ratio of 1.4. Both animals were Zebu cross-breds, originating from Pichincha province with a liveweight of about 200kg.

Dissection of the larvae and evagination revealed morphological features i.e. an unarmed scolex, typical for *T. saginata* metacestodes.

Table 4.1: Diagnosis of cysticercosis in bovines and pigs according to official inspection in the slaughterhouses in Quito (Pichincha Province) and Ibarra (Imbabura Province) and by the detection of circulating antigen in sera from bovines, pigs and humans. Detection of *Taenia* spp. by microscopical examination of faeces[a].

Province	Bovine cysticercosis		Porcine cysticercosis		Human cysticercosis	Taeniasis
	Ag-ELISA	Post mortem	Ag-ELISA	Post mortem	Ag-ELISA	Ritchie
Pichincha	2.54 (12/472)	0.46 (2/432)	-	(0/8154)	4.90 (116/2,368)	2.45 (17/695)
Imbabura	5.79 (23/397)	0.27 (1/374)	6.77 (40/591) 12.02 (53/441)	0.73 (8/1,101) 0.39 (7/1,795)	5.11 (99/1,938)	1.05 (13/1,240)
Total	4.03 (35/869)	0.37 (3/806)	9.01 (93/1,032)	0.52(15/2,896)[b]	4.99 (215/4,306)	1.55 (30/1,935)

a. Percentage positives (number of positive cases/number examined)
b. excluding data from Pichincha

4.3.2 Porcine cysticercosis

During the first survey in the slaughterhouse of Ibarra, post mortem inspection of 1,101 carcasses revealed eight positives (0.73%). Forty out of 591 blood samples (6.77%) were positive in the Ag-ELISA. In the second survey in the same slaughterhouse 7 of the 1,795 carcasses (0.39%) were found positive by the meat inspector. Results of the more thorough dissections of these seven pigs and their estimated total larval burden are given in table 4.2. In the Ag-ELISA, 53 pigs out of the 441 (12.02%) were positive, among which pigs 1 and 2 that were thoroughly dissected (table 4.2). No blood samples were collected from the five other parasitologically positive pigs. *Fasciola* spp. was found in two pigs, both seronegative for cysticercosis, hydatid cysts were found in four pigs seronegative for cysticercosis and in one seropositive pig with a ratio of 9. *Cysticercus tenuicollis* was found in six pigs, all with a ratio between 3 and 11. Discounting seven possible cross-reactions, 10.66% of the pigs were seropositive.

Table 4.2: Porcine cysticercosis in Imbabura province: results of thorough dissection of seven pigs found positive at routine meat inspection and estimation of the total number of metacestodes.

Pig no	Number of larvae in					Carcass	Estimated
	Brains	Tongue	Heart	Diaphragm	Muscle groups	Weight (kg)	(total no. of cysticerci)
1	1	1	2	3	10	30	289
2	3	4	17	6	12	60	707
3	1	78	0	21	225	30	6,445
4	5	6	0	10	59	50	2,794
5	2	23	17	0	269	25	6,364
6	1	0	5	4	23	25	551
7	2	1	5	17	60	60	3,409

[a] Results of thorough dissection of seven pigs found positive at routine meat inspection and estimation of the total number of metacestodes

4.3.3 Human cysticercosis

Cysticercus cellulosae circulating antigen was detected in 215 serum samples in a total of 4,306 serum samples (4.99%), with little variation between provinces: 116 positives out of 2,368 in Pichincha (4.90%) and 99 positives out of 1,938 samples tested from Imbabura province (5.11%). However, in the latter group 3.4% of the volunteers were positive whereas analysis by Ag-ELISA of the 646 neurological patients revealed 55 seropositives (8.51%). Brain CT-scans of these patients resulted in 52 diagnoses of neurocysticercosis, the majority, however, i.e. 36 out of 55, without any complaints

suggestive for cysticercosis, only three had a history of epileptic crises and 13 patients complained of recurrent headaches.

4.3.4   Human taeniasis

Coprological examination of 1,935 stools in total resulted in 30 positive cases (1.55%) with 17 out of 695 (2.45%) from Pichincha and 13 out of 1,240 (1.05%) from Imbabura province. In the vast majority of stools (93%), helminths (14%) or protozoan parasites (35%) or both (43%) were observed.

Praziquantel treatment resulted in recuperation of adult tapeworms from 17 hosts in Pichincha, 16 of these were identified as *T. saginata*, one as *T. solium*. In Imbabura, five hosts carried *T. saginata*, seven *T. solium* and in one case no worms could be recuperated. All hosts carried a single worm, except for one carrying a very small and two normal sized *T. saginata*.

4.4 DISCUSSION AND CONCLUSION

Some authors (Heinz and MacNab, 1965, cited by Joubert and Evans, 1997) found it striking that high rates of cases of human cysticercosis occur while the prevalence of intestinal *T. solium* in man is notoriously low and nicknamed this disproportion the "*T. solium*/cysticercosis paradox". In Ecuador, according to the scarce epidemiological data provided by the Ecuadorian Ministry of Public Health (Aguilar, 2002), in some provinces in the Sierra, like Bolivar, Cañar and Imbabura this paradoxical situation seems to exist as well, with official incidences of taeniasis/cysticercosis of 0/1.60, 0/1.36 and 0/3.57, per 100,000 habitants, respectively. Whereas, in the coastal provinces of Esmeraldas, Los Rios and Manabi the reverse has been reported i.e. the presence of adult *Taenia* with no or hardly any cases of human cysticercosis, i.e. 1.36/0, 11.11/0.30 and 1.01/0 per 100,000 habitants, respectively.

From 1985 to 2001, according to FAO, WHO and OIE reports, no data on *Cysticercus bovis* in Ecuador, are available in spite of being notifiable (Welte, 1997; Handistatus II, 2002), before 1985, it was listed as exceptional. Even, a report specifying adult *T. saginata* has never been made in Ecuador where usually prevalences of *Taenia* are given without reference to the species (e.g. López, 1969). All references

to adult *Taenia* were assumed to be *T. solium*, presumably because of the impact on public health.

This study confirms the presence of bovine cysticercosis in Ecuador, commonly assumed to be absent. This is very surprising and the latter statement may be untrue for the staff of slaughterhouses, which either did not report their observations or failed to recognise the metacestodes. Hence officially *C. bovis* was considered to be either absent or at least present but beyond the detectable limit. The comparison with the study of Dorny et al. (2000) in Belgian cattle is interesting: 0.26% positives on post mortem inspection vs. 3.09% seropositives in Belgium with 0.37% positives on carcass inspection vs. 4.03% seropositives in the present study or an almost equal ratio (12 against 11, respectively) by which the post mortem data have to be multiplied to obtain the seroprevalences.

Even with proper training and supervised inspection, as in the present study, regional variations on cysticercosis in Ecuador will remain as indicated by official data (Aguilar, 2005) on human and porcine cysticercosis.

Estimating the prevalence of porcine cysticercosis is even more difficult. Ante mortem palpation of the tongue is a common practice on the markets in the region. As long as no financial compensation for condemnation of cysticercotic carcasses, through insurance or subvention, exists, it will be unlikely that animals with palpable cysticerci in the tongue will be presented in public slaughterhouses. On the other hand, all positive carcasses had high numbers of cysticerci which may suggest that light infections might escape the attention of the inspector. This may be a partial explanation of the failure to detect no positive carcasses on a total of 8,154 pigs slaughtered in Pichincha, even more so since official meat inspection, limited to superficial inspection and a single incision only, was carried out without any intervention of the present investigators.

In any case, the limited number of complete dissections of the carcass does not allow any firm conclusion but data are not suggestive for clear-cut predilection sites of the metacestodes. The brains, however, of all seven pigs harboured cysticerci albeit in very low numbers, irrespective of the total larval load. Incidentally, routine post mortem inspection never includes the brains. The presence of porcine cysticercosis, in the Northern Andes of Ecuador as determined by Ag-ELISA lies well in the expected range

of an endemic region i.e. 5–30% (Craig et al., 1996). The monoclonal antibodies, used in the latter test, were raised against metabolic antigens of *T. saginata* metacestodes (Brandt et al., 1992), as such, if those are reacting with antigens of *T. solium* metacestodes, then a cross-reaction with *Taenia hydatigena* metacestodes is equally likely. Therefore, the six cases of *C. tenuicollis* found in the 53 Ag-ELISA positives in Imbabura were discounted.

As for the infection rates of human cysticercosis, we cannot rule out a biased sampling. Samples came from patients visiting the outpatient's hospital and their willingness or that of the volunteers to co-operate might have been linked to being suspicious of a problem. Again in this study, the occurrence of intestinal *Taenia* seems low and probably an underestimation, even when these data, obtained by microscopy have to be multiplied by 2.6 (Allan et al., 1996b). Nevertheless the official figures for 2001 (Aguilar, 2002) do not seem to be very realistic i.e. 1 and 2.15 cases of taeniasis and cysticercosis, respectively, per 100,000 habitants in Pichincha, in Imbabura 3.57 cases of cysticercosis and none for *Taenia* spp. In chapter 2, 16 adult specimens were differentiated from the same region as *T. saginata* and seven as *T. solium*. In *T. solium* endemic regions, prevalence of taeniasis is usually not higher than 1% (Gemmell et al., 1983) but can reach 2.7% (Allan et al., 1996b). As for *T. saginata*, Geerts (1990) estimated that the incidence of intestinal *T. saginata* in Belgium was equal or even higher than the cases of bovine cysticercosis detected p.a. Obviously, many factors like culinary traditions or food-safety regulations, make comparisons about the proportion of adult tapeworm carriers and metacestode infections between different countries often difficult. In addition, the limited number of samples and the diagnostic insensitivity related to intestinal *Taenia* does not allow a conclusion on the proportion in which both intestinal *Taenia* spp. are present. However, in Ecuador, our study confirms the presence of both *Taenia* spp. Given the relatively high numbers of bovine, porcine and human cysticercosis it is unlikely that current official estimates about the presence of adult *Taenia* are realistic which hinders a correct understanding of the epidemiology of the *T. solium*/cysticercosis complex.

CHAPTER 5

TAENIASIS-CYSTICERCOSIS IN SOUTHERN ECUADOR: ASSESSMENT OF INFECTION STATUS USING MULTIPLE DIAGNOSTIC TOOLS

5.1 INTRODUCTION

Taenia solium-taeniasis and cysticercosis are diseases present in developing countries, which are associated with social and environmental conditions such as lack of latrines, poor hygiene and free roaming pigs with access to human faeces (WHO, 2002). These conditions are common in the Andes, including Ecuador, where *T. solium* infections are endemic. In chapter 4, a survey carried out in Imbabura, in the north of Ecuador were described. There the temperate conditions allow keeping pigs in farming conditions characterised by tethering or corralling. In this region, the prevalence of taeniasis and cysticercosis in humans was 1.05% and 5.11%, respectively. The prevalence of porcine cysticercosis was 9.01%.

While in chapter 4, antigen detection was used to determine cysticercosis prevalence, most studies in Latin America used antibody detection for immunodiagnosis (e.g. Gonzalez et al., 1990; Diaz et al., 1992a; Garcia Noval et al., 1996; Sanchez et al., 1997; Goodman et al., 1999; Garcia et al., 2003a; Cruz-Licea et al., 2003). Both methods attempt to measure the proportion of infected individuals, however, antigen detection is restricted to the detection of viable cysticerci, while the finding of antibodies can indicate the presence of both viable and degenerated cysticerci (Dorny et al., 2004b). Moreover, Garcia et al. (2001) demonstrated that in cysticercosis-endemic areas of Peru and Colombia approximately 40% of people, seropositive by EITB had become seronegative when resampled after 1 to 3 years. This indicated that transient antibodies might occur following exposure to *T. solium* eggs.

The aim of this study was to evaluate the cysticercosis/taeniasis situation in an endemic community in the southern Andes, where conditions are prone to transmission of *T. solium,* i.e. extensive pig husbandry and few or no latrines. We aimed to sample the entire human and pig populations in a defined community. Serum samples of man were screened with an antigen detecting test, and stool samples by coprological examination. Pigs were examined by tongue palpation. In addition, antibody tests on serum samples, and response to praziquantel were performed on sub-populations of man and pigs.

5.2 MATERIALS AND METHODS

5.2.1 Study area and population

The study was done in Limones (80º21'W, 4º23'S), a rural community (about 35km^2) in the southern Andean province of Loja, bordering Peru. The region has two well-defined climatic seasons, i.e. a rainy season between December and May and a dry season during the rest of the year (average annual rainfall 472mm and average annual temperature 26ºC). Pig rearing is marginal in this arid region: the animals are left free roaming in search of feed during the day time and confined at night and usually kept only for local consumption (Benitez-Ortiz, 2001). According to preliminary censuses in January 2003, Limones is composed of 17 neighbourhoods with 222 families. In total, 1,059 inhabitants and 1,148 pigs were recorded.

5.2.2 Experimental design

The survey was carried out during the dry season (July-August) of 2003. The local authorities and community leaders were contacted and informed about the project. Each individual household was visited and all persons between 2 and 80 years were invited to participate in the survey. Vials for collecting stool samples were distributed to be collected the next day together with a blood sample taken at the local health centres.

Taenia carriers who were identified following examination of the stool samples were offered cestocidal treatment, i.e. a single oral dose of praziquantel at 10mg/kg b.w. and a tablet of laxative "Dulcolax" (Boehringer Ingelheim GmbH, Germany). Following treatment, tapeworms or their fragments were collected from the stools and stored in ethanol 70% for species differentiation. People who were negative for *Taenia* spp. eggs but appeared to be infected with gastrointestinal nematodes were offered a single dose of albendazole (400mg).

Pig owners were requested to keep their animals penned. From the estimated 1,148 total number of pigs in the community, 646 were submitted to the visual inspection of the ventral face of the tongue (Gonzalez et al., 1990). Pigs positive to tongue inspection were treated immediately with a single oral dose of oxfendazole (30mg/kg b.w.) (Gonzalez et al., 1996).

A computer-generated random sample of 100 persons was selected among the

participants. The serum samples from these persons were also analysed by EITB (Tsang et al., 1989) for the detection of circulating antibodies against *T. solium* cysticerci.

One hundred pig serum samples were collected from the external jugular vein as described by Framstad et al. (2004) and examined by EITB, including 23 tongue-positive pigs and 77 randomly selected tongue-negative pigs.

The protocol of this survey was approved by the Ethical Committee of the Central University of Ecuador.

5.2.3   Diagnostic methods

Blood samples were centrifuged and the sera were transferred at 4°C to Quito-Ecuador where they were frozen at -20°C until analysis. Tongue inspection was performed in pigs, visually and by palpating the ventral lingual surface as described by Gonzalez et al. (1990). Faecal samples were analysed by the formalin-ether method (Ritchie, 1948). Tapeworms collected from human stools following praziquantel treatment were differentiated by morphological characteristics, Glucose Phosphate Isomerase (GPI) patterns and PCR-RFLP as described in chapter 2.

Sera from humans were tested in an Ag-ELISA (Brandt et al., 1992) with the modifications, including pre-treatment of the sera by trichloroacetic acid and the interpretation of the results, as described by Dorny et al. (2000) and Erhart et al. (2002). Because a preliminary experiment, consisting in the dissection of the entire carcass of 5 pigs that were found positive in the Ag-ELISA, revealed that two pigs were infected with metacestodes of *T. solium*, another two with *T. hydatigena* and one pig with both parasites, it was decided not to use this test for serodiagnosis. *T. hydatigena* infections in pigs are known to cross-react in the Ag-ELISA (Dorny et al. 2004a).

Human and porcine sera were tested, at the Cayetano Heredia University in Lima-Peru, by EITB (Tsang et al., 1989), for antibodies against seven *T. solium* glycoprotein antigens.

5.3 RESULTS

5.3.1   Human population

From the 1,059 inhabitants, 800 serum samples and 958 stool samples could be

collected. A total of 18 sera [2.25% (CI 95%: 1.34-3.53)] were positive for *T. solium* metacestode circulating antigens (age range from 5 to 78 years). From the 958 faecal samples 14 [1.46% (CI 95%: 0.80-2.44)] were positive for *Taenia* spp. eggs by coprological examination (age range from 12 to 73 years). All except two positives were from separate households. Following treatment of these carriers, 12 worms were collected. All of these worms were identified as *T. solium.* Among these tapeworm carriers four individuals were positive by Ag-ELISA. One of these individuals who was positive in both Ag-ELISA and EITB developed clinical symptoms, i.e. severe headache and epileptic seizures, shortly after treatment of taeniasis. He was referred to a hospital in Loja city where NC was confirmed by CT-scan and where he was treated.

From the subset of serum samples collected for further analysis, six samples were positive by Ag-ELISA while 40 samples were positive by EITB. In the EITB, three samples recognised all seven glycoproteins (GP), while two samples showed 5 bands, two samples 4 bands, 16 samples three bands, 7 samples two bands and 10 samples only one band. The proteins most frequently recognised were the GP50 (85%), GP39-42 complex (88%) and GP24 (90.5%); the lower molecular weight proteins [GP21 (10%), GP18 (10%), GP14 (7.5%) and GP13 (17.5%)] were less recognised. From the six samples positive by Ag-ELISA, only two were positive by EITB, showing one (GP50) and three bands (GP50, GP39-42, GP24), respectively.

5.3.2   Pigs

From the 646 pigs examined, 23 [3.56% (CI 95%: 2.27-5.29)] had between one and 20 cysts in the sublingual surface.

In the subset of 100 pigs 74 serum samples were positive in EITB, including the 23 pigs with cysticerci at tongue inspection and 51 tongue negative pigs. Like for the human samples the proteins most frequently recognised were the GP50 (97%), GP39-42 complex (75%) and GP24 (72.5%); the lower molecular weight proteins [GP21 (5.5%), GP18 (7%), GP14 (1.5%) and GP13 (12%)) were less recognised. None of the samples recognised all seven GP, three samples reacted with six bands, one with five bands, seven with four bands, 46 with three bands, five with two bands and 12 with one band.

5.4 DISCUSSION

This study demonstrated the presence of *T. solium* in humans and pigs in a rural community in the southern Andes of Ecuador. More than 80% of the human population participated in the study and more than 56% of the pigs could be inspected by tongue examination. Therefore, we consider this sample to be representative for the human and pig populations in the surveyed community.

Taeniasis was detected in 1.46% of the human population by microscopic examination; which is slightly higher than the results from similar studies in the North of Ecuador (see chapter 4). However, all *Taenia* in this study were identified as *T. solium* whereas in the North only 8 out of 29 worms collected were *T. solium,* the other 21 worms being identified as *T. saginata*.

With this higher percentage of *T. solium* tapeworms in the population also a higher percentage of human and porcine cysticercosis cases would be expected. Nevertheless, the percentage of Ag-ELISA positives in humans was lower than in the study in the North. In contrast, the percentage of tongue-positive pigs in the South was higher (3.56%) than the number of carcass inspection-positive pigs detected in the North (0.52%). However, tongue and meat inspections have an equally low sensitivity (Dorny et al., 2004a), therefore, these figures probably seriously underestimate the real prevalence of porcine cysticercosis in both regions.

EITB performed on a sub-set of 100 randomly selected individuals indicated a very high proportion of the human population carrying antibodies against *T. solium*: among these 100 individuals only six had circulating antigens and more than 40% had circulating antibodies. The proportion of antibody positive pigs among the randomly selected tongue negatives was also very high [66% (CI 95%: 54-77)].

While Ag-ELISA measures active cysticercosis, detection of circulating antibodies indicates exposure and thus not merely active infection (Dorny et al. 2004b). Thus, a positive antibody test would indicate infection, active or past, or a transient antibody response (Garcia et al., 2001, Gonzalez et al., 2006). The high proportion of antibody positives in humans and in pigs would reflect high exposure rate in the study area. Indeed, risk factors for taeniasis-cysticercosis such as open-air defecation, free roaming of pigs and the eating of undercooked pork are very prominent in Loja province. In this

area pigs have been seen to cover wide distances for finding feed and water, which increases the risk of infection of pigs.

The data shown in this study suggest that frequency of contact with oncospheres may be an important factor in the development of endemic stability (Gonzalez et al., 2006). This has been described in other parasitoses such as malaria and lymphatic filariasis (Coluzzi, 1999; Melrose, 2002). In the context of human cysticercosis "endemic normal" (Melrose, 2002) would thus refer to people who, despite regular exposure to oncospheres and subsequent development of an antibody response, have only temporarily or never developed viable cysticerci in the CNS, hence do not show clinical evidence of disease. The clinical prevalence of NC in this region merits further studies; however, preliminary results indicate low involvement of NC in epilepsy cases.

More data are needed to support the hypothesis of endemic stability in cysticercosis, yet, these observations point to the importance to differentiate between persons with active cysticercosis and those having been exposed to oncospheres, i.e. the simultaneous use of antigen and antibody detection, ideally supported by neuro-imaging.

As a final conclusion, high exposure of humans and pigs to oncospheres of *T. solium* was demonstrated in a region where the harsh climatic conditions are a major constraint for agriculture, which directly affects pig husbandry. In spite of the limited number of samples that could be analyzed by EITB, it was demonstrated that a high percentage of humans and pigs have antibodies against the parasite. Most of the infected individuals, however, did not show any evidence of NC. In addition, our findings point out the difference of interpretation of test results, based on direct and indirect immunodiagnostic methods and suggest the use of multiple diagnostic methods to determine the true prevalence of the disease. This multi-testing based diagnosis will allow a better understanding of the epidemiology of the taeniasis-cysticercosis complex and may also support individual diagnosis.

CHAPTER 6

GENERAL DISCUSSION

6.1 INTRODUCTION

This work aimed at a better understanding of the *T. solium* complex in Ecuador and the development of the tools to achieve this. In this chapter the shortcomings in the epidemiological knowledge and the contributions of the present work are discussed. In addition, options for control of the *T. solium* complex adapted to the situation in Ecuador are critically discussed.

6.2 DETECTION AND DIFFERENTIATION OF ADULT *TAENIA* SPP. IN *T. SOLIUM* ENDEMIC REGIONS

In most *T. solium* endemic regions, all large intestinal cestode infections in man tend to be diagnosed as *T. solium*. Little attention is given to the beef tapeworm, which may co-exist. In some South American countries like in neighbouring Peru, the presence of *T. saginata* is mentioned (Wilkins et al., 1999; Mayta et al., 2000), yet with little epidemiological data. Preconceptions and the difficulties related to the differentiation between both *Taenia* spp. are probably the main reasons. We presented the first report of *T. saginata* in Ecuador. In the Northern Andes, we found prevalences of 1.5% *Taenia* spp., yet surprisingly more than 70% appeared to be *T. saginata*, whereas in the South the latter was not observed. With both *Taenia* parasites occurring in man, differentiation is important when control programmes are developed. In addition, since the presence of an adult *T. solium* is an important cause of cysticercosis in carriers and family members, (Garcia-Noval et al., 1996; Carrique-Mas et al., 2001) detection and identification is of highest medical importance.

Distinction between *T. saginata* and *T. solium* is commonly based on their morphology, alternatively on isoenzymes. More recently, PCR-based differentiation showed its great potential (Dinkel et al., 1998; Lores et al., 2002; Martinez et al., 2003; Stefanic et al., 2004). However, results were not always straightforward and often interpretation was difficult (Mayta et al., 2000; Gonzalez et al., 2002; Yamasaki et al., 2002, 2004; Nunes et al., 2003, 2005). We developed a PCR-RFLP based method to differentiate DNA extracted from parasite somata (chapter 2) to be modified later-on for extracting this from faeces. High specificity was obtained in the amplification of human cestodes and the differentiation by RFLP using *Dde*I as restriction enzyme.

Although extraction of parasite DNA from faeces followed by PCR (chapter 3) looked promising, we encountered some difficulties with non-specific reactions. DNA extraction from faeces is hampered by the presence of proteolytic enzymes, low levels of detectable DNA or DNA from many sources causing non-specific bands. Factors like diet (Morphy et al., 2003), ambient temperature at collection of the samples (Jajkova et al., 2006), transport and storage conditions (Morphy et al., 2002; Roeder et al., 2004) have been reported to affect the results of the PCR. In addition, differences were observed according to the methods used to extract the DNA. Therefore, a step-wise analysis of the protocol should be performed, thereby assessing the sample storage methods, optimizing the times and temperatures of the extractions methods and PCR protocols; alternatively the development of more specific probes should be considered.

Apart from the technical difficulties to be resolved, PCR-RFLP on faecal samples is an expensive and demanding technique. Yet, once the sensitivity of conventional techniques like e.g. repeated microscopy (Sanchez et al., 1997; Navone et al., 2005), has reached an acceptable level, PCR-RFLP on pre-selected positive faecal samples to differentiate the species involved, would be a valuable tool.

6.3 EPIDEMIOLOGY OF CYSTICERCOSIS IN ECUADOR

Serological diagnosis of cysticercosis is currently based on either antibody detection, mainly by EITB, or on a monoclonal-based ELISA for antigen detection. The availability of the current methods is not yet optimal but our work showed that the combined use of both methods is opening interesting views on the epidemiology, since in humans, antigen detection allows the search for active cysticercosis, whereas antibody detection indicates exposure to infection.

Cysticercosis has been studied in two regions of the Ecuadorian Andes. The northern region (Chapter 4) has a temperate climate, sufficient rainfall and produces various crops year-round. Consequently, pigs are tethered along fields and roadsides during daytime. The southern region of study (Chapter 5) has a dry climate with rainfall limited to a few months, fields produce very little and pigs are free roaming, covering wide distances. Given this, after the study in the North was completed it was hypothesised that the situation on NC in the South would be far worse. However, in spite of the higher prevalence of intestinal *T. solium* in the South (1.46%), in comparison to the

North (0.41%), a lower sero-prevalence of human cysticercosis as detected by Ag-ELISA was found, i.e. respectively, 2.25% in the South versus 4.99% in the North. For every adult *T. solium*, eight times less cysticercosis cases were found in the South in comparison to the North. Admittedly, diagnosis of taeniasis, though for both regions done by the same tools and staff, is not very sensitive; in addition, both studies were not done simultaneously. These findings plus the high antibody seroprevalence (>40%) as obtained by EITB for human cysticercosis in the South, suggest that exposure does not always lead to active cysticercosis. Occurrence of such "transient antibodies" was described by Garcia et al. (2001) and the prevalence of those might be inversely related with the number of cases of active cysticercosis.

Unfortunately, antibody detection by EITB could not be done on samples from the North. However, should less frequent contacts lead to lower immunoresistance, hence a higher risk of developing active cysticercosis, then proportionally a closer match between antigen and antibody prevalence in the North is within the expectations. Other surveys like the age at first contact, cohort studies and follow-up studies to evaluate the effects of anti-taeniicidal control should be included in future research.

The situation of porcine cysticercosis is more difficult to evaluate, because infection with *T. hydatigena* cysticerci in pigs causes cross-reactions in the Ag-ELISA. Autopsies revealed that prevalence of *T. hydatigena* is high in the South and low in the North. Comparison of data from visual inspection of carcasses for *T. solium* cysticercosis suggests higher prevalences in the South than in the North. Yet, the low sensitivity of the tongue palpation and the unreliability of slaughterhouse data do not allow firm conclusions. The limited data on antibody detection in pigs in the South indicate a high exposure to oncospheres, similar to the situation in humans.

6.4 OPTIONS FOR CONTROL OF NC IN ECUADOR

At face value, control of cysticercosis should not be difficult because man is the only final host. Proper disposal of human faeces should be sufficient to break the cycle. However, poor hygienic standards, mostly related to poverty and ignorance, are still prevailing and remain a key factor for the presence of a multitude of parasites, including *T. solium*. Consequently, all efforts should be used for controlling cysticercosis i.e. information campaigns, improved hygiene, improved pig husbandry, ante- or post-

mortem detection of pigs, treatment of pigs and detection and treatment of tapeworm carriers.

However, a conflicting situation might arise when an endemic stability is evident. Upsetting this situation by treating some carriers might lead to decreased contact and consequently loss of immunity of the population. Consequently, a control programme should envisage the participation of the entire population aiming to eliminate all tapeworms.

Control strategies can be organised at three levels, and ideally they should act on all three:

 (1) One of the most obvious measures is at the level of infected pigs. A control method consisting of inspection of all pigs before slaughter and of the carcasses at the abattoir and condemnation of positive carcasses would drastically reduce transmission to humans. However, information collected at slaughterhouses on porcine cysticercosis reveals another reality: we observed in the slaughterhouse a prevalence of 1%, whereas in the same region more than 3% of the pigs had a positive tongue palpation, which is not even a very sensitive method (Dorny et al., 2004a). Obviously, in traditional pig husbandry, even when the facilities are available, there is often little financial room for presenting pigs in slaughterhouses. Furthermore, in the absence of any insurance, farmers will think twice before presenting tongue positive pigs to the slaughterhouse. Consequently, in our experience in *T. solium* endemic regions, the vast majority of pigs are slaughtered at home.

(2) Preventing the intermediate host to become infected i.e. preventing pigs to have contact with human faeces. Construction of latrines is the most obvious answer (Flisser et al., 2003). However, the success rate may differ according to the region: in the Northern Andes, pigs are usually tied up during the day in the fields along roadsides and feed on garbage; at night they are penned. Access to human faeces is by no means excluded but it is less likely than in the South. In that region, pigs are left to roam around. Water scarcity and consequently, rural poverty forces people to cover wide distances, disfavouring the availability or the use of latrines. Contrary to the South, in the Northern Andes, more than 68% of households have latrines (Proaño-Perez and

Chavez-Larrea, 1999), albeit often not properly used, because of poor hygienic standards.

Recent developments on the vaccination of pigs are promising (Lightowlers et al., 2004; Gonzalez et al., 2005; Flisser et al., 2006), yet some aspects are still under investigation i.a. duration of protection and frequency of boosting. It is likely that the application on a large scale and the success rate will be determined by economical parameters.

(3) Control measures related to the final host: either mass treatment or detection and treatment of *Taenia* carriers. Participation of the population at risk is an important feature required for both options and this can hardly be expected to be 100%. Whether the entire population has to be reached and what proportion of *Taenia* carriers is necessary to maintain the infection in a region need to be investigated. Cruz et al. (1989) and Sarti et al. (2003) applied mass treatment in endemic areas and noticed one year later a decrease of taeniasis (from 1.6% to 0%) and porcine cysticercosis (from 11.4% to 2.6%) (Cruz et al., 1989). However, effects of mass treatment last for about 2 years, thereafter infection rates increase again (Carvajal y Cueva, 1999; Garcia, 2002).

6.5 CONCLUSIONS AND FUTURE PROSPECTS

In 1860 Davine wrote (cited in Pawlowski and Schultz, 1972) that no other animals than taeniids were causing so many hypotheses, discussions and misconceptions. Although this is likely to be an exaggeration, the complexity of the epidemiology of *T. solium* and of the means to evaluate this, is tempting to support this statement. To understand the epidemiology of *T. solium* in a region, finding and identifying the carriers of adult tapeworms is important. Improved conventional detection of oncospheres should be followed by techniques to differentiate *T. solium* and *T. saginata.* Copro-PCR showed promising results but should be fine-tuned.

In our study active cysticercosis in humans was found to be more frequent in the North than in the South. We are aware that we should be cautious with expanding these results to the whole region. Nevertheless, further studies to confirm these findings are highly recommended. The simultaneous use of Ag-ELISA and EITB might provide valuable data on this subject. The role of the prevalence of adult *T. solium*, hence the

degree of exposure to oncospheres, on the prevalence of human cysticercosis has to be further investigated.

Given the cross reactions with *T. hydatigena* and the difficulties to distinguish between exposure and active infection in pigs, the ante-mortem diagnosis in pigs remains problematic and requires more specific methods.

Finally, close collaboration between the medical and veterinary sectors is important in order to evaluate the public health and socio-economic impacts of the disease.

SUMMARY

Taeniasis-cysticercosis is caused by *Taenia saginata* and *Taenia solium*. Adult *Taenia* spp. are always intestinal parasites highly specific for man. Bovines and pigs act as intermediate hosts for *T. saginata* and *T. solium*, respectively. Metacestodes are located in the tissues, commonly the muscles; larvae of *T. solium* can also occur in nervous tissues and cause neurocysticercosis in man, considered the most common cause of acquired epilepsy in endemic countries. Consumption of undercooked pork, free-roaming pigs with access to human faeces, are key factors in the occurrence of *T. solium*. In Ecuador in particular, *T. solium* is endemic in the Andean region and rare or absent in most costal regions. This study focused on the understanding of epidemiology of the taeniasis-cysticercosis complex in two Andean regions of Ecuador and the improvement of the diagnostic tools to this aim.

This thesis is divided into four parts: (1) an introduction formulating the problems, underpinned by a general review of the literature leading to the description of the objectives of this study (**chapter 1**); (2) the development of highly sensitive and specific PCR-based techniques to detect and to differentiate *T. solium, T. saginata* and *Hymenolepis nana* somata (**chapter 2)** and tapeworm-DNA extracted from faecal samples (**chapter 3**); (3) an assessment of the taeniasis-cysticercosis situation in two endemic areas in the Ecuadorian Andes (**chapters 4** and **5)** and (4) a general discussion where the results are commented in a broader perspective (**chapter 6)**.

A general introduction in **chapter 1** outlines the problem of taeniasis-cysticercosis with an assessment of the situation in Ecuador and the position of the present study. A review of literature, on important cysticercosis-related topics like origin, history, morphology, life cycle and epidemiology is given with i.a. the main shortcomings on control and diagnosis. The emphasis of this review is on the epidemiological aspects and diagnostic techniques used to estimate the prevalence of taeniasis and cysticercosis, with particular reference to PCR-based assays for the diagnosis of taeniasis. At the end of this chapter, the objectives are presented, the main aim being to gain a better understanding of the taeniasis-cysticercosis problem in Ecuador by applying improved diagnostic tools.

Chapter 2 describes the development of a PCR based tool to underpin current diagnostic methods, i.e., morphological and iso-enzymatic studies of proglottids. A PCR test complemented with restriction enzyme analysis was modified by redesigning one of

the primers to reduce non-specific amplifications experienced when using field samples. The use of these new, highly specific primers and the restriction enzyme *Dde*I led to the development of a diagnostic assay allowing clear differentiation between *T. saginata* and *T. solium* proglottids in field samples. This assay confirmed the presence of *T. saginata* in Ecuador.

In **chapter 3,** based on the previous chapter, an improved PCR-RFLP method to differentiate DNA from *T. solium* and *T. saginata*, extracted from faeces, was developed. New primers were tested with improved specificity to amplify parasite DNA in faecal samples. For this purpose, three different faeces extraction protocols were compared i.e. the commercial QIAamp® DNA stool mini kit; the guanidium thiocyanate/zirconium matrix method (Boom) and DNA isolation based on a sequence-capture technique (Mangiapan). The PCR-RFLP assay was used on faecal extracts from 35 known *Taenia* carriers collected before anthelmintic treatment. Both QIAgen and Boom protocols showed non-specific bands, which in Mangiapan were not observed in negative samples from Ecuador, though they were present in negative samples from Belgium, complicating the interpretation of the PCR. The PCR-RFLP assay on faecal extracts from 35 known tapeworm carriers detected 27 samples positive for *T. solium* and 8 for *T. saginata*. Restriction with D*de*I enzyme gave clear and distinctive profiles for *T. solium*, *T. saginata* and *H. nana*; the primers developed in this work amplified neither *Hymenolepis diminuta* nor *Diphyllobothrium latum*. This new PCR-RFLP assay has potential as a supporting tool for the specific diagnosis of *Taenia* spp., following preselection of samples by standard coprological assays. However, the appearance of non-specific bands in some samples requires further improvement of the assay.

Chapter 4, describes an epidemiological study in the northern Andes with the first report of *T. saginata* in Ecuador, urging reconsideration of some assumptions in the epidemiology of the taeniasis/cysticercosis complex in this country. Therefore, data on the infection of both tapeworms in man and animals in Pichincha and Imbabura provinces in the Andean region, north of Quito, were compiled. On post mortem inspection 3 out of 806 (0.37%) bovine carcasses had *T. saginata* metacestodes, however, 35 sera out of 869 (4.03%) showed circulating antigen in a monoclonal antibody-based sandwich ELISA (Ag-ELISA). Porcine cysticercosis was detected in 15 out of 2,896 (0.52%) carcasses and 93 out of 1,032 serum samples (9.01%) were

positive in Ag-ELISA. In humans, 4.99% (215 out of 4,306) antigen positives were found, whereas coprological examination of 1,935 stools resulted in 30 positive cases (1.55%). The limited number of adult tapeworms (29) that were collected does not allow firm conclusions on the proportion of each species, but in total 21 specimens were identified as *T. saginata* and 8 as *T. solium*. These data have been discussed in view of the epidemiology of human cysticercosis.

In **chapter 5,** data of a second epidemiological study, carried out in an endemic community in the southern Ecuadorian Andes, are given. The poor living conditions in this region presumed higher prevalences of *T. solium* cysticercosis than in the North. The study was undertaken in Limones, a rural community in Loja province with a population of 1,059 people and 1,148 pigs. Participation was high: 800 serum samples and 958 stool samples of the people were collected. In addition, 646 pigs were tongue inspected. Circulating antigen was detected by Ag-ELISA in 2.25% of the human population. Intestinal taeniasis was detected in 1.46% of the samples by the formalin-ether technique; following treatment and recovery of tapeworm fragments, all were identified as *T. solium*. Porcine cysticercosis was diagnosed in 3.56% of the pigs by tongue inspection. Unfortunately antigen detection in pigs was seriously hampered by the highly prevalent *Taenia hydatigena*. In addition, enzyme linked immunoelectrotransfer blot (EITB) was performed on a subset group of 100 humans to confirm the results of the Ag-ELISA. One hundred serum samples from pigs were also analysed by EITB. It appeared that 43 and 74% of humans and pigs, respectively had antibodies against *T. solium* cysticerci. It is concluded that contrary to the high exposure of the human population to *T. solium* as suggested by EITB, the number of active cysticercosis cases, diagnosed by Ag-ELISA, was low, which may indicate endemic stability. The further use of complementary diagnostic methods for a better understanding of the epidemiology of *T. solium* is suggested.

In the general discussion (**Chapter 6)**, the results of the different chapters are discussed, highlighting the most important findings and implications for the understanding of the taeniasis-cysticercosis complex. In this chapter, the importance using highly sensitive and specific diagnostic tools based on molecular biology is emphasised. Comparison of the observations in the North and the South are indicative for an endemic stability in the southern community under study. Frequent contact

contrasted with few cases of active cysticercosis as evidenced by respectively, antibody and antigen detection. In the northern study, in spite of proportionally less adult *T.solium*, the prevalence of active cysticercosis was higher. Both these surveys are prelimary studies and need further confirmation by i.a. simultaneous use of antibody and antigen detection in various locations combined with improved detection of the carriers of adult tapeworms. Options for control of this disease are presented and their potential for use in Ecuador are discussed.

SAMENVATTING

Taeniasis-cysticercosis wordt veroorzaakt door *Taenia saginata* en *Taenia solium*. De mens, waar het volwassen stadium enkel voorkomt in de darm, is voor beide species een zeer specifieke eindgastheer. Het rund en het varken zijn tussengastheer van respectievelijk *T. saginata* en *T. solium*. De mens kan eveneens accidenteel tussengastheer zijn van *T. solium*. De metacestoden komen doorgaans in de spieren voor, maar de larven van *T. solium* kunnen ook het zenuwstelsel parasiteren en doen dit ook bij de mens en veroorzaken op die manier neurocysticercosis, één van de belangrijkte oorzaak van verworven epilepsie in endemische streken. Consumptie van onvoldoende verhit varkensvlees, vrij rondzwervende varkens met toegang tot menselijke feces zijn de belangrijkste elementen bij het voorkomen van *T. solium*. In Ecuador komt *T. solium* in het bijzonder voor in de Andes streek, zelden of niet in de kuststreken. Deze studie beoogde een bijdrage te leveren aan de kennis van de epidemiologie van het taeniasis-cysticercosis complex in twee regio's van de Ecuadoraanse Andes en tot dit doel de huidige detectie methodes te verbeteren.

Deze thesis bestaat uit vier delen: (1) een algemene inleiding met een samenvatting van de problematiek, ondersteund door een literatuurstudie, leidend tot de beschrijving van de objectieven van dit werk (**hoofdstuk 1**); (2) de ontwikkeling van een zeer gevoelige en specifieke PCR ter differentiatie van *T. solium*, *T. saginata* en *Hymenolepis nana* somata (**hoofdstuk 2**) en lintworm-DNA, geëxtraheerd uit feces monsters (**hoofdstuk 3**); (3) een beoordeling van de taeniasis-cysticercosis situatie in twee endemische streken van de Ecuadoraanse Andes (**hoofdstukken 4** en **5**) en (4) een algemene discussie waar de resultaten besproken worden in een breder perspectief (**hoofdstuk 6**).

Hoofdstuk 1 de algemene inleiding, schetst het probleem van taeniasis-cysticercosis met een overzicht van de huidige situatie in Ecuador en de plaats van deze studie hierin. Een overzicht van de literatuur over cysticercose wordt gegeven, met aandacht voor de oorsprong, morfologie, levenscyclus en de epidemiologie. Nadruk wordt gelegd op de belangrijkste zwakke punten bij de bestrijding en de diagnostische technieken welke gebruikt worden om de prevalentie van taeniase en cysticercose te bepalen. Speciale aandacht wordt hierbij gegeven aan de PCR technieken die ontwikkeld werden ter detectie van *Taenia* spp. Dit hoofdstuk wordt besloten met een voorstelling van de objectieven en het doel van de studie, namelijk een beter inzicht te

krijgen in het probleem van taeniasis-cysticercosis in Ecuador door toepassing van verbeterde diagnostische technieken.

Hoofdstuk 2 geeft een beschrijving van de ontwikkeling van een PCR, ter ondersteuning van morfologische en iso-enzymatische identificatie van proglottiden. Een PCR test met een restrictie enzym analyse werd gewijzigd door de primers te herwerken ten einde niet-specifieke amplificaties te reduceren. Deze amplificaties werden dikwijls waargenomen bij veldmonsters. Het gebruik van deze nieuwe zeer specifieke primers en van het *Dde*I restrictie enzym liet een zeer duidelijke differentiatie van *T. saginata* en *T. solium* proglottiden toe, afkomstig van veldmonsters. Tevens werd door deze test de aanwezigheid van *T. saginata* in Ecuador bevestigd.

In aansluiting met het vorige hoofdstuk wordt in **hoofdstuk 3** een verbeterde PCR-RFLP methode beschreven om DNA van *T. solium* en *T. saginata*, geëxtraheerd uit feces, te identificeren. Nieuwe primers, met een verhoogde specificiteit om parasitair DNA in feces monsters te amplificeren, werden getest. Hierbij werden drie DNA extractie methodes vergeleken, namelijk de "commercial QIAamp® DNA stool mini kit"; de guanidium thiocyanaat/zirkonium matrix methode (Boom) en DNA isolatie gebaseerd op een "sequence-capture" techniek (Mangiapan). De PCR-RFLP test werd toegepast op feces extracten van 35 geïdentificeerde *Taenia* dragers. Monsters werden verzameld vóór taeniicide behandeling. Zowel de QIAgen en de Boom extracties gaven niet-specifieke banden, deze werden niet gezien in Mangiapan extracten van negatieve monsters afkomstig uit Ecuador. Echter, Mangiapan extracten van negatieve monsters uit België, vertoonden eveneens niet-specifieke banden, die de interpretatie van de PCR bemoeilijkte. Met deze PCR-RFLP op feces extracten van 35 lintworm dragers werden 27 *T. solium* en 8 *T. saginata* geïdentificeerd. Restricties met D*de*I enzym gaven duidelijk onderscheidbare profielen voor *T. solium*, *T. saginata* en *H. nana*; de primers ontwikkeld in deze studie amplificeerden geen DNA van *Hymenolepis diminuta* noch van *Diphyllobothrium latum*. Deze nieuwe PCR-RFLP test kan een waardevol hulpmiddel bieden ter ondersteuning van de specifieke detectie van *Taenia* spp., na preselectie van de monsters door routine coprologische testen. Het vermijden van niet-specifieke banden vereist echter nog verdere aanpassingen van de test.

Hoofdstuk 4 beschrijft een epidemiologische studie in de noordelijke Andes, waarbij ook voor het eerst het voorkomen van *T. saginata* in Ecuador wordt gemeld. De

mogelijkheid dat beide *Taenia* soorten samen kunnen voorkomen dwingt tot herziening van bepaalde veronderstellingen aangaande de epidemiologie van het taeniasis/cysticercosis complex in dit land. Daarom werden de gegevens over beide lintwormen bij mens en dier uit Pichincha en Imbabura, provincies in de Andes ten noorden van Quito, vergeleken. Bij post mortem onderzoek van 806 runderkarkassen werden drie gevallen (0.37%) van *T. saginata* cysticercose gevonden. En 35 sera op 869 (4.03%) hadden circulerend antigeen, aangetoond met een monoklonale antilichaam sandwich-ELISA (Ag-ELISA). Varkenscysticercose werd gevonden in 15 van de 2896 (0.52%) karkassen en 93 op een totaal van 1032 serum monsters (9.01%) werden positief bevonden in Ag-ELISA. Bij mensen werden, 4.99% (215 op 4306) antigeen positieve sera gevonden en coprologisch onderzoek van 1935 monsters toonde daarentegen 30 positieve gevallen (1.55%) aan. Het beperkte aantal volwassen lintwormen dat kon gerecupereerd worden (29) laat geen veralgemeende besluiten toe aangaande het voorkomen per soort, maar in totaal werden 21 specimen geïdentificeerd als *T. saginata* en slechts 8 als *T. solium*. Deze gegevens werden besproken in het kader van de epidemiologie van humane cysticercose.

Hoofdstuk 5 geeft de resultaten weer van een tweede epidemiologische studie uitgevoerd in een endemische gemeenschap in de zuidelijke Andes te Ecuador. Omwille van de arme levensomstandigheden werd in die streek een hogere prevalentie van *T. solium* cysticercose verwacht dan in het noorden. Deze studie werd uitgevoerd in Limones, een landelijke gemeenschap met een bevolking van 1059 mensen en 1148 varkens. De deelname van de bevolking was vrij hoog: 800 serum en 958 feces monsters werden verzameld. Tong-inspectie werd uitgevoerd op 646 varkens. Circulerend antigeen werd gevonden d.m.v. Ag-ELISA in 2.25% van de sera van mensen. Intestinale taeniase werd gevonden in 1.46% van de monsters door de formaline-ether methode. Na behandeling en recuperatie van de lintworm fragmenten werden al deze geïdentificeerd als *T. solium*. Cysticercose werd gevonden bij 3.56% van de varkens na tong-inspectie; het veelvuldig voorkomen van *Taenia hydatigena* liet niet toe om besluiten te trekken uit de resultaten van de circulerend antigen detectie. Als aanvullende test werd de "enzyme linked immunoelectrotransfer blot (EITB)" uitgevoerd op een subset van 100 menselijke sera ter bevestiging van de Ag-ELISA resultaten. Ook werden 100 sera van varkens onderzocht d.m.v. EITB. Hieruit bleek dat 43 en 74% van respectievelijk de mensen en de varkens antilichamen hadden tegen *T.*

solium cysticerci. Dit leidde tot de voorlopige conclusie dat, ondanks de hoge graad van blootstelling aan *T. solium* – volgens de EITB resultaten – het aantal gevallen van actieve cysticercose – zoals aangetoond door Ag-ELISA -, vrij laag is. Dit zou kunnen duiden op een vorm van endemische stabiliteit, die door verdere studies met gebruik van beide technieken, zou kunnen bevestigd worden.

In de algemene discussie (**Hoofdstuk 6**), worden de resultaten van de vorige hoofdstukken besproken. De meest belangrijke observaties en implicaties voor het begrijpen van het taeniasis-cysticercosis complex worden toegelicht. Het belang van zeer specifieke en gevoelige diagnostische hulpmiddelen, op basis van PCR, wordt benadrukt.

Een vergelijk tussen de studies in het noorden en het zuiden duidt op een vorm van endemische stabiliteit in de bestudeerde zuidelijke gemeenschap. Veelvuldig contact contrasteerde met weinig gevallen van actieve cysticercose zoals aangetoond door respectievelijk antilichaam en antigeen detecties. Ondanks de naar verhouding met het zuiden, weinige gevallen van volwassen *T. solium*, is de prevalentie van actieve cysticercose er hoger. Beide studies moeten echter beschouwd worden als voorbereidend onderzoek en moeten bevestigd worden door o.m. het gelijktijdige gebruik van antilichaam en antigeen detectie in verschillende locaties en dit gecombineerd met een verbeterde opsporing van de volwassen lintwormen. Een aantal opties voor controle van de ziekte worden voorgesteld en de mogelijkheid om deze toe te passen in Ecuador besproken.

RESUMEN

El complejo teniasis-cisticercosis es causada por *Taenia saginata* y *Taenia solium* cuyo hospedador definitivo es el hombre mientras que los bovinos y los cerdos actuan como hospedadores intermediarios, respectivamente. Los metacestodos de *Taenia* spp. se ubican principalmente en el tejido muscular y la larva de *T. solium* puede afectar al tejido nervioso y puede causar la neurocisticercosis en el hombre la cual, es considerada la causa más comun de epilepsia adquirida en países endémicos. El consumo de carne de cerdo poco cocida, la cría libre de los cerdos con acceso a las heces humanas son los factores claves en la ocurrencia de la *T. solium*. En el Ecuador, en particular, *T. solium* es endémica en la región Andina y rara o ausente en la mayoría de las regiones costeras. Este estudio se enfoca en el entendimiento de la epidemiología del complejo teniasis-cisticercosis en dos regiones Andinas del Ecuador así como, en el mejoramiento de las técnicas diagnosticas como herramientas de apoyo.

Esta tesis esta dividida en cuatro secciones: (1) una introducción general donde se encuentran formulados los problemas, una revisión de literatura que apoya y conduce a la descripción de los objetivos de este estudio (**capítulo 1**); (2) el desarrollo de técnicas de PCR altamente sensibles y específicas para la detección y la diferenciación de *T. solium, T. saginata* e *Hymenolepis nana* utilizando el ADN de los parásitos adultos (**capítulo 2**) y ADN de *Taenia* extraídos de las muestras fecales (**capítulo 3**); (3) la valoración de la situación de la teniasis-cisticercosis en dos áreas endémicas de los Andes Ecuatorianos (**capítulos 4** y **5**) y (4) una discusión general donde se comentan los resultados en una perspectiva más amplia (**capítulo 6**).

En la introducción general, en el **capítulo 1**, se subraya el problema de la teniasis y la cisticercosis con un enfoque a realidad Ecuatoriana y su postura frente al presente estudio. En la revisión de literatura se proporcionan aspectos importantes relacionados con la cisticercosis: el origen, la historia, la morfología, el ciclo de vida y la epidemiología así como, las principales deficiencias en el control y el diagnóstico. Del mismo modo, la revisión enfatiza en aspectos epidemiológicos y en las técnicas de diagnóstico que se usan para estimar la prevalencia de la teniasis y la cisticercosis, con especial interés en las pruebas de PCR para el diagnóstico de la teniasis. Al final de este capítulo se presenta los objetivos que ayudan principalmente al mejor entendimiento del problema de la teniasis-cisticercosis en Ecuador a través de la aplicación de pruebas diagnósticas mejoradas.

El **capítulo 2** describe el desarrollo de una prueba basada en PCR para el apoyo de los métodos de diagnóstico de uso corriente como son la morfología y la prueba de iso-enzimas en proglótidos. La prueba modificada de PCR se complemento con el analisis de enzimas de restricción. En la prueba de PCR se rediseño de uno de sus primers para reducir las amplificaciones inespecíficas evidenciadas cuando se usaron muestras de campo. El uso de estos nuevos primers altamente sensibles y específicos y el uso de la enzima de restricción D*de*I condujo al desarrollo de una prueba de diagnóstico que permitió una clara diferenciación del ADN extraído de los proglótidos de *T. saginata* y *T. solium* obtenidas en muestreos de campo. Esta prueba confirmó la presencia de *T. saginata* en Ecuador.

En el **capítulo 3**, basado en el capítulo anterior, se desarrolló un PCR-RFLP mejorado para diferenciar el ADN de *T. solium* y *T. saginata* extraídos a partir de heces fecales. Nuevos primers fueron probados los cuales, demostraron una mejor especificidad en la amplificación del ADN parasitario presente en las heces fecales. Para este propósito, se compararon tres diferentes protocolos de extracción de ADN usados en heces fecales: El kit comercial "QIAamp® DNA stool mini kit"; el método de Boom que utiliza una matriz "guanidium thiocyanate/zirconium" y el aislamiento de ADN basado en la técnica de captura de secuencias (Mangiapan). La prueba de PCR-RFLP fue usada en extractos fecales de 35 portadores conocidos de *Taenia* spp. colectados antes del tratamiento antihelmíntico. Los protocolos de QIAgen y Boom mostraron bandas inespecíficas en muestras negativas de Ecuador mientras que, en el protocolo de Mangiapan estas bandas no fueron observadas aunque, estas fueron evidenciadas en muestras negativas de Bélgica, las que complicaron la interpretación del PCR. El PCR-RFLP en muestras fecales de 35 portadores de *Taenia* detectó 27 muestras positivas para *T. solium* y 8 para *T. saginata*. La restricción con la enzima D*de*I dio claros y distintivos perfiles para *T. solium*, *T. saginata* e *H. nana*; los primers desarrollados en este trabajo no amplifican *Hymenolepis diminuta* ni *Diphyllobothrium latum*. Esta nueva técnica de PCR-RFLP tiene un gran potencial como una herramienta de apoyo en el diagnóstico específico de la *Taenia* spp. luego de la preselección de muestras a través del uso de pruebas coprológicas estándares. Sin embargo, la presencia de bandas inespecíficas en algunas muestras requieren de futuras mejoras en esta prueba.

El **capítulo 4** describe la situación epidemiológica en el Norte de los Andes Ecuatorianos. Además, constituye el primer reporte de *T. saginata* en Ecuador, haciendo necesaria una urgente reconsideración de algunas asunciones en la epidemiología del complejo teniasis-cisticercosis en este país. Dentro de este marco, se recopilaron datos de la infestación de *T. solium* y *T. saginata* en el hombre y los animales en las provincias de Pichincha e Imbabura en la región Andina, al norte de la capital Quito. A través de la inspección post mortem se encontró que, de 806 bovinos inspeccionados, 3 carcasas bovinas (0.37%) presentaron metacestodos de *T. saginata*, sin embargo, 35 sueros de entre 869 analizados (4.03%) mostraron antígenos en circulación en un ELISA sándwich basado en el uso de anticuerpos monoclonales (Ag-ELISA). La cisticercosis porcina fue detectada en 15 de 2,896 (0.52%) carcasas inspeccionadas y en 93 de 1032 sueros analizados (9.01%) fueron positivos al Ag-ELISA. En el hombre, el 4.99% (215 de 4,306) fueron encontrados positivos a antígenos, mientras que, de la examinación coprológica de 1,935 heces resultaron 30 casos positivos (1.55%). El limitado número de parásitos adultos (29) colectados no permite afianzar conclusiones en relación a la proporción de cada especie, no obstante, un total de 21 especimenes fueron identificados como *T. saginata* y 8 como *T. solium*. Estos datos fueron discutidos en el marco de la epidemiología de la cisticercosis humana.

En el **capítulo 5** se proporcionan los datos de un segundo estudio epidemiológico ejecutado en una comunidad endémica sureña de la región Andina del Ecuador. Las condiciones de pobreza en esta región hacían presumir tasas de prevalencia de cisticercosis por *T. solium* más altas que en el Norte. El estudio fue llevado a cabo el Limones, una comunidad rural en la provincia de Loja con una población de 1,059 pobladores y 1,148 cerdos. La participación fue alta y se recolectaron 800 sueros y 958 muestras fecales. Adicionalmente, 646 cerdos fueron inspeccionados en la lengua. En la población humana, se detectó que el 2.25% de sueros presentaba antígenos en circulación diagnosticados por Ag-ELISA. La teniasis fue detectada en el 1.46% de las muestras analizadas por la técnica de formol-éter; luego del tratamiento y la recuperación de fragmentos de *Taenia*, todos fueron identificados como *T. solium*. La cisticercosis porcina fue diagnosticada en el 3.56% de los cerdos examinados en la lengua; desafortunadamente, por las reacciones cruzadas con *Taenia hydatigena* que presenta la técnica de Ag-ELISA no fue utilizada en cerdos. Adicionalmente, el

"enzyme linked immunoelectrotransfer blot" (EITB) fue aplicado en un subgrupo de 100 humanos para confirmar los resultados de Ag-ELISA al igual que cien sueros de cerdos. De entre estos resultados, el 43 y el 75% de sueros humanos y porcinos, respectivamente, tuvieron anticuerpos contra cisticercosis de *T. solium*. Se concluye que, contrariamente a la alta exposición de la población humana a *T. solium* como fue sugerido por el EITB, el número de casos de cisticercosis activa, diagnosticados por Ag-ELISA fue bajo, lo cual podría indicar una estabilidad endémica. Se sugiere en un futuro el uso de métodos de diagnóstico complementarios para un mejor entendimiento de la epidemiología de *T. solium.*

En la discusión general, (**capítulo 6**), se discute los resultados de los capítulos, destacando los más importantes hallazgos e implicaciones para la comprensión del complejo teniasis-cisticercosis. En este capítulo, se enfatiza la importancia en el uso de herramientas de diagnóstico altamente sensibles y específicas basadas en biología molecular. Las comparaciones entre las observaciones encontradas en el Norte y el Sur indican la presencia de una estabilidad endémica en la comunidad sureña estudiada. El contacto frecuente contrastado con la presencia de pocos casos de cisticercosis activa fue evidenciada por la detección de anticuerpos y antígenos, respectivamente. En el estudio realizado en el Norte, a pesar de que proporcionalmente existen menos adultos de *T. solium*, la prevalencia de cisticercosis activa fue alta. Los dos estudios realizados en esta tesis son preliminares y necesitan una futura confirmación sea por el uso simultaneo de técnicas de detección de anticuerpos y antígenos en varias localidades combinados con la detección mejorada de los portadores de *Taenia.* Finalmente se presentan y discuten las opciones para el control de esta enfermedad y su potencial uso en el Ecuador.

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