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The epidemiology of trichinellosis in Northern Vietnam

Vu Thi Nga

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Promoters:

Prof. Dr. Pierre Dorny

Prof. Dr. Nguyen Van De

Dr. Sarah Gabriël

Laboratory of Parasitology
Department of Virology, Parasitology and Immunology
Faculty of Veterinary Medicine, Ghent University
Salisburylaan 133, B-9820 Merelbeke

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LIST OF ABBREVIATIONS

AUC	Area under curve
bp	Base pair
CI	Confidence interval
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
dpi	Days post infection
EU	European Union
FAO	Food and Agriculture Organization
ICT	International Commission on Trichinellosis
IgA	Immunoglobulin isotype A
IgE	Immunoglobulin isotype E
IgG	Immunoglobulin isotype G
IgM	Immunoglobulin isotype M
kD	Kilodalton
OD	Optical density at a wave length
P	Probability
lpg	Larvae per gram
NCVD	National Centre for Veterinary Diagnosis
NIMPE	National Institute of Malariology Parasitology and Entomology
SDS - PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Spp.	Species
Sub DAH	Sub Department of Animal Health
WHO	World Health Organization
wpi	Weeks post infection

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CHAPTER 1

TRICHINELLOSIS - A LITERATURE REVIEW

1.1. General introduction

Trichinellosis is an important foodborne parasitic zoonosis caused by nematodes belonging to the genus *Trichinella*. These parasites are among the most widespread zoonotic pathogens in the world (Murrell and Pozio, 2011; Pozio et al., 2009).

Trichinella spp. was first discovered in 1835, by James Paget and Richard Owen in human muscles (United Kingdom, London) and was found later in 1846 by Joseph Leidy in swine muscles (Philadelphia, USA) (Murrell et al., 2000; Pozio and Zarlenga, 2005). Trichinellosis has formerly been classified as a list B disease by the World Organization for Animal Health (OIE, 2000). *Trichinella* spp. have adapted to an extremely wide range of hosts including domestic animals, wildlife, and humans. Most species only infect mammals, but non-encapsulating species can also infect birds and reptiles (Pozio and Murrell, 2006). Until now, nine species and three genotypes have been identified (Krivokapich et al., 2008; Mitreva et al., 2011; Pozio and Zarlenga, 2013) and the genus can be divided in two distinct groups, characterized by non-encapsulated and encapsulated clades (Pozio et al., 2009; Pozio et al., 2001).

Trichinella spp. are transmitted by ingestion of raw or undercooked meat that contains infective larvae. In human, while most cases have only mild symptoms after infection, ingestion of high numbers of larvae can cause serious disease, as well as death. Trichinellosis has been historically associated with pork; however, it is now emerging as a more widespread foodborne zoonosis as the consumption of game meat increases (Murrell and Pozio, 2011). Human trichinellosis has been reported in 55 countries around the world. *Trichinella* spp. have been found in domestic animals (mainly pigs) of 43 countries and in wildlife of 66 countries (Pozio, 2007). Trichinellosis is not only a public health hazard (Murrell and Pozio, 2011), it also represents an economic problem in animal production and food safety (Gajadhar and Gamble, 2000; Gajadhar et al., 2009). The global incidence of human trichinellosis was 65,818 cases with 42 deaths reported from 42 countries in a 24 year period (1986-2009) (Murrell and Pozio, 2011). The global number of disability- adjusted life years (DALYs) due to trichinellosis was estimated to be 76 per billion persons per year (Devleesschauwer et al., 2014). The global cost of detecting *Trichinella* infection in pigs was estimated to be \$3.00 (€2.10) per pig, which equals \$570 million (€388 million)/year for 190 million domestic pigs slaughtered annually in 15

European countries (Gottstein et al., 2009). In the EU, the budget spent on control of *Trichinella* infections in domestic pigs is disproportionate to its occurrence, which has become negligible in controlled housing conditions (Torgerson, 2013). Therefore, the EU is aiming at moving from systematic controls at slaughter to a risk-based approach.

Trichinellosis is endemic in some areas of the world where failure of veterinary control due to human errors or social upheavals occurs (Gajadhar and Gamble, 2000; Pozio, 2007). With the expansion of global trading and the increase in animal production, there is a potential for outbreaks of trichinellosis in virtually any country, where the veterinary and medical services are often not familiar with the infection (Murrell and Pozio, 2011). Political, economic changes, and wars can contribute to an increase of *Trichinella* infection among various hosts including human (Gajadhar and Gamble, 2000; Gajadhar et al., 2009).

The aim of this literature review is to present an overview of the current knowledge on *Trichinella* spp. infections, with emphasis on the taxonomy, epidemiology, molecular epidemiology, immunological response, diagnosis, treatment, and control. In chapters 2, 3, and 4 the experimental work of this thesis is presented. In chapter 5, the findings, options for control and future perspectives are discussed.

1.2. *Trichinella* spp.

1.2.1. Taxonomy

The genus *Trichinella* belongs to the phylum Nematoda, class Adenophorea, order Trichocephalida, and family Trichinellidea (Soulsby, 1982). Since *T. spiralis* was discovered by Paget and Owen 1835 (Murrell et al., 2000) 180 years ago, nine species as well as 3 genotypes have been described in the *Trichinella* genus (Pozio and Zarlenga, 2013). Within the genus two distinct groups are recognized, characterized by clades of species that produce encapsulated and non-encapsulated muscle larvae (Pozio et al., 2009; Pozio and Zarlenga, 2013) (Table 1.1). The taxonomy of genus *Trichinella* is now readily established and isolates of each genotype can be easily propagated under laboratory conditions (Murrell et al., 2000; Pozio and Zarlenga, 2013).

The morphology of all species and genotypes of the genus *Trichinella* are indistinguishable at all developmental stages (Pozio and Zarlenga, 2005). The usual

morphological criteria for *Trichinella* species have not proved adequate for *Trichinella* spp. except for non-encapsulating species (Pozio et al., 1992a) (Table 1.2). Identification of *Trichinella* species and genotypes are now primarily based on major biochemical and molecular characteristics (Murrell et al., 2000; Pozio and Zarlenga, 2013).

The encapsulated clade of parasites includes six species: *Trichinella spiralis*, *Trichinella nativa*, *Trichinella britovi*, *Trichinella murrelli*, *Trichinella nelsoni* and *Trichinella patagoniensis*. Three additional genotypes (*Trichinella* T6, T8, T9) are yet to be defined taxonomically. *Trichinella* T6 has not yet been assigned a taxonomic position because of its biological and genetic similarity to *T. nativa*. *Trichinella* T8 is capable of interbreeding with *T. britovi* under laboratory conditions. *Trichinella* T9 is phylogenetically related to *T. murrelli* (Pozio and Zarlenga, 2013). All these species/genotypes induce the development of a collagen capsule around the larva during the muscle phase of the infection (Krivokapich et al., 2008; Murrell et al., 2000; Pozio and Zarlenga, 2005). The host species of the encapsulated group comprise only mammals (Pozio et al., 2004b; Pozio and Zarlenga, 2005). The clade of *Trichinella* spp. that produces non-encapsulating muscle larvae comprises *Trichinella pseudospiralis*, *Trichinella papuae*, and *Trichinella zimbabwensis*. These species do not encapsulate following muscle cell differentiation (Gottstein et al., 2009; Pozio and Zarlenga, 2013). One (*T. pseudospiralis*) of the three species infects mammals and birds while two species (*T. papuae* and *T. zimbabwensis*) infect mammals and reptiles (Pozio et al., 2004a; Pozio and Zarlenga, 2013).

Among all these species, *T. spiralis* is the most important one, both in terms of distribution and zoonotic potential. *T. spiralis* has a cosmopolitan distribution and is transmitted to humans through eating raw or undercooked meat (Mitreva and Jasmer, 2006; Murrell and Pozio, 2011; Pozio, 2013). This species has been detected in a broad range of mammal hosts: domestic animals (pigs, cats, dogs), sylvatic animals (wild boars, sylvatic carnivores) and synanthropic animals (brown rat, armadillo) (Pozio et al., 2001; Pozio, 2013; Pozio and Zarlenga, 2013). *T. spiralis* is the most important etiological agent of human trichinellosis that leads to high morbidity around the world. The pathogenicity of *T. spiralis* is higher than that of other species due to the higher number of newborn larvae produced by the females and the stronger immune reaction induced in humans (Mitreva et al., 2011; Pozio and Zarlenga, 2013).

Table 1.1. Taxonomy, distribution, and pathogenicity of the 12 *Trichinella* genotypes currently identified (Ancelle et al., 1988; Bruschi and Murrell, 2002; Capo and Despommier, 1996; Dworkin et al., 1996; Garkavi, 1973; Gottstein et al., 2009; Hulinska et al., 1985; Krivokapich et al., 2008; Nagano et al., 1999; Nelson et al., 2003; Owen et al., 2005; Pozio et al., 2009; Pozio and Zarlenga, 2013; Pozio and La Rosa, 2000; Pozio et al., 1999a; Pozio et al., 1999b; Pozio and La Rosa, 1998; Pozio and La Rosa, 1991a; Pozio, 1991b; Schellenberg et al., 2003; Serhir et al., 2001; Zarlenga et al., 1999).

Year	<i>Trichinella</i> species and genotype	Distribution	Host	Infectivity for pigs	Pathogenicity in humans
1835	<i>T. spiralis</i> (T1)	Cosmopolitan	Domestic and sylvatic mammals	++++	High
1972	<i>T. nativa</i> (T2)	Arctic and subarctic areas of America, Asia, Europe	Sylvatic carnivores	-	Moderate
1992	<i>T. britovi</i> (T3)	Temperate areas of Europe and Asia, Northern and Western Africa	Sylvatic mammals and seldom domestic pigs	+	Low
1972	<i>T. pseudospiralis</i> (T4)	Cosmopolitan	Sylvatic mammals and birds, domestic pigs	+	Low
2000	<i>T. murrelli</i> (T5)	United States and Southern Canada	Sylvatic carnivores	-	Moderate
1992	T6	Canada, Alaska, Rocky Mountains, and Appalachian Mountains in US	Sylvatic carnivores	Unknown	Low
1972	<i>T. nelsoni</i> (T7)	Sub-saharan Africa	Sylvatic carnivores	+	High
1992	T8	South Africa and Namibia	Sylvatic carnivores	Unknown	Unknown
1999	T9	Japan	Sylvatic carnivores	Unknown	Unknown
1999	<i>T. papuae</i> (T10)	Papua New Guinea, Thailand	Wild boars, salt water crocodiles	+	High
2002	<i>T. zimbabwensis</i> (T11)	Zimbabwe, Mozambique, Ethiopia, South Africa	Nile crocodiles, monitor lizards	+	Unknown
2008	<i>T. patagoniensis</i>	Argentina	Cougars	Low	Unknown

Table 1.2. Major characteristics of *Trichinella* species (Bruschi and Murrell, 2002; Gottstein et al., 2009; Hurnikova, 2004; Krivokapich et al., 2012; Murrell et al., 2000; Pozio et al., 2002a; Pozio et al., 1992b; Pozio and La Rosa, 2000; Pozio and Zarlenga, 2005)

	<i>T. spiralis</i>	<i>T. nativa</i>	<i>T. britovi</i>	<i>T. nelsoni</i>	<i>T. murrelli</i>	<i>T. pseudospiralis</i>	<i>T. papuae</i>	<i>T. zimbawensis^a</i>	<i>T. patagoniensis</i>
Morphology (length in mm)									
Adult male	1.0-1.8	1.0-1.8	0.99-1.91	1.0-1.8	0.91-1.089	0.6-0.9	0.81-1.06	1.0-1.3	0.6-0.9
Adult female	1.37-3.7	1.3-3.7	2.2-3.4	1.3-3.7	1.55-1.81	1.26-2.10	0.88-1.31	1.7-1.9	0.9-1.6
Muscle larvae	0.61-1.0	0.61-1.0	0.86-1.0	0.61-1.0	0.84-0.92	0.62-0.76	0.88-1.38	1.06-1.09	0.6-0.97
Encapsulated in muscle	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes
Newborn larval production in 72h	110±2.6	29.8±2.0	47.4±1.2	47.0±3.2	30.6±1.9	48.5±3.1	Unknown	Unknown	25.91±8.86
Nurse cell development (dpi)	16-34	20-30	24-42	34-60	24-70	N	N	N	N
Freeze resistance	0 day at -30 °C	1.5-2.5 days at -30 °C	1.0 day at -30 °C	0 day at -30 °C	0 day at -30 °C	0 day at -30 °C	Unknown	4 weeks at -5 °C	3 months at -5 °C

^a: Morphological features of *T. zimbawensis* collected from the gut of mice infected with larvae isolated from crocodile muscles.

N: Not applicable.

1.2.2. Life cycle

All stages of the life cycle of the genus *Trichinella* occurs within a single host (Gottstein et al., 2009; Mitreva and Jasmer, 2006) (Figure 1.1). The cycle starts by the ingestion of muscle larvae; these are released from the surrounding tissue and their capsules by the action of pepsin and hydrochloric acid in the stomach of the new host. The larvae are then carried to the small intestine where they invade the columnar epithelium and penetrate a row of columnar epithelial cells. This migration is followed by four molting cycles of the larvae (between 10 and 28 h post ingestion), which leads to a transformation into the adult worm. The adult stage develops within 2 days after ingestion of the larvae. After copulation, the females begin to produce newborn larvae (NBL) on the sixth or seventh day post ingestion. The number of NBL shed depends on the immune status of the infected host, the host species, and on the infecting species of *Trichinella* (Capo and Despommier, 1996; Pozio et al., 1992b). It is estimated that one female adult worm can produce as many as 500 to 1,500 newborn larvae during its life span before a combination of immune responses forces their expulsion from the small intestine. The time period from the larvae being released from the cyst in the stomach of the new host up to the production of the new generation is called the enteral or intestinal phase of the life cycle (Capo and Despommier, 1996).

NBL migrate directly into the intestinal submucosa and are carried by the circulatory system (both lymphatic and blood vessels) to various organs, including the myocardium, brain, lungs, retina, lymph nodes, pancreas, and the cerebrospinal fluid. However, only the larvae that invade the cells of the skeletal muscles will survive (Capo and Despommier, 1996; Gottstein et al., 2009). *Trichinella* larvae induce the muscle cells to modify their structure into “nurse cells” in which the larvae will grow. Within the nurse cells, NBL develop to the infective muscle-stage larvae without molting (size is 0.65 to 1.45 mm in length and 0.026 to 0.040 mm in width). In most *Trichinella* species they gradually encyst and develop into the infective stage in about 21 to 30 days after infection. In humans, calcification of the cyst begins about five months after infection and is usually completed after 18 months (Bruschi and Murrell, 2002). For *T. spiralis*, the calcification of encapsulated larvae may take place later, after 6 months. Calcification of the collagen capsule occurs first, followed by the nurse cell and the larva. The whole process may lead to the death of the larvae, but not all larvae, as some larvae may survive for years in the same host (Gottstein et al., 2009).

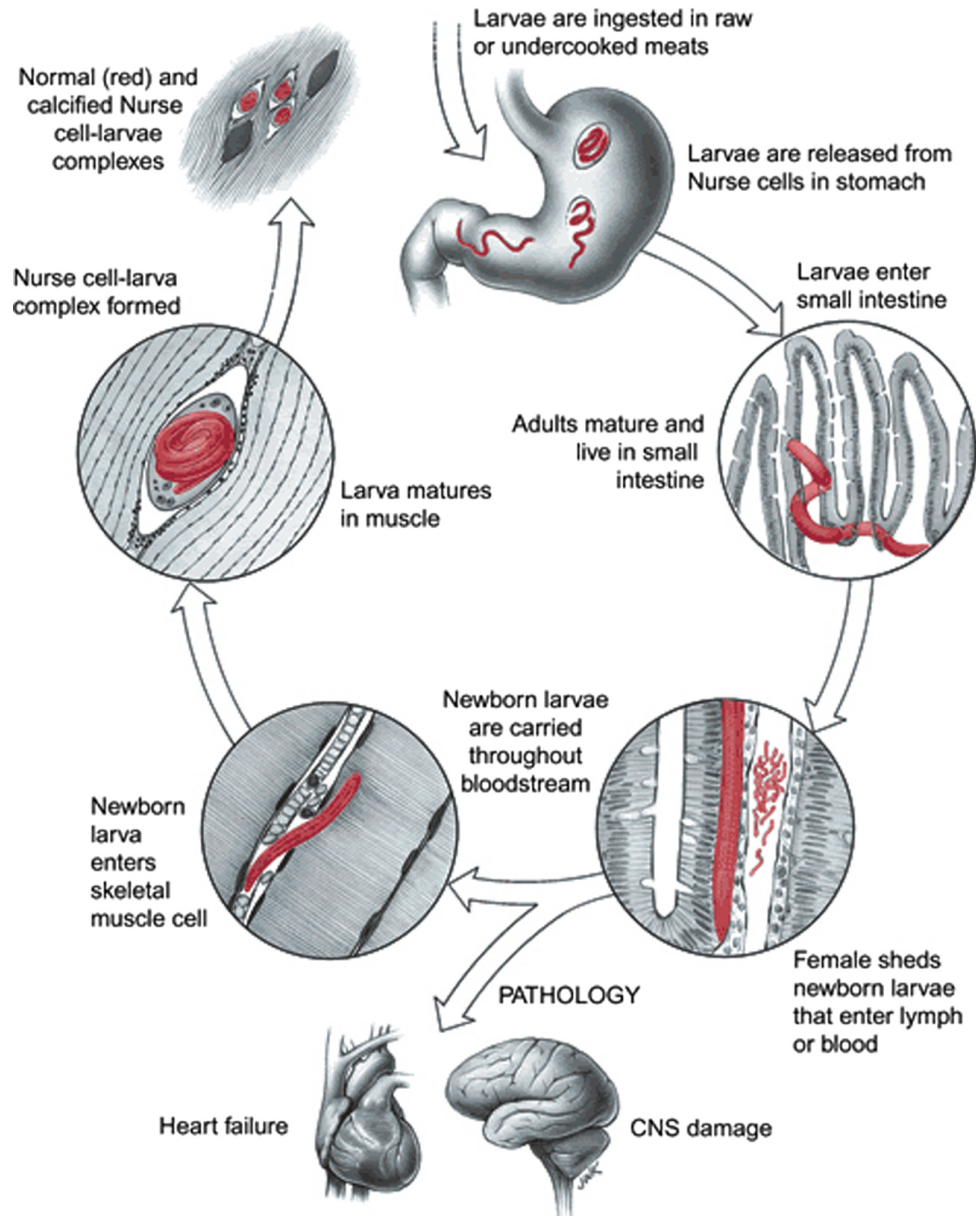


Figure 1.1. *Trichinella* spp. life cycle (Mitreva and Jasmer, 2006).

In three species, *T. pseudospiralis*, *T. papuae*, and *T. zimbabwensis*, the muscle larvae do not induce the formation of a collagen capsule. The larvae appear to be non-pathogenic for the natural hosts (excluding humans) unless very large numbers are involved (Pozio, 2013; Pozio and Zarlenga, 2005). Larval infectivity can be retained for many years, depending on the host species: longevity can be as long as 39 years in humans or over 20 years in polar bears (Froscher et al., 1988; Gottstein et al., 2009; Kumar et al., 1990). The time period from the NBL penetration in the muscle cell up to larval survival in the muscles is called the muscle phase of the life cycle. This hypobiotic stage is maintained until muscles containing larvae are being ingested by a new host (Gottstein et al., 2009).

1.2.3. Clinical disease

1.2.3.1. Clinical disease in humans

Trichinellosis is a serious disease with a wide variation of clinical features, causing both acute and chronic disease stages (Kociecka, 2000). The clinical signs and symptoms in humans relate to larvae in all phases of the life cycle (Capo and Despommier, 1996; Mitreva and Jasmer, 2006) and are affected by the number of larvae ingested, the sex, age, and immune status of the patient (Bruschi and Murrell, 2002). The main clinical signs and symptoms of trichinellosis are myalgia, diarrhea, fever, facial edema, and headache (Mitreva and Jasmer, 2006) (Figure 1.2). Clinical signs and symptoms will disappear within 2-8 weeks after treatment (Capo and Despommier, 1996; Murrell and Pozio, 2011).

1.2.3.1.1. Incubation period

Based on the disease severity, five clinical forms of trichinellosis can be recognized: severe, moderately severe, benign, abortive, and asymptomatic forms. The length of the incubation period depends on the severity of disease. When the course of infection is more severe, the incubation period is very short and death may occur (Bruschi and Murrell, 2002). The incubation period is less than one week for the severe form, two weeks for the moderately severe form, and at least 3-4 weeks for the benign and abortive forms (Dupouy-Camet et al., 2002).

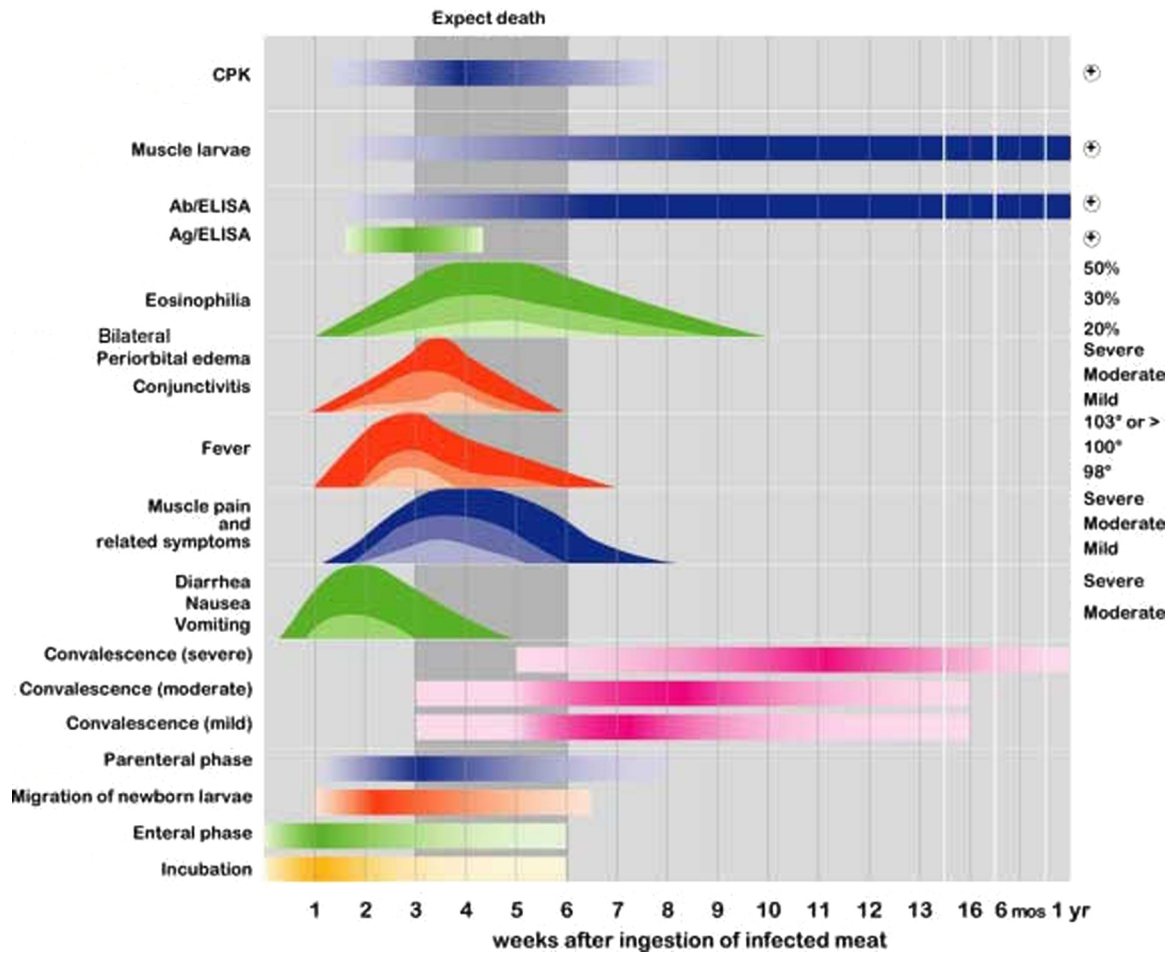


Figure 1.2. Summary of the main clinical signs and symptoms, laboratory findings, and diagnostic test results for patients suffering from mild (light color) to severe (most intense color) clinical of trichinellosis (Mitrev and Jasmer, 2006).

1.2.3.1.2. Acute phase

The clinical course of the acute phase of the infection is characterised by an intestinal and a muscle phase. Clinical signs and symptoms are observed for at least two to seven days, but may persist for many weeks (Bruschi and Murrell, 2002). At the start, non-specific signs and symptoms are manifested such as, uneasiness, headache, fever, fever-associated chills, and gastrointestinal disorders (Gottstein et al., 2009). These signs and symptoms are associated with the intestinal phase of the life cycle. They are followed by clinical signs and symptoms that are associated by the muscular phase, consisting of persistent fever, facial edema, muscle pain, and severe asthenia that may last for several weeks (Dupouy-Camet et al., 2002). The fever usually persists for 1 to 3 weeks, depending on the infection dose and the severity of the disease (Gottstein et al., 2009).

Neurological complications are rather rare in trichinellosis patients. Anisocoria and facial nerve paresis have been observed in such severe cases. Most brain abnormalities visualized by computed tomography or magnetic resonance imaging as well as the clinical signs and symptoms disappear in 4 to 8 wpi (Gottstein et al., 2009).

1.2.3.1.3. Chronic phase

Encephalitis, bronchopneumonia, and sepsis are the results of bacterial super-infections that may appear at a late stage of acute disease (Gottstein et al., 2009).

Whether or not a chronic phase of trichinellosis exists is actually still under debate (Dupouy-Camet et al., 2002). Chronic trichinellosis may consist of feelings of general discomfort that can persist for months or even years after the acute stage. Also, formication, numbness, excessive sweating, as well as impaired muscle strength, conjunctivitis, impaired coordination, and persistence of specific IgG antibodies have been reported in some patients up to ten years post infection; whereas in other patients, viable larvae were detected in muscles without presentation of clinical signs or symptoms up to 39 years post infection (Dupouy-Camet et al., 2002; Gottstein et al., 2009).

1.2.3.2. Clinical disease in animals

Trichinella spp. infections occur in many carnivorous and omnivorous animal species, but infections in animals only rarely result in clinical signs (Schuppers et al., 2010b). In pigs given different infection dosages of *T. spiralis* larvae, no significant differences were observed in rectal temperature and cardiac and respiratory rates when compared to uninfected control animals (Gamble et al., 2000; Ribicich et al. 2007). In contrast, significant differences were detected in blood hemoglobin concentration and eosinophil counts, as well as in values of liver enzymes. Also, inoculated pigs showed significantly lower weight gains when compared with uninoculated controls (Ribicich et al. 2007). Mild gastrointestinal disturbances were seen in dogs and cats during the first week of infection after experimental inoculation with 7500 larvae of *T. spiralis* (Bowman et al., 1993). In horses, transient myalgia and an increase in rectal temperature were observed only after inoculation of a massive dose of first stage *T. spiralis* larvae (Soule et al., 1989).

1.2.4. Immunological response

1.2.4.1. Immunodominant antigens

During the *Trichinella* spp. life cycle, distinctive antigens are produced by each stage of the parasite, including newborn larvae, muscle larvae, and adult worms. *Trichinella* stage-specific antigens can induce specific host immune responses, including eliciting a host's protective immune response. Among *Trichinella* antigens, *Trichinella* muscle larva antigens were shown to participate in the induction of immune responses that may protect, at least in part, the host from a subsequent *Trichinella* infection (Bien et al., 2013).

Trichinella antigens can be divided into surface, excretory/secretory (E/S), and somatic components. Surface antigens are mainly constituents of the outer cuticle although secretions from inner parts of the body wall as well as from the oesophagus can temporarily accumulate on the surface. E/S antigens come mainly from the excretory granules of the stichosome and the cuticles (membrane proteins) (Wang et al., 2014), whereas somatic constitutive antigens come from the internal parts of the worms (Dea-Ayuela and Bolas-Fernandez, 1999). Several antigens are useful in the immunodiagnosis of trichinellosis (see Section 1.2.5.4.1).

1.2.4.2. Immunological response in the host

1.2.4.2.1. Antibody response in the intestine

Most hosts exhibit a strong immunity to reinfection to *Trichinella* and this immunity generally occurs at the level of the intestine, resulting in the rejection of first stage larvae. The intestinal immunity and the time taken to expel larvae vary between host species (Azab et al., 1999). In horses, total immunity to reinfection is shown at the intestinal level (Boireau et al., 2000). However, the mechanisms regulating the immune response to a primary infection are less clear in humans (Bruschi, 2002). In mice, the levels of serum antibody are low during the intestinal phase of infection (Wakelin et al., 1994).

Primarily IgA, IgM, and IgG are found in the mucus (deVos et al., 1992). IgE antibodies are important in the early response to cysts, mediating both the recruitment of eosinophils to encysted larvae in muscles and reducing the numbers of muscle cysts. Binding of IgE to the

muscle larvae and evidence of increased cyst necrosis supported a role for IgE in immunity to primary infection with *T. spiralis* (Gurish et al., 2004).

1.2.4.2.2. Antibody response in blood

The time of seroconversion after a primary *Trichinella* infection is dependent upon the host species and the host' individual immune response, but also on the *Trichinella* species involved and the number of ingested larvae (Gottstein et al., 2009).

In humans, *Trichinella* antibodies can be detected from 15 to 60 dpi and may persist for more than 30 years after infection (Gomez-Morales et al., 2008). In the rat model, seroconversion was observed at 30 dpi and increased until 80 dpi (Franssen et al., 2011). In pigs, seroconversion does not occur before 3 to 4 wpi (Kapel, 2005; Mitreva and Jasmer, 2006; Nockler et al., 2000), with antibodies persisting for at least 6 months after infection with no decline in titre (Gajadhar et al., 2009). The wild boar antibody response to *Trichinella* resembles the domestic pig response (Kapel, 2001). *Trichinella* antibodies can be detected up to 8 months following infection in horses (Sofronic-Milosavljevic et al., 2005).

1.2.5. Diagnostic methods

Trichinellosis can be diagnosed using direct or indirect techniques. With direct methods, first-stage muscle larvae are visualized by microscopic examination of tissue (trichinoscopy) or on digested muscle sample. Indirect methods are based on testing for specific antibodies. The species level of *Trichinella* can be identified by molecular methods. Diagnostic methods are applied for individual diagnosis or for epidemiological studies on humans and animals, and for food safety testing on meat products (Gottstein et al., 2009).

1.2.5.1. Trichinoscopy

Trichinoscopy is the earliest method carried out for *Trichinella* meat inspection. It was introduced during the 19th century (Beck et al., 2005; Seidel, 1954; Venturiello et al., 1998). The method has been used to examine meat to protect consumers from pork-transmitted *Trichinella* infections (Gajadhar and Gamble, 2000). According to the OIE “Manual of Standards for Diagnostic Tests and Vaccines”, a small piece of muscle is compressed between two glass plates until it becomes translucent and then examined for the presence of

Trichinella muscle larvae using a dissecting stereo-microscope at 15-40 X magnification (Gajadhar et al., 2009; Gamble et al., 2000).

The advantage of this method is the low technical requirements for the laboratory; it is a simple procedure that is carried out with basic equipment. The disadvantages are the time-consuming procedure, the need for a trained technician for the microscopy work, and the lack of sensitivity of the method. Trichinoscopy is less sensitive than artificial digestion for the detection of larvae in tissues containing low numbers of *Trichinella* larvae. The sensitivity and specificity of trichinoscopy with 6 or less larvae per gram were estimated at 43.4 and 88%, respectively (Beck et al., 2005). Also, larvae of non-encapsulating species such as *T. pseudospiralis* are very difficult to detect by this technique. Because of these limitations, trichinoscopy is not recommended by the ICT, OIE or EU for the routine examination of carcasses from pigs, wild boar, and horses (Gajadhar et al., 2009). However, it is still the method of choice in many Southeast Asian countries (Barennes et al., 2008; Intapan et al., 2011).

1.2.5.2. Digestion methods

The artificial digestion method was applied for *Trichinella* detection as early as in 1897, when larvae were isolated from muscle tissue by pepsin-hydrochloric acid digestion (Gamble, 1998). Several modifications of the artificial digestion method have been published. Of these, six digestion methods for inspection of meat samples have been approved by EU legislation (Webster *et al.*, 2006). Up to date, the magnetic stirrer digestion method is preferred as the reference method (Official Journal of the European Union, 2005; Webster *et al.*, 2006), and has become the method of choice for routine slaughter inspection in most industrialized countries. The technique is widely used on pooled samples and can be employed in a variety of circumstances with a minimum of equipment (Webster et al., 2006). The official method usually uses the diaphragm pillars or tongue as sample specimens. The amount of muscle to be digested depends on the host and the predilection sites (Nockler et al., 2004). Samples of tissue are digested in an artificial gastric fluid containing 1% pepsin and 1% hydrochloric acid (final concentration 0.12 N). The ground or diced samples are stirred or shaken in the fluid at 44-46 °C for 30 min or longer. After letting the digest settle, the larvae are detected in the sediment by stereomicroscope or trichinoscope (Gamble, 1998). The length of the digestion step depends on the host species

and on the muscle sampled. Tongue, masseter muscle, and muscles from carnivores have to be digested for at least 1 h, not for 30 min as is standard (Nöckler, 2007).

The artificial digestion method can be used for testing a 10 g sample of diaphragm muscle from individual animals or a pooled sample of 100 g. In pigs, the standard sample size used for pooling is 1 g, but in horses at least 5 g samples are recommended (Boireau et al., 2000; Nockler et al., 2000). In endemic countries, the pooling of samples for digestion may not be feasible because of the likelihood of the need to re-test each sample individually in order to identify infected carcasses (Gajadhar et al., 2009). The isolation of larvae from muscle samples may not be carried out before 17 to 21 dpi because before this time larvae are not yet resistant to digestion. When non-encapsulating species are suspected as the aetiological agents, digestion methods must be performed carefully (Bruschi and Murrell, 2002).

The artificial digestion is more sensitive, efficient, and reliable than the trichinoscope method (Webster et al., 2006). The digestion method has a theoretical detection limit of less than 1 larva per gram muscle tissue, but it is usually set at 1-3 larvae per gram of tissue (Nockler et al., 2000; Forbes et al., 2003; Gajadhar et al., 2009). However, some studies indicate that the sensitivity of the muscle digestion method seems to be lower than that cited in the legislation (Beck et al., 2005; Gajadhar and Forbes, 2002; Gamble, 1999; Schuppers et al., 2010a; Vallee et al., 2007).

1.2.5.3. PCR

All species and genotypes of the genus *Trichinella* are morphologically indistinguishable at all developmental stages. Many methods have been developed for identification of *Trichinella* species and genotypes. Isoenzyme analyses were the first methods used (Flockhart et al., 1982; Zarlenga et al., 1999), but have been replaced throughout the years by polymerase chain reaction (PCR) based methods (Pozio and La, 2003; Pozio and Zarlenga, 2005; Zarlenga et al., 1999). They can be used for diagnostic confirmation of suspected larvae, for the taxonomic investigation of the *Trichinella* genus, for phylogenetic studies, and for studying the genetic variability at the level of a single larva (Zarlenga and La, 2000; Mitreva and Jasmer, 2006; Pozio and La, 2003). PCR-based methods include Random Amplified Polymorphic DNA-PCR (RAPD), multiplex PCR, Single Strand Conformational Polymorphism (SSCP), nested PCR, real time PCR, and PCR followed by

Restricted Fragment Length Polymorphism (RFLP) (Hunt, 2011; Guenther et al., 2008; Pozio and La, 2003; Zarlenga and La, 2000).

Three genes (nuclear small-subunit rDNA, mitochondrial large-subunit rDNA, and cytochrome oxidase I DNA) are commonly used for species identification (Zarlenga et al., 2006) (Table 1.3). The nuclear DNA elements such as, the internal transcribed spacers (ITS), are useful in species identification (Audebert et al., 2000). Currently, a sensitive and reproductive diagnostic method using mitochondrial rDNA as a reliable genetic marker has proven to correctly identify *Trichinella* species (Blaga et al., 2009). The cytochrome oxidase I DNA (CO1) gene has been referred as a DNA barcode, which is highly conserved; it has frequently been used for investigating intra and inter specific variations in *Trichinella* (Callejon et al., 2009; Nagano et al., 1999; Yang et al., 2008).

Table 1.3. Characteristics of the 9 identified *Trichinella* species and 3 genotypes (Bruschi and Murell, 2002; Nagano et al., 1999; Pozio et al., 2002a; Pozio et al., 2009).

<i>Trichinella</i> species	Molecular markers (bp) by multiplex PCR (Gene)
<i>T. spiralis</i>	173 (ESV)
<i>T. britovi</i>	127, 253 (ESV, ITS1)
<i>T. pseudospiralis</i>	310, 340, 360 (ESV)
<i>T. papuae</i>	240 (ESV)
<i>T. nativa</i>	127 (ESV)
<i>T. nelsoni</i>	127, 404 (ESV, ITS2)
<i>T. murrelli</i>	127, 316 (ESV, ITS2)
<i>T. zimbawensis</i>	264 (ESV)
<i>T6</i>	127, 210 (ESV, ITS1)
<i>T8</i>	127, 253 (ESV, ITS1)
<i>T9</i>	127, 253 (ESV, ITS1)
<i>T. patagoniensis</i>	127 (ESV)

ESV (Expansion segment 5), sequence belonging to domain 4 of the nuclear ribosomal gene.

ITS1 (Internal transcribed spacer 1), interspaced sequence 1 of the nuclear ribosomal gene.

ITS2: (Internal transcribed spacer 1), interspaced sequence 2 of the nuclear ribosomal gene.

1.2.5.3.1. *Random amplified polymorphic DNA-PCR (RAPD)*

The RAPD was the first PCR based method for *Trichinella* species identification. The method can be used to identify the species of a single *Trichinella* larva by using only a single arbitrary primer under nonspecific amplification conditions (Bandi et al., 1993; Mitreva and Jasmer, 2006). The advantages of the RAPD method are its speed, simplicity, and sensitivity (Pozio et al., 2009; Zarlenga and La, 2000), but due to using short PCR primers this method lacks specificity and is prone to contamination; moreover, the complex banding profiles complicate the differentiation (Zarlenga et al., 1999).

1.2.5.3.2. *Multiplex PCR*

Multiplex PCR was developed 15 years ago to identify five species of *Trichinella* and two additional genotypes (Zarlenga et al., 1999). This technique can distinguish at the level of a single larva, variations in the ITS region as well as in the gap region of the ESV of the large subunit ribosomal DNA (Gottstein et al., 2009; Zarlenga et al., 1999; Zarlenga and La Rosa, 2000). Based upon a single amplification and specific primers for each region, each genotype can be recognized by a specific amplification profile (Pozio and La, 2003; Zarlenga et al., 1999). The multiplex PCR is a sensitive, inexpensive, and rapid molecular method (Gottstein et al., 2009). However, when applied on samples of pooled larval DNA, this method can lead to ambiguous results due to concurrent infection with several *Trichinella* species in the same host (Zarlenga and Higgins, 2001).

1.2.5.3.3. *Real time PCR*

The first real time PCR technique for *Trichinella* detection was developed by Guenther in 2008. It uses either SYBR Green or Taq-man probe technologies and is based on the hybridization of specifically designed probe for that species (Cuttell et al., 2012; Mercedes Alonso, 2011). The real time PCR assay can be useful for pooled samples (Atterby et al., 2009; Cuttell et al., 2012; Guenther et al., 2008) or for the detection of *Trichinella* in meat samples (Mercedes Alonso, 2011).

The real time PCR method is highly sensitive and specific. It not only allows a qualitative detection and/or a quantitative measurement of parasite DNA (Mercedes Alonso, 2011; Cuttell et al., 2012), but also the simultaneous differentiation of isolates to the species or

genotype level (Cuttell et al., 2012; Guenther et al., 2008). It is a powerful tool in the food quality and security control (Mercedes Alonso, 2011). However, the main disadvantage of the real time PCR method is the high cost (Atterby et al., 2009; Cuttell et al., 2012; Guenther et al., 2008).

1.2.5.3.4. *Other molecular methods*

The PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism) method can also be used to differentiate *Trichinella* spp. (Kwon et al., 2001). The disadvantages of PCR-RFLP are its labour intensiveness and the safety measures that have to be taken because of the use of hazardous reagents such as, ethidium bromide or similar intercalating dyes that are mutagenic and dangerous for human health (Zarlenga et al., 1999)

The SSCP (Single Strand Conformational Polymorphism) method has also been used to differentiate species and to study intraspecies variation of *Trichinella* (Gasser et al., 1998). SSCP involves genotype-specific primers. Therefore, it generally produces unequivocal and reproducible results (Zarlenga et al., 1999). SSCP is a laborious method and needs radioisotope markers for visualization of the results (Zarlenga and La, 2000).

1.2.5.4. Serological assays for antibody detection

Serology has a great diagnostic value for human trichinellosis (Gomez-Morales et al., 2008). In animals, serological tests can be used for the presence of anti-*Trichinella* antibodies in the serum or in the meat juice for both ante mortem and post mortem examinations (Nockler et al., 2000). Serological methods are at present not recommended as a substitute for direct (pooled sample digestion) methods of individual carcasses at slaughter because they fail to detect early stages of infections (Kapel, 2005; Mitreva and Jasmer, 2006; Nockler et al., 2000). Serological methods are recommended for herd surveillance programmes and can be used in detecting ongoing transmission of *Trichinella* at the farm level (Gamble et al., 2004). They are also used for diagnostic confirmation in human patients and in epidemiological studies (Gomez-Morales et al., 2008; Gottstein et al., 2009).

1.2.5.4.1. Antigen

E/S antigens

The E/S proteins of *T. spiralis* muscle larvae are the most commonly used diagnostic antigens to detect anti-*Trichinella* IgG in serological assays. However, host antibodies to E/S antigens are generally not detectable until 4-5 wpi and therefore are not useful for detecting early infections (Gottstein et al., 2009; Liu et al., 2013). The sensitivity and specificity of serodiagnosis are dependent on the quality of the antigen used in the test. E/S antigen proved higher diagnostic potential than somatic antigen and higher sensitivity than tyvelose antigens. Most previous studies proved the importance of E/S antigen in accurate diagnosis of *Trichinella* infection (Gottstein et al., 2009; Escalante et al., 2004).

E/S antigens are recommended for use in ELISA and Western Blot (WB) for detecting anti-*Trichinella* antibodies by the International Commission on Trichinellosis (Wang et al., 2014). These predominant antigen epitope recognized by animals and humans infected with *T. spiralis* is the so-called TSL-1 (*Trichinella spiralis* first larvae) group (Gottstein et al., 2009). The quality of E/S antigens depends on the methods used for the cultivation of *Trichinella* muscle larvae and on the purification of the antigen (Gamble et al., 1983; Gamble et al., 1988; Korinkova et al., 2008; Murrell et al., 1986; Nockler et al., 1995). Using E/S antigens of muscle larvae of *T. spiralis*, the immunoblot format proved to be more specific than the conventional ELISA (Enzyme Linked Immunosorbent Assay) (Venturiello et al., 2000; Gottstein et al., 2009).

Tyvelose antigen

The principal constituents of E/S antigens are glycoproteins, glycan carriers that contain a unusual sugar, the tyvelose (3,6-dideoxy-D-arabinohexose). Tyvelose is one of the major highly specific immunodominant epitopes of *Trichinella* (Forbes et al., 2004; Gamble et al., 2004; Pozio et al., 2002b) and has proven to be specific for immunodiagnosis of trichinellosis (Escalante et al., 2004). The tyvelose antigen offers a synthetic alternative to E/S antigens in ELISA for trichinellosis diagnosis (Goyal et al., 2002; Moller et al., 2005) and it can be used for surveillance where specificity of detection is more relevant (Moller et al., 2005).

Antigenic recombinants

Antigenic recombinants derived from *T. spiralis* larvae have been applied in vaccine development and *Trichinella* antibody detection in humans and pigs (Nagano et al., 2008; Nuamtanong et al., 2012; Sun et al., 1994; Wang et al., 2013b). Many antigenic recombinants have been produced and tested in ELISA and WB, such as Ts21 protein, *T. spiralis* serpin (TsSERP), T668 recombinant protein (newborn larvae stage-specific gene of *T. spiralis*), serine proteases, and 53-kDa proteins (Li et al., 2013; Niu et al., 2005; Nuamtanong et al., 2012; Wang et al., 2009). The antigenic recombinant 53 kDa proteins are useful for early immunodiagnosis of trichinellosis (Nagano et al., 2008). However, the possibility of cross-reactions of recombinant antigens with other helminth diseases has so far been neglected (Wang et al., 2009).

Many serological diagnostic assays have been developed for detecting *Trichinella* antibodies. These include, immunofluorescence antibody test, ELISA, WB, complement fixation test, hemagglutination test, etc. (Nockler et al., 2000; Gottstein et al., 2009).

1.2.5.4.2. ELISA

In humans and animals, ELISA is the most commonly used serological test for detection of *Trichinella* infection. It measures the level of specific *Trichinella* antibodies in serum or muscle juice samples (Nockler et al., 2009). The most commonly used antigens in ELISA are E/S antigens released from *Trichinella* muscle larvae (Gottstein et al., 2009). The E/S ELISA shows a high sensitivity, but the specificity may be affected by cross-reactions with other parasitic diseases, in humans mainly if these persons originate from developing countries (Gomez-Morales et al., 2008; Gottstein et al., 2009) (Table 1.4). E/S ELISA allows the detection of as few as 1 larva per 100 g of muscle tissue (Gottstein et al., 2009).

ELISA has the advantage of being a rapid, simple, and relatively inexpensive method (Mitreva and Jasmer, 2006). However, a seropositive result in ELISA should be subjected to a confirmatory test before a final decision is made. Confirmation can be done by artificial digestion of a muscle sample in animals (Gottstein et al., 2009) or by an appropriate confirmatory serological method such as WB in humans and animals (Gomez-Morales et al., 2012; Frey et al., 2009).

1.2.5.4.3. Western Blot

WB is a useful method for the differential diagnosis of trichinellosis. A WB using E/S antigen from *T. spiralis* is generally used as the confirmatory test for ELISA positive samples (Nockler et al., 2009; Frey et al., 2009).

WB has been used for the detection of anti-*Trichinella* antibodies in animals, mostly in pigs (Frey et al., 2009) and horses (Sofronic-Milosavljevic et al., 2005; Frey et al., 2009). Many studies indicate that WB shows high sensitivity and specificity (Nockler et al., 2009; Frey et al., 2009; Yera et al., 2003) (Table 1.4) with a pattern of specific bands ranging from 48-72 kDa for positive pigs (Gomez-Morales et al., 2012). The disadvantages of the WB method are labor, high cost, and time intensiveness (Cuttell et al., 2012; Gottstein et al., 2009).

In humans, WB is routinely used as a confirmatory test to distinguish between patients with *Trichinella* infections and other helminth infections (Frey et al., 2009; Yera et al., 2003). It has been suggested that WB is suitable for early diagnosis, thus allowing early treatment, which is known to reduce the clinical complications of trichinellosis (Dupouy-Camet et al., 2002; Yera et al., 2003). Normally, a pattern of specific bands ranging in size between 53 - 72 kDa for positive humans is considered diagnostic for *Trichinella* infection (Gomez-Morales et al., 2012).

Table 1.4. Serological tests for detection of *Trichinella* antibodies.

Method	Antigen	Sensitivity	Specificity	Reference
ELISA	Crude antigen	99% (human) 100% (pig)	60% (human) 91% (pig)	(Andiva et al., 2002) (Tattiyapong et al., 2011)
ELISA	E/S antigen	98-100% (pigs) 99-100% (human)	98% (pigs) 95-100% (human)	(Moller et al., 2005; Yepez-Mulia et al., 1999) (Gomez-Morales et al., 2008; Escalante et al., 2004)
ELISA	Tyvelose	98% (pigs)	99% (pigs)	(Gamble et al., 1997; Pozio et al., 2002b)
WB	Crude antigen	98% (pig) 98% (human)	100% (pig) 98% (human)	(Nockler et al., 2009) (Yera et al., 2003)
WB	E/S antigen	100% (pig) 100% (human)	100% (pig) 100% (human)	(Gomez-Morales et al., 2012) (Gomez-Morales et al., 2012)

1.2.5.5. Muscle biopsy

Muscle biopsy is a method applied to diagnose human trichinellosis. It is recommended particularly when the results of serological testing are unclear (Bruschi and Murrell, 2002). However, this invasive method is painful for patients (Gottstein et al., 2009). Typically, the biopsy of the deltoid or another skeletal muscle obtained from a patient (2-4 mm³) is used for testing (Bruschi and Murrell, 2002; Capo and Despommier, 1996). The biopsy sample can be examined for the presence of muscle larvae by the trichinoscopy method, histologically, or it can be digested with pepsin and hydrochloric acid. Histological examination is more sensitive than trichinoscopy at an early stage of muscle invasion, when larvae are very small and can not easily be differentiated from muscle fibers (Gottstein et al., 2009). Histological examination may reveal live or dead larvae, increased vascularity, small haemorrhages, and accumulation of inflammatory cells (Bruschi and Murrell, 2002; Capo and Despommier, 1996; Gottstein et al., 2009). The basophilic transformation of muscle cells represents a valuable diagnostic criterion of *Trichinella* invasion even when no larvae have been detected (Gottstein et al., 2009). Because of the small sample size examined, this method has a limited sensitivity (Capo and Despommier, 1996), depending on the degree of infection (Wang et al., 2013a). Therefore, a negative result does not exclude an infection (Bruschi and Murrell, 2002).

1.2.6. Epidemiology

1.2.6.1. Global epidemiology

Since the 19th century, trichinellosis has been found in all continents, with the current exception of Antarctica (Gottstein et al., 2009; Pozio and Zarlenga, 2013). Human trichinellosis has been documented in 55 (27.8%) countries, especially in those countries where eating habits include the consumption of raw or undercooked meat (Murrell and Pozio, 2011; Pozio, 2007). The global number of disability-adjusted life years (DALYs) due to trichinellosis was estimated to be 76 per billion persons per year (Figure 1.3). The global incidence rate was 469.2 to 985.3 cases per billion persons per year, and a global mortality rate of 0.300 to 0.828 per billion persons per year. The global burden of disease was estimated at 523 DALYs in 2010 (Devleeschauwer et al., 2014). Human trichinellosis affects primarily adults (median age 33.1 years) and about equally affects men (51%) and women (Murrell and Pozio, 2011). *Trichinella* infection has been

documented in domestic animals, in wildlife of 43 (21.9%), and 66 (33.3%) countries, respectively around the world (Murrell and Pozio, 2011; Pozio, 2007). The species most frequently associated with human infection is *T. spiralis*, the species that is normally found in domestic pigs (Bruschi and Murrell, 2002). Some countries have no or an inadequate national reporting system of epidemiological data, which could lead to underreporting (Gajadhar and Gamble, 2000; Pozio, 2007).

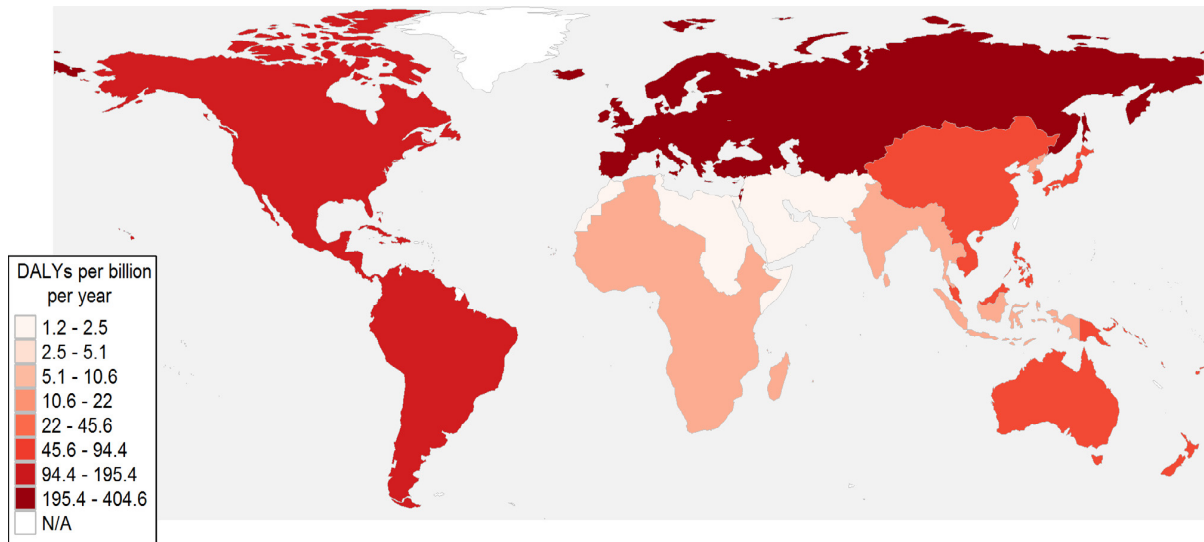


Figure 1.3. Global burden of trichinellosis per billion persons per year (Devleesschauwer et al., 2014).

Trichinellosis is still endemic in developing countries (Southeast Asia, Central, and South America) where a lack of knowledge of the disease, poor farm management practices and sanitary regulations, and inadequate veterinary infrastructure prevail (Gajadhar and Gamble, 2000; Ortega-Pierres et al., 2000; Takahashi et al., 2000). Trichinellosis is an emerging or re-emerging foodborne disease in some parts of the world. With the expansion of global trading, there is a potential for outbreaks of trichinellosis in virtually any country, particularly from the consumption of pork products (Gajadhar and Gamble, 2000). Economic changes, revolutions, and wars, such as in Eastern Europe in the 1990s have contributed to an increase in *Trichinella* prevalence among the affected human populations (Pozio, 2014). Also, climate changes such as increasing temperatures may allow some potentially infected animals to survive winters in larger numbers, increase their population as well as expand their habitat thus increasing the opportunity for transmission of *Trichinella* infection to humans (Hueffer et al., 2013).

The most typical feature of the parasite's epidemiology is the obligatory transmission by ingestion of meat (Bruschi and Murrell, 2002). The global distribution of *Trichinella* in conjunction with varying cultural eating habits represents the main factor favouring human infections in the world (Murrell and Pozio, 2011; Pozio, 2007). The infection is strongly related to traditional dishes based on raw or undercooked meat of different animals infected with larvae of *Trichinella* species (Murrell and Pozio, 2011). In recent decades, eating habits have changed worldwide and the migratory flow of humans with their own food practices including the consumption of raw or undercooked meat has led to the emergence of parasitic zoonoses (Macpherson, 2005). In some countries, trichinellosis occurs only among ethnic minorities and tourists who acquired *Trichinella* spp. infections after traveling or hunting in endemic areas and subsequently developed disease after their return to their home countries. In countries where most of the population is Muslim, *Trichinella* infection is rare and may not be reported at all (Houze et al., 2009; Murrell and Pozio, 2011).

Trichinella spp. infections have been documented in a wide range of animal hosts, including mammals, birds, and reptiles (Gottstein et al., 2009). The epidemiological cycles of nematodes of the genus *Trichinella* are the domestic cycle, with transmission predominantly between domestic animals, and the sylvatic cycle, which generally involves wild carnivores (Pozio, 2000). In both cycles, humans acquire the infection by consuming raw or undercooked meat containing larvae of *Trichinella* (Gottstein et al., 2009; Zimmer et al., 2008). The major sources of *Trichinella* spp. infection for humans are domestic and wild pigs (Figure 1.4) (Gajadhar and Gamble, 2000; Gottstein et al., 2009; Murrell and Pozio, 2011). Domestic pigs can be infected when they are raised under free-ranging conditions or when they are fed with infected meat scraps or other contaminated feed (Pozio, 2014). Typically, outbreaks of trichinellosis occur in situations of consumption of meat from backyard pigs and unregulated home slaughter in underdeveloped regions (Gajadhar and Gamble, 2000). The separation of cycles into the domestic or synanthropic cycle, involving primarily pigs (Pozio, 2014), and the sylvatic cycle, involving wild animals, does not preclude risk of exposure of pigs to species of *Trichinella* typically found in wild animals (Ribicich et al., 2010).

The spread of infected meat in the environment by humans seems to be important for *Trichinella* infection in rats. The role of the rat in the epidemiology of *Trichinella* species

continues to be debated as either a reservoir or an accidental host. It is now accepted that infected rats represent an offshoot of the domestic cycle, being recipients of infection from that cycle (Hill et al., 2010; Pozio, 2014).

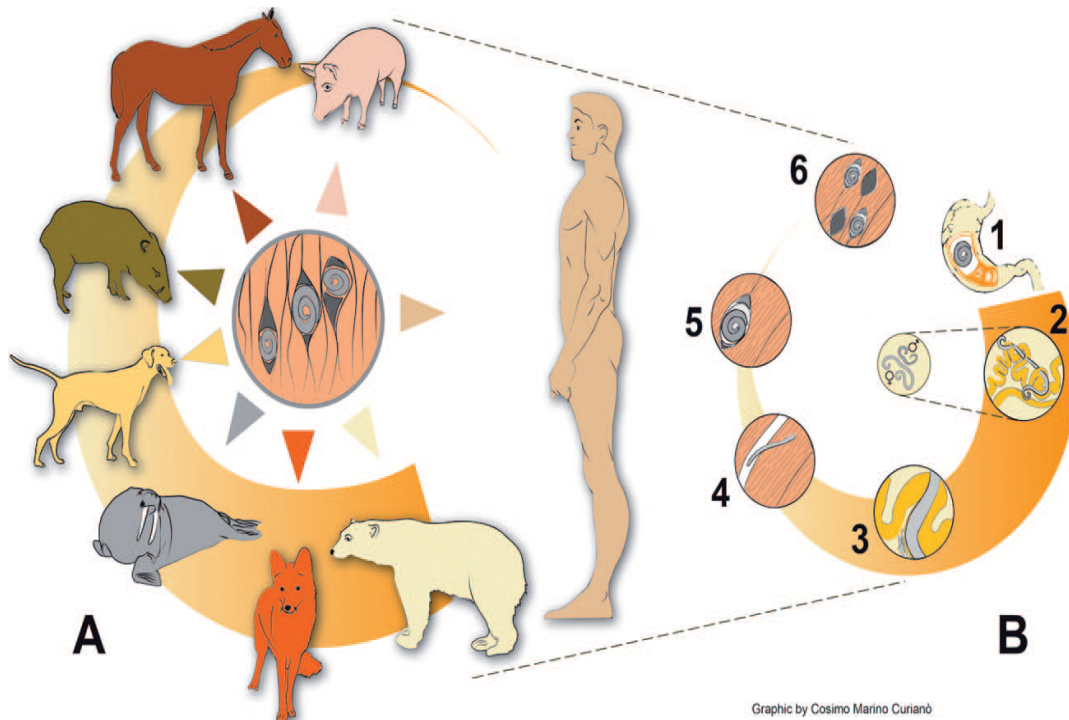


Figure 1.4. Main sources of *Trichinella* infections (Gottstein et al., 2009).

1.2.6.2. Epidemiology of trichinellosis in Southeast Asia and China

Since 1923, there have been relatively few published reports on *Trichinella* infection in Southeast Asia (Le Louet and Broudin, 1923). In this region *Trichinella* spp. infections have been documented in domestic animals (mainly pigs) in six countries (Indonesia, Lao PDR, Malaysia, Myanmar, Thailand, Vietnam), in wildlife in Thailand, and in humans in five countries (Cambodia, Indonesia, Lao PDR, Thailand, Vietnam) (Pozio, 2007) (Table 1.5). The main sources of human infection were reported to be free-roaming pigs and wild boars (Khamboonruang, 1991; Pozio, 2001; Pozio, 2007; Taylor et al., 2009); also jackal, turtle, squirrel, monitor lizard, and black bear were associated with human infection (Doege et al., 1969; Kaewpitoon et al., 2008; Pozio and Darwin, 2006; Suriyanon and Klunklin, 1972).

From 1962 to 2009, there were 136 outbreaks of trichinellosis in Southeast Asia, about 8162 people were affected and 105 patients died (Barennes et al., 2008; Kaewpitoon et al.,

2008; Pozio, 2007; Pozio, 2001; Sayasone et al., 2006; Taylor et al., 2009). The main outbreaks of trichinellosis have been reported in Thailand, Lao PDR, and Vietnam (Murrell and Pozio, 2011; Pozio, 2007). Among these countries the highest number of trichinellosis outbreaks occurred in Thailand. In that country, from 1962 to 2005, 130 outbreaks were reported, involving 7392 patients and 97 deaths, with a morbidity rate of 0.04 per 100,000 people (Kaewpitoon et al., 2008; Kaewpitoon et al., 2006; Pozio, 2007; Takahashi et al., 2000); seroprevalence in pigs was reported to be as high as 4% in Thailand (Barennes et al., 2008). Trichinellosis data in Lao PDR are limited, with only two outbreaks reported. There were 35 identified cases of *Trichinella* infection and no deaths were recorded (Barennes et al., 2008; Sayasone et al., 2006).

In Southeast Asian countries, three *Trichinella* species have been identified from both the domestic and sylvatic cycles. These include encapsulated and non-encapsulated species, namely *T. spiralis*, *T. pseudospiralis*, and *T. papuae* (Barennes, 2013; Barennes et al., 2008; Intapan et al., 2011; Jongwutiwes et al., 1998; Kaewpitoon et al., 2008; Kaewpitoon et al., 2006) (Table 1.5). *T. spiralis* is the most common species and has been identified in Thailand, Lao PDR, and Indonesia (Chomel et al., 1993; Intapan et al., 2011; Pozio and Khamboonruang, 1989; Tantrawatpan et al., 2012) (Figure 1.5). *T. pseudospiralis* has been reported in Thailand in 1998 (Jongwutiwes et al., 1998; Pozio et al., 2009) and *T. papuae* was identified as the etiologic agent of human trichinellosis in Thailand and Malaysia (Intapan et al., 2011; Tantrawatpan et al., 2012).

In China, *Trichinella* infections in humans and in animals have been documented in 33 of 34 provinces (Liu and Boireau, 2002; Cui et al., 2011). During 2004-2009, 15 outbreaks of human trichinellosis, consisting of 1387 cases and four deaths, were reported in the three southwestern provinces (i.e Yunnan, Inner Mongolia, and Sichuan). The overall seroprevalence of human trichinellosis was 3.19%. Pork is the predominant source of human trichinellosis in China (Cui et al., 2011).

Table 1.5. *Trichinella* infection in Southeast Asian countries and China.

Countries	<i>Trichinella</i> species	Domestic animals	Sylvatic wildlife	Human	Reference
Cambodia	Unknown	ND	ND	Yes	(Pozio, 2001)
Indonesia	<i>T. spiralis</i>	Yes	ND	Yes	(De Carneri and Di Matteo, 1989; Pozio, 2001)
Malaysia	<i>T. papuae</i>	Yes	ND	ND	(Intapan et al., 2011; Kurup et al., 2000; Pozio, 2007)
Myanmar	Unknown	Yes	ND	ND	(Pozio, 2001; Watt et al., 2000)
Lao PDR	<i>T. spiralis</i>	Yes	ND	Yes	(Barennes et al., 2013; Pozio, 2001; Sicard et al., 1976)
Thailand	<i>T. spiralis</i>	Yes	Yes	Yes	(Kaewpitoon et al., 2008; Kaewpitoon et al., 2006; Khamboonruang, 1991)
	<i>T. pseudospiralis</i>	ND	Yes	Yes	(Jongwutiwes et al., 1998)
	<i>T. papuae</i>	ND	Yes	Yes	(Khumjui et al., 2008; Kusolsuk et al., 2010)
Vietnam	Unknown	Yes	ND	Yes	(Blanc et al., 1956; Le Louet and Broudin, 1923; Merlea, 1957; Pozio, 2001; Pozio, 2007)
China	<i>T. spiralis</i>	Yes	Yes	Yes	(Cui et al., 2011)
	<i>T. nativa</i>	Yes	ND	ND	(Fu et al., 2009)

ND: Not documented

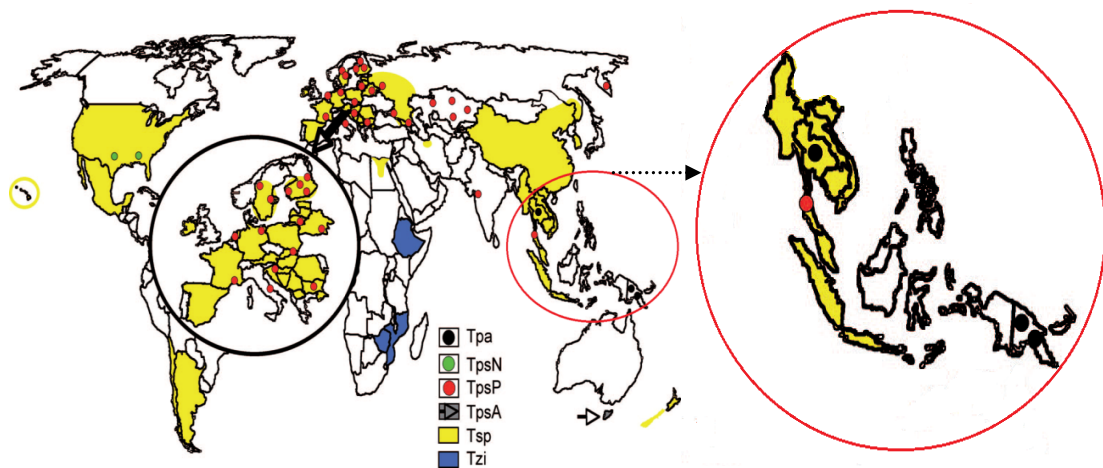


Figure 1.5. The distribution of *T. spiralis* (Tsp), *T. pseudospiralis* (TpsP), *T. papuae* (Tpa) in Southeast Asian countries (red circle) (Gottstein et al., 2009).

Based on these reports it can be concluded that trichinellosis is an endemic foodborne zoonosis in Southeast Asian countries and China. The need to urgently implement veterinary and educational programs was recognised. Epidemiological investigations reveal that the outbreaks have occurred mostly in mountainous regions among indigenous people. These populations mostly practice a free-roaming pig husbandry system. Outbreaks often occurred following traditional festivals such as New Year and wedding ceremonies (Kaewpitoon et al., 2008; Kaewpitoon et al., 2006; Murrell and Pozio, 2011), during which local pigs are slaughtered and pork is often consumed raw or undercooked (Kaewpitoon et al., 2008; Kaewpitoon et al., 2006; Murrell and Pozio, 2011). Epidemiological data on human trichinellosis are likely to be underreported because diagnostic facilities in local health centres do not allow for definitive diagnosis, and epidemiologic investigations are insufficient (Barennes et al., 2008; Sayasone et al., 2006). In addition, populations in mountainous and rural areas have limited knowledge about the disease (Barennes et al., 2008; Sayasone et al., 2006). Additionally, travel of people to endemic areas where they consume local dishes and movement of livestock and wildlife may contribute to spreading of the infection in the region (Murrell and Pozio, 2011).

In the last five decades, there have been changes in the distribution, prevalence, and impact of trichinellosis in Southeast Asian countries (Grace et al., 2011; Pozio, 2001).

Major difficulties in assessing the changing disease status in this area are encountered due to a lack of reported data on the occurrence of trichinellosis, a lack of well functioning public health systems that enable precise reporting of disease outbreaks, and a lack of the knowledge of the disease, sanitary regulations, and adequate veterinary infrastructure (Chomel et al., 1993; Gajadhar and Gamble, 2000; Pozio, 2007). Globally, the direct impacts of trichinellosis may actually be increasing, because in a globalized and highly interconnected world, the effects of disease may extend far beyond the endemic regions. Travel, migration, and trade may continue to promote the spread of infections into new populations. In the case of trichinellosis, not all slaughtered pigs are examined for *Trichinella* larvae in many Southeast Asian countries such as, Vietnam, Lao PDR, and Cambodia (Kaewpitoon et al., 2008; Kaewpitoon et al., 2006; Murrell and Pozio, 2011; Pozio, 2001).

1.2.7. Trichinellosis in Vietnam

1.2.7.1. General introduction

Vietnam is located on the Indochina peninsula in Southeast Asia. The country occupies about 331,051 square kilometers, and the population was estimated at 87 million in 2010 (adapted from <http://www.gso.gov.vn>). Vietnam has a long land border of 4,550 km, bordering China in the North, Lao PDR and Cambodia in the West, and the South China Sea in the East. Vietnam is stretching from 23°23' to 8°27' North latitude. The country is divided into the Northern and Southern regions (adapted from <http://www.mofa.gov.vn>). North Vietnam has four distinct seasons with hot and humid summers, receiving the majority of rainfall, cool, and relatively dry winters. In the winter, the temperatures are the lowest in December and January; in some areas temperatures can drop to around 0 °C and it can occasionally snow in the Northern mountainous regions where most of the ethnic minorities live. In contrast, the weather in Southern Vietnam is always hot with dry and rainy seasons (Blair et al., 2011). Mountainous areas make up 3/4 of the country; lower areas are located along the Pacific seaside of the country and are densely populated (adapted from <http://english.doanthanhvien.vn>).

1.2.7.2. Epidemiology of trichinellosis in Vietnam

Trichinellosis is an emerging or re-emerging zoonotic disease in Vietnam. There have been a number of early suspicions and reports of human and animal trichinellosis cases in Vietnam. *Trichinella* spp. was detected for the first time in 1923 in two (0.04%) out of 4,952 pigs tested in Hanoi (Le Louet and Broudin, 1923), then human trichinellosis was diagnosed in six soldiers in Saigon in 1953; a wild pig was found to be the source of infection (Blanc et al., 1956; Merlea, 1957). During 1970-2001 (31 years), there were two outbreaks of human trichinellosis and from 2001-2008 (8 years) two outbreaks occurred. These outbreaks occurred in two northern provinces namely, Dien Bien and Son La (Figure 1.6) and involved 91 people, of which eight died (Murrell and Pozio, 2011; NIMPE, 2008; Pozio, 2007; Taylor et al., 2009). This makes Vietnam one of the Southeast Asian countries contributing to a high mortality (8.2%) observed in comparison to the average number of deaths at world level, which is 1.0% (Murrell and Pozio, 2011; Pozio, 2007). The Northern Vietnamese countries where the outbreaks occurred are near endemic south-west China (Yunnan province) and Lao PDR, where a high prevalence of *T. spiralis* in domestic pigs was reported (Barennes et al., 2008; Cui et al., 2011).

All outbreaks of human trichinellosis since 1923 involved the consumption of pork (Pozio, 2007). Most of infected people belonged to ethnic minorities. All infected people had consumed the infected raw pork at wedding, funeral or New Year parties, where local dishes such as, “thit chua”, “lap”, “goi”, that contain raw or undercooked pork are served (Son La subDAH, 2008). All four outbreaks occurred in mountainous districts where the hygienic conditions and pig breeding practices are poor: free-roaming pigs have access to pork scraps scattered in the environment after slaughtering, carcasses of dogs and cats, or they may prey on rodents or other animals; backyard slaughter is a general practice (NIMPE 2008; Taylor et al. 2009).

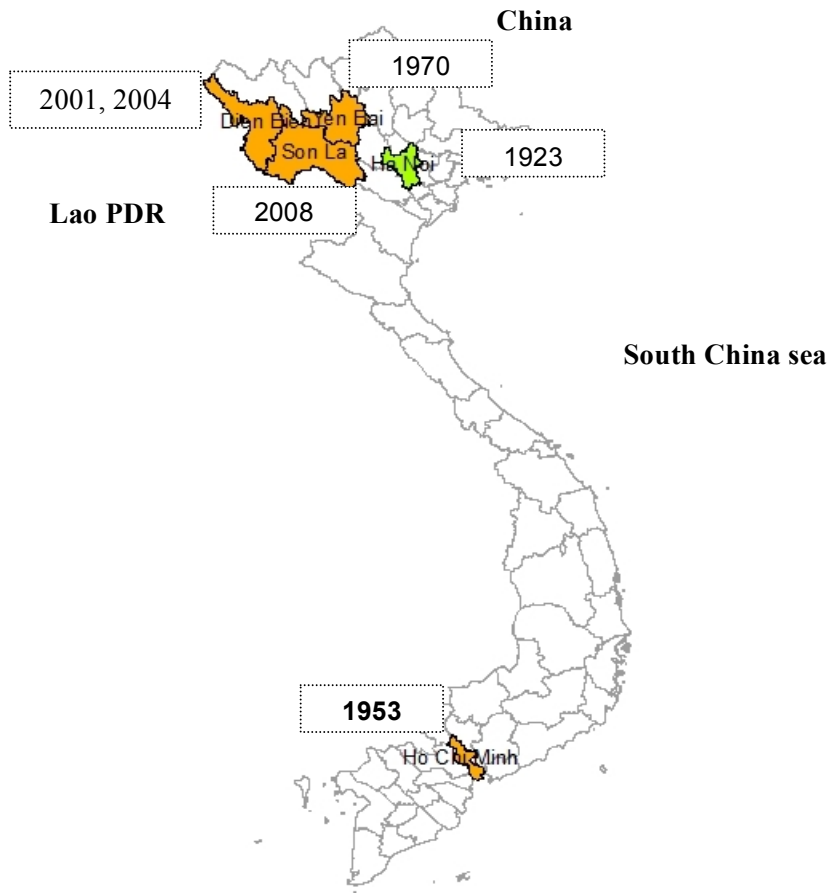


Figure 1.6. Map of *Trichinella* infection in Vietnam (the orange color indicates where outbreaks of human trichinellosis were reported and the green color where a serosurvey on pig *Trichinella* infection was done).

In these regions, marketing of animals is uncontrolled and animals may be transported over provincial and country borders, favouring the spread of *Trichinella*-infected pigs in the area. Inspection of *Trichinella* larvae in meat is not mandatory in Vietnam. There has not been any reported information of *Trichinella* infections in other domestic animals such as dogs and cats, and very little information is available on *Trichinella* infection in pigs. The unavailability of equipment for diagnosis in the slaughterhouses and laboratories are the main reasons for the limited achievements in studies on *Trichinella* spp. in Vietnam until now. Currently, it is not known whether *Trichinella* parasites are restricted to the three northwestern provinces where outbreaks occurred in the last decades or whether it is more widespread in the country. The economic and public health impacts of this parasitic infection have also not yet been considered in Vietnam.

1.2.8. Control - prevention and treatment

1.2.8.1. Control and prevention

1.2.8.1.1. Prevention of trichinellosis in humans

The prevention of trichinellosis in humans is based on several measures, such as awareness and improvement of hygiene through health education, improvement of pig husbandry systems, rodent control, meat inspection, and processing. One of the main approaches is education of the consumer about the risk of consumption of raw or undercooked meat from both domestic and sylvatic animals (Gottstein et al., 2009). To prevent the consumption of viable *Trichinella* larvae, there are some methods for the preparation of meat and meat products that are considered safe. Cooking meat to an internal temperature of 60 °C for at least one minute is advised (Bruschi and Murrell, 2002). Freezing is another method to inactivate *Trichinella* larvae in meat; however some *Trichinella* species, such as *T. nativa* are freeze-resistant. Fortunately, freeze-resistant species have a low infectivity for pigs, but they can infect wildlife such as bears (Gottstein et al., 2009). In general, pork should be frozen at either -15 °C for 20 days, -23 °C for 10 days, or -30 °C for six days if the meat is less than 15 cm thick (Bruschi and Murrell, 2002). Pieces of meat up to 50 cm thick should be frozen for more than 4 weeks. At levels that have been shown to inactivate larvae (0.3 kilogray), irradiation is an acceptable method for rendering meat safe for human consumption for sealed packaged food in countries where irradiation methodology is allowed (Gottstein et al., 2009). Curing, drying, and smoking processes are not recommended by the International Commission on Trichinellosis because of the variability in the salt concentrations, temperatures used, and drying times.

1.2.8.1.2. Control of *Trichinella* infections in animals

Based on the understanding of the epidemiology of *Trichinella* infections, it is well known that this zoonosis can be controlled by preventing parasite transmission at the farm level and by correctly applying standard hygienic procedures at the abattoir (Gamble et al., 2000). Measures at the farm level include, proper feed storage, rodent control, farm hygiene including proper disposal of dead animals, contact with rats should be closely controlled, and purchase of piglets from farms with controlled housing condition should

be promoted. Wildlife species are reservoir hosts for all species of *Trichinella*. The only measure that can be implemented to reduce the prevalence of infection in wildlife is to instruct hunters to avoid leaving animal carcasses in the field after skinning or removing and discarding the entrails (Gottstein et al., 2009). Another important factor is to implement active and passive surveillance systems in animals (Hueffer et al., 2013).

When humans fail to implement a proper management of domestic animals and wildlife, *Trichinella* infection is transmitted from the sylvatic environment into the domestic one, sometimes through synanthropic (intermediary between domestic and sylvatic) animals. In addition, some species can be transferred from domestic animals to wildlife when carrion of domestic animals is not properly disposed off or carcasses of dead free-ranging pigs are not appropriately removed from the field (Gottstein et al., 2009).

1.2.8.2. Trichinellosis treatment

Treatment of human trichinellosis has to be initiated as early as possible to limit muscle invasion by larvae and to reduce muscle damage. The efficacy of anthelmintics is very low once the larvae are encapsulated (Gottstein et al., 2009). A general rule is that therapeutic plasma levels of the drug should be maintained for an extended period, rather than that a high plasma level is reached for a short period (Bruschi and Murrell, 2002). Drugs administered in trichinellosis patients include anthelmintics, anti-inflammatory drugs (glucocorticoids), and preparations that compensate for protein and electrolyte deficits (Gottstein et al., 2009) (Table 1.6). Anthelmintics include benzimidazoles (albendazole, mebendazole), and pyrantel.

Albendazole and mebendazole were reported to be more efficient than other anthelmintics. Benzimidazoles are contraindicated in pregnant women (Bruschi and Murrell, 2002; Kociecka, 2000) but these drugs can be used in the second trimester of pregnancy (de Silva et al., 1999; Dupouy-Camet et al., 2002; Haider et al., 2009). Pyrantel is given in a single dose of 10 to 20 mg/kg of body weight, repeated for 2 to 3 days. In contrast to benzimidazoles, pyrantel is not absorbed from the intestinal lumen and acts by paralyzing parasites (Bruschi and Murrell, 2002; Kociecka, 2000). Pyrantel may be used in pregnant women and children, but it is active only against worms in the gut; it has no effect against newborn and muscle larvae (Bruschi and Murrell, 2002; Gottstein et al., 2009).

The success of treatment is evident from the clinical improvement of the patient's symptomatology. Factors such as the *Trichinella* species involved, intensity and length of infection, and host response can aid in deciding on the treatment course. Light infections do not require treatment. The treatment goal for the very early infection phase is to limit muscle invasion by larvae; when this has already occurred the goal is to reduce muscle damage, which is responsible for the major clinical manifestations (Bruschi and Murrell, 2002; Dupouy-Camet et al., 2002; Gottstein et al., 2009).

Table 1.6. Treatment for intestinal and muscle stages of trichinellosis (Bruschi and Murrell, 2002; Gottstein et al., 2009).

Symptomatic treatment	Specific treatment	Recommended dose
Analgesic drugs	Mebendazole	200-400 mg: 3 times/day for 3 days Followed 400-500 mg: 3 times/day for 10 days
Antipyretic drugs	Albendazole	400 mg/day for 8-14 days
Corticosteroid	Prednisolone	30-60 mg/day for 10-15 days

1.3. Conclusions

Since the 19th century, many studies have shown that trichinellosis occurs in humans, and domestic, sylvatic, and synanthropic animals in a very wide geographical area. *Trichinella* species have been found in various mammals, birds, and reptiles. Up to date, nine species and three genotypes have been identified but *T. spiralis* is the most cosmopolitan species and it is most often associated with zoonotic infection. It is likely that data on trichinellosis are underestimated due to absence or inadequate national reporting systems and due to lack of knowledge of the disease in some regions in the world. The epidemiology of trichinellosis can vary due to climate change, animal breeding systems as well as to increased travel and changing eating habits of people. The lack of adequate veterinary infrastructure and sanitary regulations as well as the behaviour to consume raw pork dishes are the main reasons why trichinellosis is endemic in some countries. Recently, epidemiological studies have provided a more detailed picture of the transmission ways. The diagnosis of trichinellosis in humans is difficult, because of misdiagnosis with other helminthiasis and other infections due to the lack of

pathognomonic signs and symptoms, and because there is a lack of diagnostic facilities in hospitals at national, provincial, and commune level in endemic areas. Control based on improvement of farm management and on meat inspection has proven to be very effective as *Trichinella* infection has virtually been eliminated in domestic animals in the EU and the USA. Unfortunately, the control and prevention of *Trichinella* infection is more difficult in endemic regions of developing countries.

Trichinellosis is not a very well known foodborne disease in Vietnam and information on the epidemiology of the parasite is lacking. The disease is known from sporadic studies on human trichinellosis reported in national hospitals in Ha Noi from 3 mountainous provinces of northwest Vietnam. With 3/4 provinces of Northern Vietnam being mountainous provinces, trichinellosis has to be considered a potential public health problem.

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RATIONALE AND OBJECTIVES

Rationale

Recent trichinellosis outbreaks in Northern Vietnam have generated a number of research questions:

1. What is the occurrence of *Trichinella* in domestic animals in and outside outbreak areas and which *Trichinella* species can be identified in domestic animals in Northern Vietnam?
2. Does *Trichinella* occur in wildlife in Northern Vietnam?
3. Is *Trichinella* endemic in this region? Which *Trichinella* species occur in humans? Which are clinical signs and symptoms associated with human trichinellosis in Northern Vietnam?

Objectives

General objective

To study the epidemiology and the zoonotic importance of trichinellosis in Northern Vietnam, by assessing the prevalence, identifying the prevailing species of *Trichinella* that are involved in human and animal infections, and by studying risk factors for infection.

Specific objectives

1. To estimate the prevalence of *Trichinella* infections in domestic pigs, dogs, and cats in Northern Vietnam and identify the causative *Trichinella* spp.
2. To identify which *Trichinella* spp. circulate in wildlife and estimate the prevalence of *Trichinella* infections in wild boars and synanthropic rats in Northern Vietnam.
3. To assess the prevalence of trichinellosis in humans in the context of outbreak situations and in provincial hospitals; determine the clinical signs and symptoms associated with trichinellosis and determine the *Trichinella* spp. causing human infections.

CHAPTER 2

***TRICHINELLA* INFECTIONS IN DOMESTIC ANIMALS**

Part 1: High prevalence of anti-*Trichinella* IgG in domestic pigs in a community where an outbreak of trichinellosis occurred in northwest Vietnam and species identification of *Trichinella* spp.

Based on the manuscript: **Vu, T.N.**, Dorny, P., La Rosa, G., To, L.T., Nguyen, V.C., Pozio, E., 2010. High prevalence of anti-*Trichinella* IgG in domestic pigs of the Son La province, Vietnam. *Vet. Parasitol.* 168, 136-140.

2.1.1. Introduction

In South East Asia, infection is endemic in both humans and animals in China and in the Lao Popular Democratic Republic (PDR), mainly in the regions bordering Vietnam. In these two countries trichinellosis is almost exclusively attributable to the consumption of raw pork from backyard or outdoor pigs (Wang et al., 2006; Sayasone et al., 2006).

Although trichinellosis is considered rare in Vietnam, a few outbreaks have been reported: the first outbreak was diagnosed in six soldiers in Saigon in 1953 (Merlea, 1957); and a second outbreak, attributed to the consumption of pork imported from Lao PDR, was reported in 1970 (De et al., 2006). From 1970 to 2004, three outbreaks occurred, involving 68 persons, with six deaths; these outbreaks occurred in the provinces of Yen Bai and Dien Bien (former Lai Chau province) (both in northwestern Vietnam) and were attributed to the consumption of pork of unknown origin (Pozio, 2007). In June 2008, an outbreak involving 22 persons, with two deaths, occurred in the commune of Lang Cheu, in the Bac Yen district of the Son La province, which borders the two above-mentioned provinces; the epidemic was attributed to the consumption of a local dish known as “lap”, which is made with raw pork, in this case from a local pig, and which had been served in a private home (NIMPE, 2008; Taylor et al., 2009). This outbreak showed that once again trichinellosis was re-emerging in Vietnam and suggested that *Trichinella* spp. parasites circulate among pigs in northwestern Vietnam. However, no information is available on the circulation or prevalence of *Trichinella* spp. in domestic pigs or other animals in Vietnam.

The objective of the present work was to estimate the prevalence of *Trichinella* infection in domestic pigs reared in the commune where the 2008 human outbreak occurred and in another three neighbouring communes of the Bac Yen district.

2.1.2. Materials and methods

2.1.2.1. Study area

The Son La province is located in northwestern Vietnam and has low per capita income in the country (www.sonla.gov.vn/sonla/Vietnam/VH/DT/index2.htm). The province's Bac Yen district is a highland area (average altitude: 700m above sea level) located in the

northern part of the province; it is characterized by a continental climate, with an average annual temperature of 21⁰C (IMH, 2008). In Bac Yen, the human population is characterized by seven ethnic minorities, with the H'Mong ethnic group representing 50% of the total population. This ethnic group lives in 11 of the district's 16 communes, and most people are illiterate (www.sonla.gov.vn/sonla/Vietnam/VH/DT/index2.htm). In these communes, all pigs are free-roaming or kept in crudely constructed pens. The H'Mong people often consume raw pork (lap) (Taylor et al., 2009) (Figure 2.1).

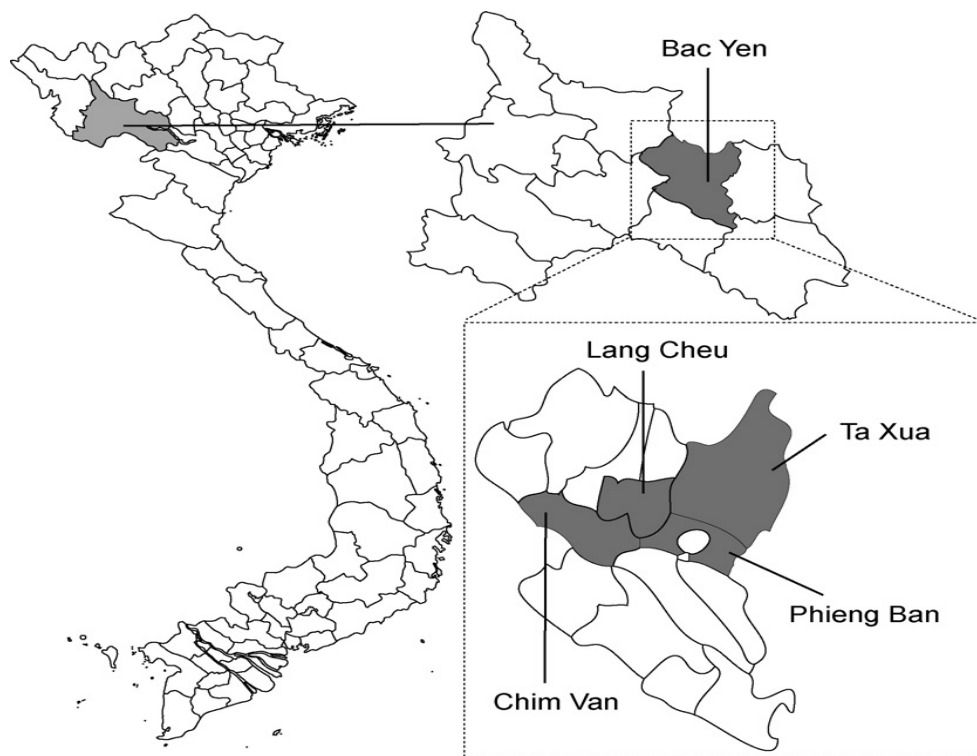


Figure 2.1. Map of the communes of pig origin (Chim Van, Lang Cheu, Phieng Ban and Ta Xua) of the Bac Yen district, Son La province, Vietnam

2.1.2.2. Study design and sampling

The study area consisted of four communes in the Bac Yen district: Lang Cheu (where the 2008 outbreak occurred), Chim Van, Phieng Ban, and Ta Xua (Figure 2.1). The survey was conducted on all of the free-roaming pigs in these communes. A blood sample (2-3 ml) and muscle sample (50 g in total of the diaphragm, tongue and masseter) were collected from each pig, from December 9, 2008 to April 30, 2009. The blood sample was collected from the heart during evisceration, stored in ice boxes, and immediately transported to the National Centre for Veterinary Diagnosis in Ha Noi, where it was

centrifuged to separate the serum; the serum samples were stored at -20°C until analysis. Muscle samples were preserved in ice boxes, sent to Ha Noi, stored at $2-8^{\circ}\text{C}$, and tested within 7 days of collection.

2.1.2.3. Serodiagnosis

Serum samples were tested at the National Centre for Veterinary Diagnosis with a commercial ELISA kit using excretory/secretory (E/S) antigens (PrioCheck® *Trichinella* Ab ELISA, Prionics, Schlieren-Zurich, Switzerland), in accordance with the manufacturer's instructions. Serum samples were tested only at a 1:40 dilution according to the manufacturer's instructions. The microtiter plates were read at 450 nm within 15 min. Positive and negative control sera were those supplied by the manufacturer. The ELISA results were expressed as the ratio sample/positive control (S/P) (%) by comparing the optical density (OD) of the sample to the mean OD of the positive control serum, tested in duplicate. The result was considered to be valid when the mean OD of the positive controls was >1.0 , the mean percentage of positivity (PP) of the weak positive controls was $>35\%$, and the mean OD of the negative controls was <0.2 . If these criteria were not met, the results were considered to be not valid and the samples were tested again. The cut-off of the ELISA was 15 PP. Serum samples were considered to be positive when the PP was equal to or greater than the cut-off, whereas serum samples were considered to be negative when the PP value was below the cut-off.

2.1.2.4. Pathological diagnosis and parasite identification

For economic reasons, the parasitological diagnosis was performed on a randomly selected sample of 76 pigs that were positive for anti-*Trichinella* IgG. For each of these pigs, 50 g in total of muscle tissue from the diaphragm, tongue and masseter were tested for the presence of *Trichinella* sp. larvae by artificial digestion, in accordance with the Commission Regulation No. 2075/2005 of the European Union (European Commission, 2005). *Trichinella* sp. larvae collected after digestion were preserved in 2 ml conical vials with 90% ethanol and forwarded to the European Union Reference Laboratory for Parasites, Istituto Superiore di Sanita, Rome, Italy, for the species identification of single larvae by multiplex PCR, in accordance with a previously published protocol (Pozio and La Rosa, 2003).

2.1.2.5. Data analysis

Logistic regression was used to analyse the prevalence of infection among pigs, by commune of pig origin, sex and age. Stata software version 13.1 (Stata Cooperation, College Station, TX, USA) was used for the statistical analysis. A p value of <0.05 was considered to be significant.

2.1.3. Results and discussion

Serum and muscle samples were collected from all 1035 free-roaming pigs (41.6% males and 58.4% females). The age distribution of these pigs was as follows: 89 (8.6%) younger than 2 months, 406 (39.2%) between 2 and 8 months, 441 (42.6%) between 9 and 36 months, and 99 (9.6%) older than 36 months. Of the 1035 pigs, 206 (19.9%) were seropositive, and seropositive pigs were found in all four communes. The highest seroprevalence (25.7%) was detected in the Lang Cheu commune (117 of the 456 pigs tested), where the human outbreak of trichinellosis had occurred in 2008. In the other three communes (Chim Van, Ta Xua and Phieng Ban), the seroprevalence was, 20.0% (27/135), 16.8% (46/274), and 9.4% (16/170), respectively. A logistic regression showed that the seroprevalence was not significantly different between Lang Cheu and Chim Van communes ($p > 0.05$). However, there were significant differences in seroprevalence of infection between Lang Cheu, Ta Xua and Phieng Ban communes ($p < 0.05$).

The seroprevalence increased with the age of the pigs: in particular, it was 1.1%, 1.2%, 32.9% and 55.6% for, respectively, pigs younger than 2 months, between 2 and 8 months, between 9 and 36 months, and older than 36 months. There was no significant difference in the seroprevalence between pigs younger than 2 months and those 2-8 months of age ($p > 0.05$), whereas there was a significant difference between pigs up to 8 months of age and those older than 8 months ($p < 0.0001$). The overall prevalence of infection did not significantly differ by gender ($\chi^2 = 1.92$, 1 d.f., $p = 0.165$).

Muscle samples from the 76 randomly selected seropositive pigs (which ranged in age from 7 months to 10 years) were examined by artificial digestion to confirm infection. The randomly selected pigs were from the communes of Phieng Ban, Chim Van, and Lang Cheu. *Trichinella* larvae (from 2 to 19 larvae) were detected in only 11 (14.5%) animals, which originated from the communes of Chim Van and Lang Cheu. The age of these 11

pigs ranged from 24 to 120 months. All larvae were identified as belonging to *Trichinella spiralis*.

The domestic pig is one of the main reservoirs of *T. spiralis* worldwide, and it was the means through which *T. spiralis* colonized most of the continents, acting as a sort of Trojan horse (Rosenthal et al., 2008), together with the wild boar (*Sus scrofa*), from which the domestic pig originates (Larson et al., 2005). The present study clearly shows for the first time that *T. spiralis* is widespread in northwestern Vietnam and explains the occurrence of human outbreaks of trichinellosis. Northwestern Vietnam is located between northern Lao PDR and southeastern China (Yunnan province), which are two areas with a high prevalence of *T. spiralis* infection in domestic pigs (Barennes et al., 2008; Wang et al., 2006). Furthermore, the H'Mong people, who make up a large part of this area's population, often consume raw pork, and they are unaware of the basic means for preventing the transmission of foodborne diseases.

The results of this study reveal that *T. spiralis* is widespread among free-roaming pigs, with a prevalence ranging from 9.4% to 25.7%. For the outbreaks of human infection in the Yen Bai province in 1970 and in the Dien Bien province in 2001 and 2004 (both of which border the Son La province), we do not know whether the outbreaks were due to the consumption of local pigs or of illegally imported pigs or pork products from Lao PDR or China.

The overall prevalence of infection among pigs was 19.9% (206/1,035), yet only 11 (14.5%) of the 76 pigs tested had *Trichinella* larvae. There are several plausible reasons for this discrepancy. In particular, the artificial digestion test could be insufficiently sensitive for detecting larvae in muscle tissues with a low worm burden (e.g., <1-3 larvae/g); this is consistent with the very low number of larvae (from 2 to 19) detected in 50 g of positive samples. Furthermore, the specificity of the ELISA kit could be lower than expected, even though E/S antigens were used. A recent serological investigation on human serum samples using ELISA and E/S antigens revealed a high number of cross-reactions with other human pathogens (Gómez Morales et al., 2008), suggesting that the specificity of the E/S antigens may be questionable. Finally, the ELISA kit used in the present study was evaluated with serum samples from European pigs, and it is well known that the immune response can differ by breed (World Organisation for Animal Health, 2008).

Although serologically positive pigs were found for all four age categories, most of them were older than 36 months. This is not surprising, since *Trichinella* infection shows a typical cumulative effect. In fact, sows and boars live longer than fattening pigs and thus have an increased risk of exposure to *Trichinella* infection.

In the Bac Yen district, the hygienic conditions of breeding are very poor which is one of the major factors in the circulation of parasitic diseases in both humans and pigs. In fact, *Trichinella* infection in pigs is consistently associated with poor hygienic conditions and breeding, in which pigs, particularly free-roaming pigs, are allowed to eat *Trichinella*-infected pork scraps scattered in the environment after slaughtering, to prey on rodents or other animals, and to eat carcasses of dogs and cats, mainly when the pig diet is poor in protein (Liu and Boireau, 2002; Pozio and Murrell, 2006; Sapkotal et al., 2006).

The high number of infected pigs in the investigated area may represent a risk for human infection, and it is possible that the documented outbreaks are only the tip of the iceberg. Actually, in endemic areas with poor sanitary conditions, inhabitants may have difficulties in accessing health care facilities. Moreover, trichinellosis can be clinically mild and resemble other diseases, in that it does not have pathognomonic signs or symptoms, and in endemic areas, if people frequently eat *Trichinella*-infected meat, they can develop an asymptomatic form of the disease (Owen et al., 2005). For these reasons, diagnosis should be based on several clinical and laboratory features, as suggested by the algorithm proposed by Dupouy-Camet and Bruschi (2007).

The high serological prevalence among pigs in the Bac Yen district suggests that investigations in neighbouring districts and provinces should be performed, despite the discrepancy between the serological and parasitological results of our study, which should also be investigated. The occurrence of *Trichinella* infection in both pigs and humans indicates that the people living in this area should be made aware of the risk of this disease and be encouraged to adopt adequate livestock-breeding practices.

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CHAPTER 2

***TRICHINELLA* INFECTIONS IN DOMESTIC ANIMALS**

Part 2: The seroprevalence of *Trichinella* infections in domestic animals

Based on the manuscript: **Thi, N.V.**, De, N.V., Praet, N., Claes, L., Gabriel, S., Dorny, P., 2013. Seroprevalence of trichinellosis in domestic animals in northwestern Vietnam. *Vet. Parasitol.* 193, 200-205.

2.2.1. Introduction

Trichinellosis is considered an important emerging or re-emerging zoonotic disease in some Southeast Asian countries (Cui et al., 2011; Kaewpitoon et al., 2008; Taybouavone et al., 2009). The occurrence of human infections is related to the widespread tradition of eating raw or undercooked pork in these countries (Conlan et al., 2011). *Trichinella spiralis* is the most common species associated with outbreaks in Southeast Asia, but in Thailand *T. pseudospiralis* and *T. papuae* have also been isolated (Pozio, 2007).

In Vietnam, four outbreaks of human trichinellosis were reported in the northwestern provinces (Dien Bien and Son La) during 1970-2008, which were related to pork consumption (Pozio, 2007; Vu et al., 2010). A survey on *Trichinella* infections in pigs was conducted in a rural district in Son La province, where an outbreak of human trichinellosis had occurred in the previous year (Taylor et al., 2009). A seroprevalence of 19.9% was found in pigs in this village and *Trichinella* larvae could be demonstrated by artificial digestion in the carcasses of 11 of 86 serologically positive pigs. Risk factors for transmission of *Trichinella* in northwestern Vietnam are present, such as free roaming of animals, feeding of animals with kitchen wastes, consumption of raw and undercooked meat and the lack of screening for *Trichinella* infection at slaughter.

In order to better understand the epidemiology of *Trichinella* infection in northwestern Vietnam and assess the importance of the domestic life cycle, a seroprevalence study was set up in pigs, dogs and cats in two provinces where outbreaks previously occurred.

2.2.2. Materials and methods

2.2.2.1. Study area and sample design

The study was conducted in all districts of two provinces, Dien Bien and Son La, located in northwestern Vietnam (20⁰39' - 22⁰33' north latitude, 102⁰10' - 105⁰20' east longitude) (IMH, 2008).

The sample sizes in both provinces were calculated on the basis of an expected conservative prevalence of 10% in pigs and of 1% in cats and dogs (95% CI, 5% error, Win Episcopo 2.0) (Branscum et al., 2006). The number of calculated samples was increased by two times for each animal species to increase the reliability. The animal

population in those provinces is estimated at, 676,900 pigs (Son La: 449,500, Dien Bien: 227,400), 352,600 dogs (Son La: 187,600, Dien Bien: 165,000) and 167,300 cats (Son La: 83,000, Dien Bien: 84,300), respectively (Dien Bien and Son La Sub DAH, 2010). A cross sectional randomized sampling approach was chosen. A total of 558 pigs, 125 dogs and 98 cats was sampled from 239 households in 32 communes of all 20 districts of the two provinces. At least 26 pigs, 6 dogs and 4 cats were sampled in each district. Sex and age distributions for the sampled animals were: pigs (N = 558), male 249 (44.6%), female 309 (55.4%), between 4 and 48 months; dogs (N = 125), male 71 (56.8%), female 54 (43.2%), between 5 and 36 months; cats (N = 98), male 83 (84.7%), female 15 (15.3%), between 12 and 54 months. Approval for this study was obtained from the National Center for Veterinary Diagnosis in Ha Noi.

2.2.2.2. Blood collection

Whole blood samples from the pig jugular vein and, from the dog and cat femoral veins were collected using disposal syringes, transported to the lab in a cool box, and allowed to clot for 30 min at 37 °C to separate serum. Serum was stored at -70 °C until saturation on filter paper. Pig positive controls were from naturally infected animals while positive dog and cat control samples were from animals that were experimentally infected with a Vietnamese *T. spiralis* isolate (Bowman et al., 1993; Nareaho et al., 2000). Negative control serum samples were from Vietnamese industrial pig farms and from domestic dogs and cats from the urban environment of Ha Noi, where the risk for transmission of *Trichinella* is minimal.

2.2.2.3. Filter paper samples

Before forwarding the serum samples to the Institute of Tropical Medicine, Antwerp, Belgium, they were saturated on filter paper (Whatman No. 4). The volume adsorbed on the filter disc was determined by saturation with known amounts of serum to be able to perform ELISA and WB for trichinellosis. After adsorption, filter papers were dried overnight at room temperature, sealed in aluminum foil, and heated in an electrical household oven at 60 °C for 30 min with the aim to inactivate potential viral contamination. Following collection, filter papers wrapped in aluminum foil were left to thoroughly cool, then transferred to labeled plastic bags containing silica gel. Filter papers were stored at 4 °C until tested (Wilmaerts, 2010).

2.2.2.4. Elution of antibodies from serum samples on filter paper

Filter paper discs were cut from the filter papers impregnated with serum. Elution of samples was done in phosphate buffered saline - PBS plus 0.05% Tween 20 (PBS-T) with 5% skimmed milk (SM) on a vortex shaker for 1 h at room temperature. A final dilution of 1:200 of serum was obtained. Samples were mixed again prior to testing (Wilmaerts, 2010).

2.2.2.5. Production of first stage larval excretory/secretory (E/S) antigen

Trichinella spiralis first stage larvae (TSL) were liberated by the hydrochloroacetic acid pepsin artificial digestion method (EG/2075/2005) from the muscles of male rats that were infected with 1500 TSL at least 6 weeks before euthanasia. The larvae were washed 3 times with phosphate buffered saline (PBS) before being resuspended in Roswell Park Memorial Institute (RPMI) - 1640 medium complemented with l-glutamine 200 mM and gentamycin (50 mg/ml) at a concentration of 2000 - 2500 larvae/ml culture fluid. Next, the larval suspension was incubated in a sterile culture flask during 19 h at 37 °C in 5% CO₂. After incubation, the supernatant was collected, passed through a 0.2 µm disposable filter (Sartorius Stedium Minisart® Syringe filter) and mixed with protease inhibitors (Protease inhibitor cocktail tablets (Roche)). Finally, the protein concentration of the product was measured using the Bradford method.

2.2.2.6. Enzyme-linked immunosorbent assay (ELISA)

The detection of anti-*Trichinella* antibodies in animal samples using E/S antigens was done by ELISA according to Vercammen et al. (2002) with slight modifications.

Briefly, 100 µl of E/S *T. spiralis* antigens, diluted at 1 µg/ml was coated on the wells of ELISA plates (NuncMaxiSorp, Denmark) and incubated for 30 min at 37 °C. The plates were washed once with PBS-T then wells were blocked with 150 µl of blocking solution (PBS-T with 5% skimmed milk (SM)) for 15 min at 37 °C with shaking. After discarding the blocking solution and without washing, 100 µl of samples and controls at a 1:200 dilution were applied on the plates and incubated at 37 °C for 15 min, before being washed five times. Bound antibodies were detected by incubation for 15 min at 37 °C with 100 µl of peroxidase-conjugated antibodies: anti-pig Ig diluted at 1:20,000 (Sigma 5670), anti-dog Ig diluted at 1:9000 (Jackson 304-035-003) and anti-cat Ig diluted at 1:30,000

(Jackson 102-035-003). After washing five times, 100 µl of chromogenic substrate, prepared by mixing 0.04% (v/v) hydrogen peroxide (Merck 107209) and 0.03% (w/v) o-phenyldiamine (OPD, Dako S2045) in deionized water, was added to each well. After an incubation of 15 min at 30 °C in the dark, the reaction was stopped with the addition of 50 µl 4N H₂SO₄ to each well and the plate was read at 490/655 nm.

The optical density of each serum sample tested was compared with a sample of negative serum samples (N = 8) at a probability level of P = 0.001 to determine the result in the test (Sokal and Rohlf, 1981). Two positive control serum samples were run on each plate.

The performance of the ELISA was assessed for sensitivity on serial serum samples from six experimentally infected pigs (oral dosages of 100-3000 *T. spiralis* first stage larvae), and for specificity on serum samples from 100 commercially reared Belgian pigs collected at the slaughter line (control pigs), and on 48 pigs that were experimentally or naturally infected with heterologous helminth infections (single infections with *Ascaris suum* (N = 20), *Trichuris suis* (N = 13) *Taenia solium* (N = 23), mixed infection with *A. suum*, *T. suis* and *Oesophagostomum dentatum* (N = 9), *Sarcoptes scabiei* (N = 4) and *Trypanosoma congolense* (N = 6). All *T. spiralis* infected pigs seroconverted between 3 and 4 wpi. All control pigs had OD values under the cut off, and pigs that received heterologous infections were also negative except for one pig with natural *T. solium* cysticercosis (Zambia) and one pig with experimental *A. suum* infection (Belgium).

In dogs and cats the ELISA was assessed on 5 experimentally infected animals (1000 TSL/kg body weight) and on 5 negative controls of each species.

2.2.2.7. Western Bot (WB)

All samples positive in the ELISA were re-examined using WB (Yera et al., 2003) for confirmation. E/S antigens were separated on a 10% SDS-PAGE gel under reducing conditions and transferred onto a nitrocellulose membrane (HybondTM-C extra, 0.45 µm pore size, code RPN 2020, Amersham Bio Science, UK). After blotting, the membranes were blocked in 5% SM in TNT buffer (2.42 g Tris, 8.77 g NaCl, 0.5 ml Tween 20, 1 L deionized water, pH 7.5) for 1 h, washed three times with 0.1% TNT buffer and one time with PBS. After drying, the membranes were cut into small wide strips, then incubated with the samples and the controls eluted 1:200 in 5% SM in TNT buffer for 1 h. Before the sample liquid was removed, the strips were washed three times with TNT buffer and

once with PBS. Anti-pig Ig (Sigma 5670) and anti-dog Ig (Jackson 304-035-003) peroxidase conjugates were diluted 1:18,000 and 1:20,000 with 3% SM, respectively. The strips were incubated with conjugate for 1 h at room temperature. After incubation, all strips were washed three times with TNT buffer and one time with PBS. Subsequently, the peroxidase substrate TMB (KPL 50-77-03) was added and incubated for 5 min at room temperature. The strips were washed with deionized water. The banding pattern was evaluated by comparison with the positive and negative controls and a molecular weight ladder.

The performance of the WB was evaluated on serum samples from *T. spiralis* experimentally infected pigs and dogs, and on a selection of non-infected controls and from pigs that had heterologous parasitic infections (see Section 2.2.2.6). In WB, five bands between 36 and 72 kDa (36, 40, 45, 55 and 72 kDa) appeared in *T. spiralis* infected animals, while these bands were not recognized by control pigs and dogs and heterologous infection pigs (including the *T. solium* and *A. suum* infected pigs that were false positive in ELISA).

2.2.2.8. Data analysis

Stata software version 11.0 (Stata Cooperation, College Station, TX, USA) was used for the statistical analysis. Exact binomial 95% confidence intervals for proportions were calculated.

2.2.3. Results

The results of the ELISA and WB are presented in Table 2.1. ELISA showed 6.8% (95% Exact Binomial confidence Interval (CI): 4.9-9.2), 4.0% (95% CI: 1.3-9.1) and 0% (95% CI: 0.0-3.7) positives in pigs, dogs and cats, respectively. From the ELISA positive samples 31/38 and 5/5 were confirmed by WB in pigs and dogs, respectively, giving a confirmed prevalence of 5.6% (95% CI: 3.8-7.8), 4% (95% CI: 1.3-9.1) and 0% (95% CI: 0.0-3.7) in pigs, dogs and cats, respectively.

Table 2.1. Serological analysis by ELISA and Western blot of serum samples from pigs, dogs and cats from northwestern Vietnam

	ELISA +ve (%(95% CI ^a))	WB +ve (%(95% CI ^a))	Total sampled
Pigs	38 (6.8 (4.9–9.2))	31 (5.6 (3.8–7.8))	558
Dogs	5(4 (1.3–9.1))	5(4 (1.3–9.1))	125
Cats	0(0 (0–3.7))	0 (0 (0–3.7))	98

^a95% exact binomial confidence intervals

Pig and dog samples positive in WB were characterized by a pattern of specific bands localized between 36 and 72 kDa (36, 40, 45, 55 and 72 kDa) (Figure 2.2). Positive pig and dog samples were shown in 8 and 5 of the 20 surveyed districts in the two provinces, respectively (Figure 2.3).

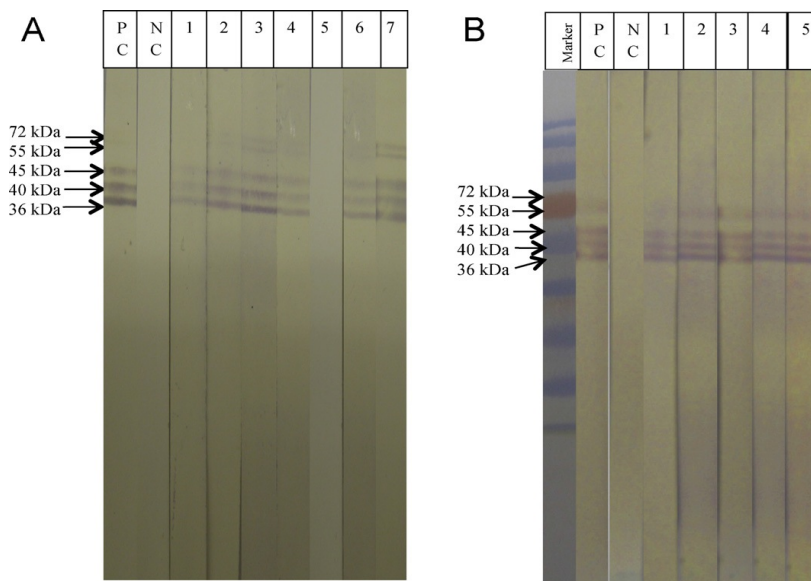


Figure 2.2. Western Blot analysis of serum samples from pigs (A) and dogs (B) from northwestern Vietnam that were positive in *Trichinella* ELISA. PC: positive control; NC: negative control; kDa: molecular weight ladder in kilodalton. (A) Lanes 1, 2, 3, 4, 6, 7 positive samples; lane 5 negative sample and (B) lanes 1, 2, 3, 4, 5 positive samples.

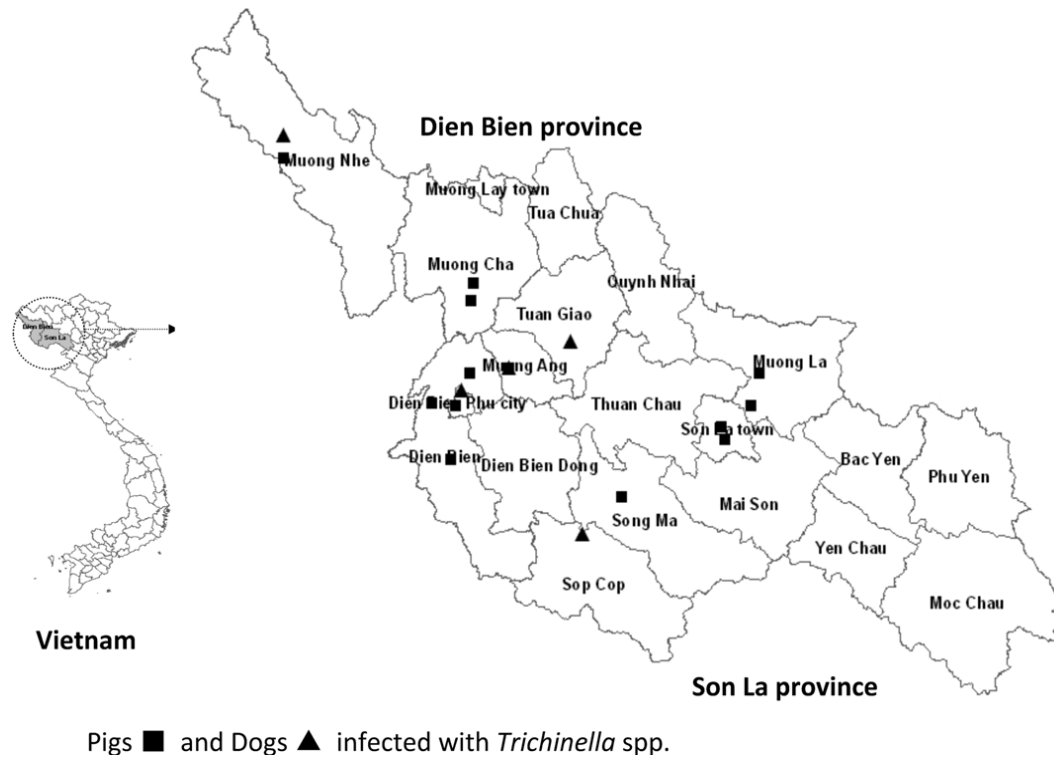


Figure 2.3. Map of Dien Bien and Son La provinces in northwestern Vietnam, showing the distribution of *Trichinella* infections in pigs and dogs

2.2.4. Discussion

Trichinellosis has recently been identified as an emerging zoonosis in three northwestern Vietnamese provinces i.e. Yen Bai, Dien Bien and Son La. These outbreaks were the result of consumption of undercooked pork. The seroprevalence of *Trichinella* infection in pigs in a district of Son La province where the most recent outbreak occurred (Taylor et al., 2009) was 19.9% (Vu et al., 2010). This high seroprevalence was not unexpected since northwestern Vietnam is located between northern Lao PDR and south-eastern China (Yunnan province), which are two areas with a high prevalence of *T. spiralis* infection in domestic pigs (Barennes et al., 2008; Wang et al., 2006). The aim of the present study was to collect baseline data on the seroprevalence of trichinellosis in pigs and domestic carnivorous animals in these provinces. A cross sectional randomized sampling approach was chosen and sampling was done in all districts of these two provinces to get more accurate distribution data, not only in outbreak areas. Samples were first screened by ELISA using E/S antigens, followed by confirmation by WB of the ELISA positive samples. ELISA is the most commonly used method for the detection of *Trichinella*

infection, mainly due to a sensitivity that allows detection of one larva per 100 g of muscle tissue and because it can be applied as an ante-mortem test, in contrast to the artificial digestion method on muscle samples (Gamble et al., 1983). However, because the specificity of E/S antigens has recently been questioned (Gómez-Morales et al., 2008; Vu et al., 2010), WB confirmation is essential to rule out false positive reactions. WB using E/S products of *T. spiralis* as antigens was reported to have a specificity of 100% (Nöckler et al., 2009). The molecular weight of bands that were recognized in pigs in this study ranged from 36 to 72 kDa. In previous studies, the sizes of fractions ranged from 43 to 64 kDa (Reiterova et al., 2007), 43 to 102 kDa (Nöckler et al., 2009) and 35 to 110 kDa (Picherot et al., 2007). Variations in the molecular weight for specific protein fractions of the different studies can be explained by the use of different protocols for preparing antigens (Pozio et al., 2002) or by different protocols of the SDS-PAGE electrophoresis, where parameters such as reducing versus non-reducing conditions, sample buffer composition, boiling versus non-boiling etc. can affect the outcome of the banding pattern (Wee et al., 2001; Nöckler et al., 2009). There is a need to standardize these methods.

We used filter papers to transport serum samples. Storage and transport of serum samples on filter paper has been successfully used for antibody detection in a number of diseases, without significantly affecting results in serological tests when compared with serum samples stored at low temperature (Banks, 1985; Beebe and Briggs, 1990; Behets et al., 1992; Chitambar and Chadha, 2000). Serum adsorbed on filter paper provides a safe, cheap and practical manner to transport samples. Heat treatment of filter papers to reduce the risk of potential viral contamination does not affect the results of the *Trichinella* ES-ELISA (Wilmaerts, 2010).

The results show a lower overall seroprevalence in pigs compared to the study in the outbreak area (Vu et al., 2010); a corrected seroprevalence after WB confirmation of ELISA positive results was 5.6%. The result is within the lower range of what has been reported in neighboring China where seroprevalence ranged between 0.01% and 29.95% (Cui et al., 2011). Positive samples were shown in 8 of the 20 surveyed districts in the two provinces, suggesting that *Trichinella* is distributed over most of the area (Figure 2.3). A large part of this area's population is inhabited by ethnic minorities that often consume raw pork and are unaware of the basic means for preventing the transmission of food-

borne diseases. Feeding the pigs with kitchen leftovers is a common practice both in extensive and semi-intensive husbandry systems.

Interestingly, 4% of the dogs were found positive in ELISA and these were all confirmed in WB. The same molecular weight bands were recognized as with pig serum samples. None of the sampled cats showed evidence of infection. In China the seroprevalence in dogs ranges from 1.2% to 44.8% (Pozio, 2007), and trichinellosis in dogs was also reported in Thailand (Dissamarn and Indrakamhang, 1985) and Lao PDR (Barennes et al., 2008). In rural areas of Vietnam dogs and pigs mostly share the same environment and food. Scavenging behavior in dogs is more likely than in cats that feed more on preys. The consumption of dog meat is part of the culinary traditions of North Vietnam. However, in contrast to pork, dog meat is usually well cooked before consumption so that it is a less probable source of infection of trichinellosis in humans.

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CHAPTER 3

TRICHINELLA INFECTIONS IN WILD ANIMALS

Based on the manuscript: **Thi, N.V.**, Nguyen, V.D., Praet, N., Claes, L., Gabriel, S., Huyen, N.T., Dorny, P., 2014. *Trichinella* infection in wild boars and synanthropic rats in northwest Vietnam. Vet. Parasitol. 200, 207-211.

3.1. Introduction

In southeast Asia, trichinellosis is a food-borne parasitic zoonosis causing several outbreaks each year, especially in the mountainous regions of Cambodia, southwest China, Lao PDR, north Thailand, and northwest Vietnam (Sayasone et al., 2006; Barennes et al., 2008; Cui et al., 2011; Nguyen et al., 2012). *Trichinella spiralis* is the most common species associated with human disease in the region, but cases of infection with non-encapsulated species (*Trichinella pseudospiralis*, *Trichinella papuae*) were also described in Thailand (Jongwutiwes et al., 1998; Kusolsuk et al., 2006). Data on the occurrence of *Trichinella* sp. in wildlife in the region are scarce.

In Vietnam, reports of trichinellosis outbreaks are a rather recent phenomenon. During 1997-2012, five outbreaks of human trichinellosis were described in mountainous provinces of northern Vietnam (Pozio, 2007; Taylor et al., 2009; Nguyen et al., 2012). All but one of these outbreaks could be attributed to the consumption of raw pork dishes; in the last outbreak the probable source was wild boar meat (Vu et al., 2010); but also outside outbreak villages. In Dien Bien and Son La provinces, serological evidence of *Trichinella* infection was found in pigs and dogs (Vu Thi et al., 2013). In neighbouring China, *T. spiralis* has been identified in wildlife (Cui et al., 2011), but identification of *Trichinella* species from Vietnam has not yet been done.

In order to better inform the local population on the risks of infection, a better knowledge of the local epidemiology is required. This includes information on the synanthropic and sylvatic reservoirs of *Trichinella*. The objectives of the present research were (1) to study the occurrence of *Trichinella* infection in hunted and farm-bred wild boar (*Sus scrofa*) and in synanthropic rats in Dien Bien and Son La provinces in Northwest Vietnam, by using parasitological and serological methods and (2) to identify the parasite species isolated from these animals by molecular methods.

3.2. Materials and methods

3.2.1. Study site

The study was conducted in Dien Bien and Son La provinces located in northwest Vietnam (20°39' - 22°33' north latitude, 102°10' - 105°20' east longitude (IMH, 2010)),

where outbreaks of trichinellosis occurred in the last decade (Pozio, 2007; Taylor et al., 2009; Nguyen et al., 2012) (Figure 3.1). The area has a subtropical climate with distinctive rotation of four seasons and an average annual temperature of 21-23⁰C (IMH, 2010). These mountainous provinces have a forest cover of 37% of the total land area (SRVN (Socialist Republic of Viet Nam, Government portal), 2013).

3.2.2. Sampling design

Two samplings were organized in the framework of this study. First, restaurant owners and hunters were asked to collect skeletal muscle samples (diaphragm, masseter, and legs) (Kapel, 2001) from hunted wild boars (*Sus scrofa*) during 2010 - 2013. A total of 62 wild boar muscle samples could be collected. Except for the hunting site, which was recorded, other information such as, sex and age data could not be recorded systematically. In addition, 261 serum samples from farm-bred wild boars (*Sus scrofa*) raised in seven farms located in the same provinces were collected. Sex and age data of these animals were recorded systematically. Second, 820 rats were trapped in rice fields in the vicinity of villages. The traps used were made locally from steel grid, and measure approximately 10 to 30 cm; a bait was put inside to attract the rats. The sample size for each province was based on a conservative 50% estimated prevalence (95% CI, 5% error) (Branscum et al., 2006). In both provinces, care was taken to collect an equal number of rats from each of the 20 districts. The latitude and longitude of the origin of rats were recorded, but the rat species was not determined.

3.2.3. Magnetic stirrer artificial digestion

Wild boar and rat muscle samples were stored at -20 ⁰C before being subjected to the magnetic stirrer artificial digestion method (Gamble et al., 2000) to determine the presence of *Trichinella* larvae. Individual digestions of 37±15 g skeletal muscles were done for each wild boar. In rats muscle samples of 5 g from 10 animals were pooled before being subjected to digestion. In the case of a positive result, individual muscle samples were retested. The recovered larvae were stored in 90% ethyl alcohol until species identification.

3.2.4. *Molecular identification*

DNA was extracted from single larvae according to the guideline of the European Union Reference Laboratory for Parasites (EURLPISS, 2013). Single larvae from International Trichinella Reference Centre (ITRC) isolate *T. spiralis* (code ISS 599) and *T. pseudospiralis* (code ISS13) were used as controls.

A nested, multiplex PCR protocol was carried out in a final volume of 30 μ l according to Zarlenga et al., 1999: the entire internal transcribed spacer region as well as the gap region of the expansion segment V of the large subunit ribosomal DNA are amplified concurrently in a first-round PCR using primer sets specific for each region, followed by a multiplex PCR for final diagnosis. A volume of 10 μ l crude DNA from a single larva was amplified in 20 μ l of 5 units of master mix (Promega code M7505) containing 1 μ l Taq polymerase, 25 μ l 5x PCR buffer, 8 μ l MgCl₂, 1 μ l dNTPs, 61 μ l pure water and 4 of primers (I-V primer sets) (10 pmol/ μ l of each primer). Amplification consisted of 39 cycles, each consisting of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. The fragments amplified from purified DNA were separated on a 1.5% agarose gel (Eurogentec, code EP-0010-05) with TAE buffer (Promega, code V4271), stained with syb safe (Invitrogen, code S33102) and visualized under a blue light transilluminator (Safe imager TM2.0, Invitrogen).

3.2.5. *Enzyme-linked immunosorbent assay*

Serum samples from wild boars were tested to detect anti-*Trichinella* IgG by ELISA using excretory/secretory antigens, according to a published previous protocol (Vu Thi et al., 2013). The cut off on each plate was calculated based on the optical densities (OD) of eight negative pig samples using a Student's t-test at a probability of $p < 0.001$. Serum samples from experimentally *Trichinella* infected pigs were used as positive controls.

3.2.6. *Data analysis*

Exact binomial 95% confidence intervals for proportions were calculated using STATA software package, version 11.0 (Stata Corp., College Station, TX, USA).

3.3. Results

Trichinella sp. larvae were found in two (3.2%; 95% CI: 0.8-4.8) of the 62 hunted wild boars. These two wild boars had been shot in the same commune, Ta Xua in Bac Yen district, Son La province (Figure 3.1). The parasite burdens in the muscle tissues were 0.1 and 0.03 larvae/g, respectively. Five larvae collected from the two infected animals (four and one larvae, respectively) were identified as *T. spiralis* by multiplex PCR.

Of the 261 serum samples from farmed wild boars tested by ELISA, none was positive for *Trichinella* antibodies (0%; 95% CI: 0-0.014). The age of these wild boars ranged from 10 to 48 months; 148 (56.7%) were male and 113 (43.3%) were female.

Twenty three (2.8%; 95% CI: 13.7 - 32.3) out of the 820 rats, originating from 7/20 districts studied were positive by the magnetic stirrer artificial digestion (Figure 3.1). In rats, the number of larvae per gram recovered was between 0.1 and 7 larvae/g (average 0.6 larvae/g). All 102 recovered larvae subjected to multiplex PCR were identified as *T. spiralis*.

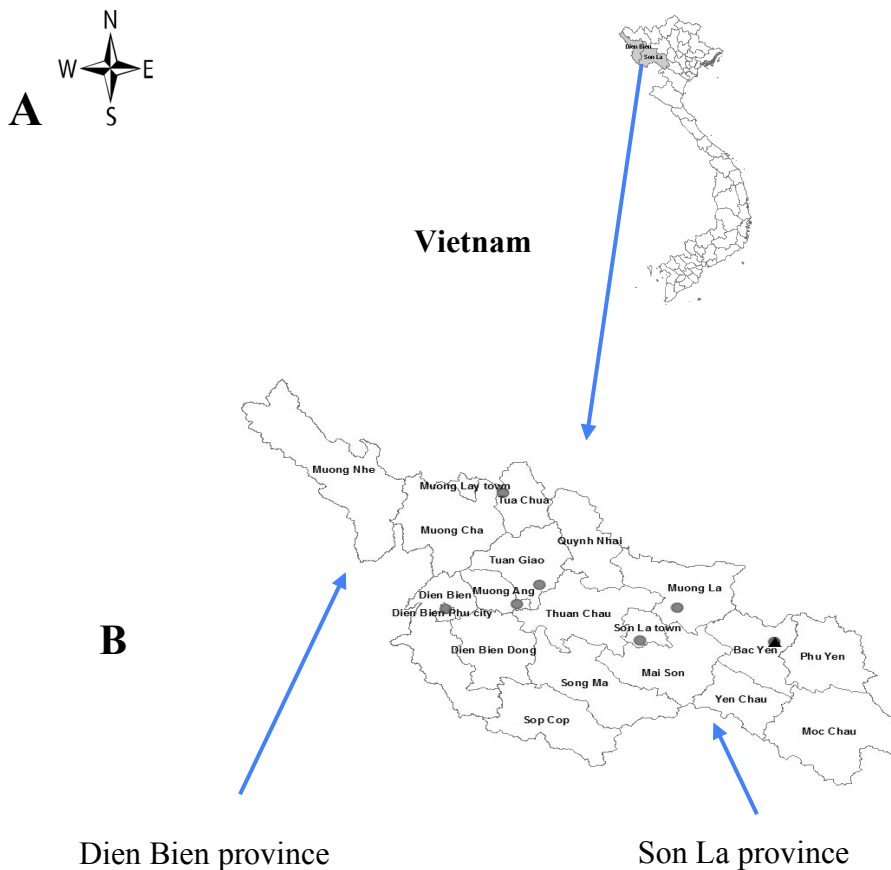


Figure 3.1. Maps of Vietnam (A) and Dien Bien and Son La provinces in northwest Vietnam (B) showing the origin of the 23 synanthropic rats and 2 wild boars infected with *T. spiralis*. Filled circles: synanthropic rats infected. Filled triangle: wild boars infected.

3.4. Discussion

This is the first report of *T. spiralis* infection in wild boars and rats in Vietnam. *T. spiralis* is the only species identified so far in the country in human and porcine infections (Vu et al., 2010; Nguyen et al., 2012). The finding of *T. spiralis* in hunted wild boars is consistent with reports of its presence in wild animals of neighboring countries (Sayasone et al., 2006; Barennes et al., 2008; Cui et al., 2011). Infection levels of *T. spiralis* in wild boar in our study were low, which has also been observed in other studies in different countries (Malakauskas et al., 2007; Cohen et al., 2010). A low parasite load of between 0.03 and 0.1 larvae/g was found in the two infected animals. Prevalence and parasite load might have been underestimated as lower sensitivity of the magnetic stirrer artificial digestion method has been reported when applied on frozen meat samples. Long-term storage of muscles by freezing affects larval integrity and consequently the recovery of intact larvae by the digestion method (International Commission for Trichinellosis, 2012). Although the parasite burden found in the tested samples was low, the results indicate that the parasite is circulating in the sylvatic reservoir with a potential for spillover to the domestic environment and the possibility of sporadic outbreaks due to the consumption of meat from animals with a higher larval burden.

The recent outbreak of human trichinellosis in neighboring Thanh Hoa province that was probably caused by the consumption of wild boar meat (Nguyen et al., 2012) shows the potential of infection from the sylvatic reservoir in Northwest Vietnam. Hunters may contribute to the spread of *Trichinella* as they often discard the carcasses of hunted wild boars in the environment, without following sanitary regulations (Pozio and Murrell, 2006). No evidence of infection was found in wild boars that were farm-raised in this area. Given the rather low prevalence of the parasite in domestic and wild animals in the region, no conclusions can be drawn on whether or not farm-bred wild boars are at risk for *Trichinella* infection.

In China, the prevalence of *T. spiralis* infection in rats ranged from 1.98% to 15.06% in six provinces or autonomous regions (Wang and Cui, 2001). Those results are consistent with the finding of 2.8% positives in this study. The distribution of *Trichinella* infection in rats in 7 out of 20 districts of Dien Bien and Son La confirms the results in pigs of a previous survey that showed evidence of *Trichinella* infection in these provinces (Vu Thi et al., 2013). Some rat species, such as the Greater Bandicoot rat (*Bandicota indica*), represent a common source of meat in Vietnam. However, the species of rats sampled in this study was not determined; therefore, the presence of *Trichinella* larvae in rats caught for human consumption could not be confirmed.

In conclusion, this study has demonstrated *T. spiralis* infection in hunted wild boars and synanthropic rats in northwest Vietnam, though both the proportion of positives and the infection load were low. The results confirm the endemicity of this zoonotic parasite in this region and indicate that the local population and health centers should be made aware of the risks of eating raw or undercooked meat dishes prepared from wild animals.

3.5. References

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CHAPTER 4

HUMAN TRICHINELLOSIS

Part 1: Molecular identification of *Trichinella* species from humans in Northern Vietnam

Based on the manuscript: De, N.V., Trung, N.V., Ha, N.H., **Nga, V.T.**, Ha, N.M., Thuy, P.T., Duyet, I., V, Chai, J.Y., 2012. An outbreak of trichinosis with molecular identification of *Trichinella* sp. in Vietnam. Korean J. Parasitol. 50, 339-343.

(Contribution of Nga, V.T: Testing of the serum samples and discussion of the results).

4.1.1. Introduction

Trichinella spiralis adult worms parasitize the intestine and their larvae encyst in muscles of humans and animals (Cook, 2013; De et al., 2006; Intapan et al., 2011; Miyazaki, 2013). In Asia, *Trichinella* spp. infection has been documented in humans in 18 countries, in domestic animals (mainly pigs) in 9 countries, and in wildlife in 14 countries (Owen et al., 2005; Pozio, 2007). Molecular identification of *Trichinella papuae* was reported in Thailand (Intapan et al., 2011) and *T. spiralis* in Korea (Sohn et al., 2003), but no reports are available in Vietnam.

During 1970 - 2008, four outbreaks of human trichinellosis occurred in three northern provinces of Vietnam and involved 90 infected cases, of which eight died (De et al., 2006; Vu et al., 2010). A fifth outbreak occurred in February 2012 in a mountainous area of Thanh Hoa province involving 24 patients who ate raw pork at a lunar Year party (6 of them are reported in this paper) (Figure 4.1). However, additional data are needed to provide a better understanding of the genetic characteristics of the Vietnamese *Trichinella* species.



Figure 4.1. Map of the endemic areas of trichinellosis in Vietnam.

4.1.2. Case record

In February 2012, 24 of 27 people, who ate raw pork at a lunar Year party in Muong Lat district, Thanh Hoa province, Vietnam, were diagnosed of having trichinellosis by ELISA using *T. spiralis* E/S antigen. All patients showed similar clinical symptoms, i.e., fever and muscle pain, and were treated with albendazole at a dosage of 800 mg/day for 10 days. Among them, six patients visited hospitals in Ha Noi, Vietnam. Their clinical presentation are as follows:

The six patients included three men and three women aged between 30 and 43 years. The clinical signs and symptoms included fever, muscular pain, difficult moving, edema in the leg/hand, edema around the eyes and difficult swallowing in all six patients. Loss of weight and difficulty in breathing were recorded in five of six patients, itching in three of six patients, and diarrhea in one of six patients (Table 4.1). Laboratory findings in these patients included eosinophilia from 18.5% to 51.8% in five of six patients; increased serum glutamic oxaloacetic transaminase (SGOT) in all six patients (range 65-219 U/L; normal values ≤ 37 U/L in man and ≤ 31 U/L in woman) and for Alanine transaminase (ALT) in all six patients (range 74-471 U/L; normal values ≤ 40 U/L in man and ≤ 31 U/L in woman). A positive ELISA result was found at 30 days after having eating raw pork in all six patients, and *T. spiralis* larvae were detected by muscle biopsy in all six patients. A *Leptospira* test and bacterial culture were negative in all six patients (Table 4.2). The six patients were treated with albendazole at a dosage of 800 mg/day for 10 days, resulting in the disappearance of the symptoms within one month.

Table 4.1. Clinical signs and symptoms in six trichinellosis patients.

Symptoms	Patient 1		Patient 2		Patient 3		Patient 4		Patient 5		Patient 6	
	Man	Woman	Man	Woman	Man	Woman	Man	Woman	Man	Woman	Man	Woman
Gender	Man	Woman	Man	Woman	Man	Woman	Man	Woman	Man	Woman	Man	Woman
Age (years)	39	41	43	41	30	30	30	30	30	30	30	30
Fever	+	+	+	+	+	+	+	+	+	+	+	+
Muscular pain	+	+	+	+	+	+	+	+	+	+	+	+
Edema in leg/hand	+	+	+	+	+	+	+	+	+	+	+	+
Edema in eyes	+	+	+	+	+	+	+	+	+	+	+	+
Difficult moving	+	+	+	+	+	+	+	+	+	+	+	+
Difficult swallowing	+	+	+	+	+	+	+	+	+	+	+	+
Difficult breathing	+	+	+	+	+	+	-	-	+	+	+	+
Loss of weight	+	+	+	+	+	+	-	-	+	+	+	+
Itching	+	+	+	+	+	+	-	-	+	+	-	-
Diarrhea	+	+	-	-	-	-	-	-	-	-	-	-
Time of symptom appearance (days)	5	7	6	7	6	8	5	8	5	5	5	5

Table 4.2. Laboratory findings in six trichinellosis patients.

Findings	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Erythrocyte/mm ³	4.2	5.5	4.8	3.3	4.5	3.4
Leukocyte/mm ³	17.0	13.6	12.4	12.4	11.1	10.2
Eosinophils (%)	51.8	18.6	5.1	15.9	28.4	18.5
SGOT (U/L)	219	115	219	65	106	112
ALT (U/L)	471	314	471	74	146	106
Serum ELISA for <i>Trichinella</i>	+	+	+	+	+	+
Biopsy on <i>Trichinella</i> larvae	+	+	+	+	+	+
<i>Leptospira</i> test	-	-	-	-	-	-
Bacterial culture	-	-	-	-	-	-

Table 4.3. Sequencing of the cox 3 of *T. spiralis* isolates from GenBank compared with Vietnamese *T. spiralis*

Notation	Origin	Host	Length	Species	GenBank no.	Author
ATGX-VN	Vietnam ^a	Human	200 bp	<i>T. spiralis</i>	-	De et al. ^a
Tspi1	China	<i>Sus scrofa</i>	200 bp	<i>T. spiralis</i>	GU339148.1	Rosenthal et al., 2008
Tspi2	China	<i>Sus scrofa</i>	200 bp	<i>T. spiralis</i>	GU339147.1	Rosenthal et al., 2008
Tspi3	China	<i>Sus scrofa</i>	200 bp	<i>T. spiralis</i>	GU339146.1	Rosenthal et al., 2008
Tspi4	China	<i>Sus scrofa</i>	200 bp	<i>T. spiralis</i>	GU339145.1	Rosenthal et al., 2008
Tspi5	USA	<i>Mephitis mephitis</i> ¹	200 bp	<i>T. spiralis</i>	GU339142.1	Rosenthal et al., 2008
Tspi6	Spain	<i>Sus scrofa</i>	200 bp	<i>T. spiralis</i>	GU339139.1	Rosenthal et al., 2008
Tspi7	Bulgaria	<i>Sus scrofa</i>	200 bp	<i>T. spiralis</i>	GU339135.1	Rosenthal et al., 2008
Tspi8	Finland	<i>Sus scrofa</i>	200 bp	<i>T. spiralis</i>	GU339134.1	Rosenthal et al., 2008
Tspi9	Genbank	<i>Sus scrofa</i>	200 bp	<i>T. spiralis</i>	GU386314.1	Webb et al., 2012

¹ *Mephitis mephitis*: Striped skunk

Molecular studies

The muscle larvae isolated from patients (Figure 4.2) were identified by molecular methods using the mitochondrial cytochrome *c* oxidase subunit III (*cox3*) gene in comparison with that of strains deposited in GenBank (Table 4.3). Comparison of 200 nucleotides of a portion of *cox3* mitochondrial genome between the Vietnamese (ATGX-VN) and other geographical isolates of *T. spiralis*, including Chinese (Tspi1, Tspi2, Tspi3, and Tspi4), US (Tspi5), Spanish (Tspi6), Bulgarian (Tspi7), Finnish (Tspi8), and GenBank (Tspi9) isolates showed that the nucleotide homology between the Vietnamese and Chinese isolates were 100% and that there was 99% homology between the Vietnamese and the other isolates. A phylogenetic tree was constructed for *T. spiralis* Vietnam and other isolates using *cox3* nucleotide sequences as estimated by neighbor-joining (NJ) using MEGA4.0 (Tamura et al., 2004); Vietnamese *T. spiralis* clustered in the same group with the other *T. spiralis* isolates (Figure 4.3).

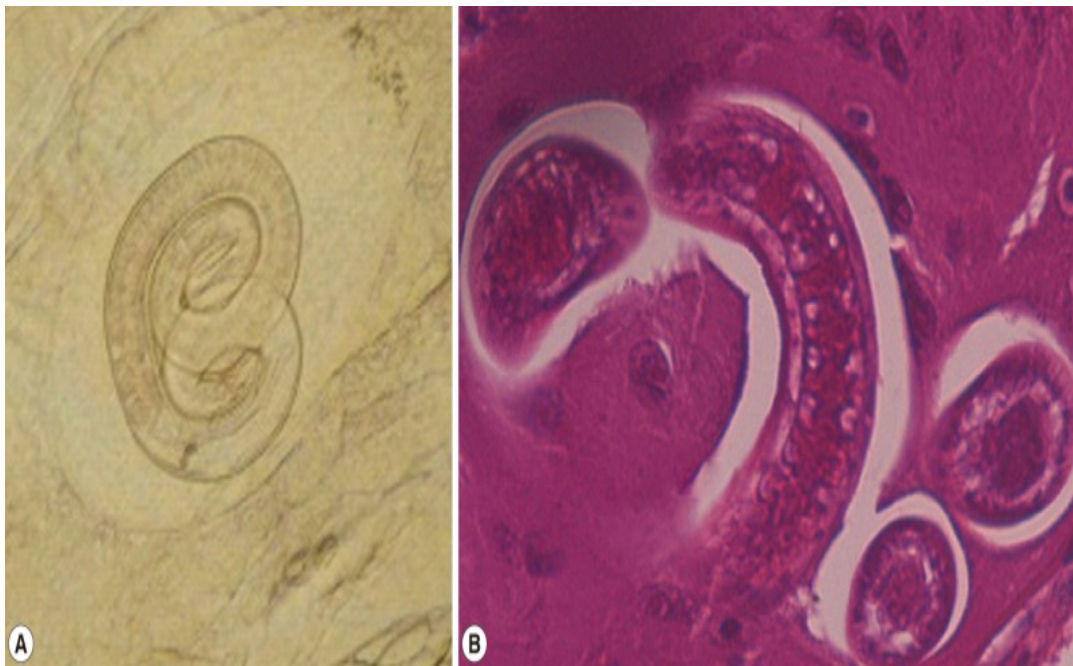


Figure 4.2. *Trichinella spiralis* larva in the human muscle (A) and section of a *T. spiralis* larva in the muscle (B).

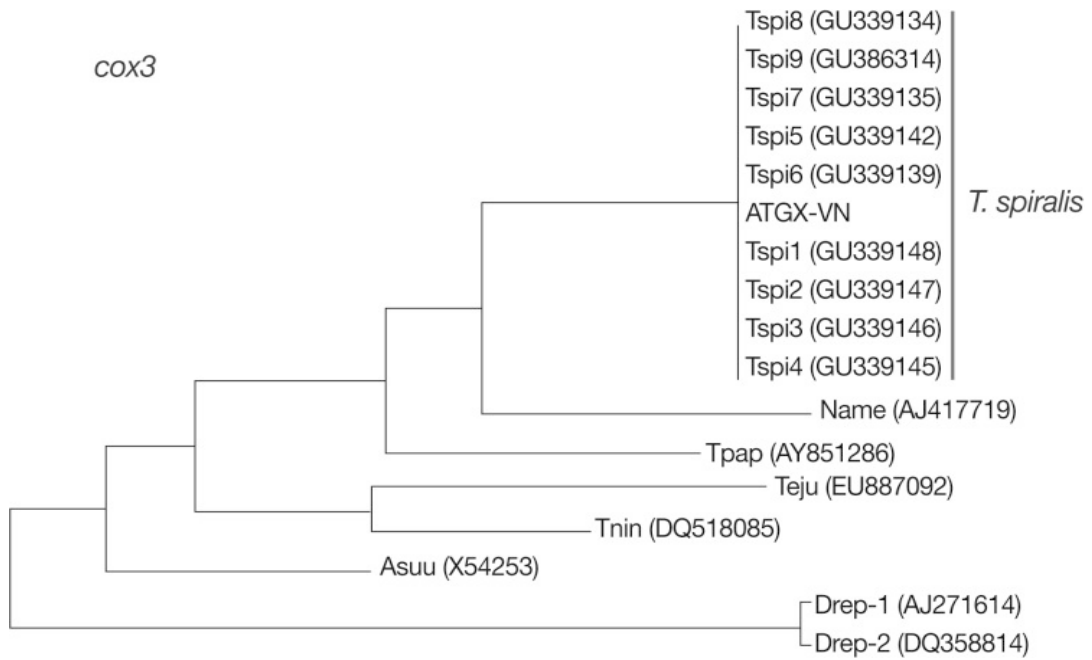


Figure 4.3. Phylogenetic tree of *Trichinella spiralis* Vietnam and other isolates from a part of *cox3* nucleotide sequence estimated by Neighbor-Joining (NJ) method using MEGA 4.0 [6]. Note: ATGX-VN= Vietnamese; Tspi1, Tspi1, Tspi2, Tspi3, Tspi4=Chinese; Tspi5=US, Tspi6=Spain; Tspi7= Bulgarian; Tspi8=Finland; Tspi9= GenBank

4.1.3. Discussion

All five trichinellosis outbreaks that occurred in the last decades in Vietnam occurred in mountainous regions of the northern part of the country (Figure 4.1). In this region, the local people have the habit of practicing free-roaming of pigs and of eating raw pork, particularly at parties. These habits are comparable to practices found in Thailand, Laos, India, and other parts of Asia and the Southern Pacific (Miyazaki., 2013). The second outbreak in Vietnam occurred 31 years after the first outbreak (De et al., 2006). It can be questioned why there were no other cases of trichinellosis for such a long time. Parasitic diseases, such as trichinellosis may be neglected or misdiagnosed by local health authorities due to a lack of knowledge and of diagnostic facilities. Actually, all the trichinellosis patients in five outbreaks of Vietnam were misdiagnosed as leptospirosis at first, because this disease also causes clinical symptoms, such as fever and muscle pain, and were treated with antibiotics and not with anthelmintic drugs. Only after some patients died, the local health services contacted the national health level, whereafter diagnosis of trichinellosis was made. Moreover, trichinellosis can be clinically mild and can resemble other diseases, because it does not have pathognomonic signs or symptoms, and in endemic areas, if people frequently eat *Trichinella*-infected meat, they can develop an asymptomatic form of the disease (Owen et al., 2005).

The main clinical signs and symptoms in the six patients in this study were fever, muscle pain, difficult moving, edema and difficult swallowing (Table 4.1), which is similar to observations in 98 trichinellosis patients in three previous outbreaks in Vietnam (De et al., 2006). In those patients symptoms developed within 1-30 days after eating infected raw pork (7.9 days in average). The symptoms included fever (100% of patients), muscle pain (100%), difficult moving (90.9-100%), edema (90.9-95.5%), difficult swallowing (11.5-90.9%), weight loss (90.0-90.9%), itching (85.0-86.4%), difficulty in breathing (50.0-80.0%), diarrhea (50.0%), lisping (40.0-68.2%), abdominal pain (35.0-100%), and stool with blood (0-9.1%); six patients died.

In our report, the biopsy for detection of larvae in the muscles of patients showed 100% positive results for *Trichinella* larvae (Figure 4.2). The result of ELISA with *T. spiralis* E/S antigen was positive in all six patients and in 18 other patients in this outbreak. The total number of leukocytes, and the percentage of eosinophils, increased remarkably. Transaminases increased also in all six patients.

In each of the reported outbreaks, patients ate raw pork from domestic pigs, and the pigs were found to be infected with *T. spiralis* larvae. In the first outbreak, the pig of which the meat was the source of the infection was a female animal of eight years old that weighed 50 kg and that was infected with 879 larvae/g muscle (De et al., 2006). Another seven years old male pig in this area was infected with 70 larvae/g muscle. In the second outbreak, the patients ate raw pork from a five years old female pig, weighing 70 kg that was confirmed to be infected with *T. spiralis* larvae. In the third outbreak, the patients consumed raw pork from a three years old female pig, weighing 60 kg. In the fourth outbreak, the patients ate raw pork from a five years old female pig weighing 70 kg that was infected with 1-3 larvae/g muscle (De et al., 2006; Vu et al., 2010). In that fourth outbreak, 206 (19.9%) of 1,053 domestic pigs (free roaming) were positive in *T. spiralis* ELISA. Muscle samples from 76 serologically positive pigs were tested by artificial digestion, and *Trichinella* larvae were detected in 11 (14.5%) of them (chapter 2; Vu et al., 2010). Appearance of clinical symptoms in our cases was not longer (6.0 days in average) than in cases of previous outbreaks (7.9 days in average). In our study, the larvae from humans were identified as *T. spiralis* by molecular methods. This is the first confirmed report of *T. spiralis* infection in humans in Vietnam based on molecular methods.

4.1.4. References

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CHAPTER 4

HUMAN TRICHINELLOSIS

Part 2: The hidden burden of trichinellosis in Vietnam

Based on the manuscript: **Vu, T.N.**, Trung, D.D., Litzroth, A., Praet, N., Nguyen, T.H., Nguyen, M.H., Dorny, P., 2013. The hidden burden of trichinellosis in Vietnam: A postoutbreak epidemiological study. *Biomed. Res. Int.* 2013, 149890.

4.2.1. Introduction

During 1997-2012, five outbreaks of human trichinellosis were described in mountainous provinces of North Vietnam (i.e., Son La, Dien Bien and Thanh Hoa provinces; Figure 4.6) (Pozio, 2007; Taylor et al., 2009; Van et al., 2012). Diagnosis of the cases from these outbreaks was made in national hospitals many weeks after the onset because there is a lack of disease knowledge and facilities for diagnosis at provincial and district hospital levels (NIMPE, 2012).

On February 24, 2012, a cluster of 6 patients was hospitalized in Bach Mai hospital and the National Hospital for Tropical Diseases, Hanoi, with main clinical symptoms consisting of fever, muscular pain, difficult moving, facial oedema, and pain of the masseter muscles (Van et al., 2012). All patients tested positive in a *Trichinella* antibody ELISA and all cases showed positive muscle biopsy results for *Trichinella* larvae. The larvae were identified as *Trichinella spiralis* by molecular analysis. The patients' histories suggested that they all had consumed meat from a locally hunted wild boar during the Vietnamese lunar year celebration (Tet) in Muong Lat town (Muong Lat district, Thanh Hoa province; Figure 4.4), about one month before hospitalization in Hanoi. The objective of this study was to conduct a postoutbreak study in Muong Lat town shortly after diagnosis of trichinellosis in the 6 patients in order to determine if more inhabitants had non diagnosed trichinellosis in this community. Ethical approval for the study was reviewed and approved by the Institutional Review Board of the Hanoi Medical University of the Vietnamese Ministry of Health (approval no. 100/HMU IRB).

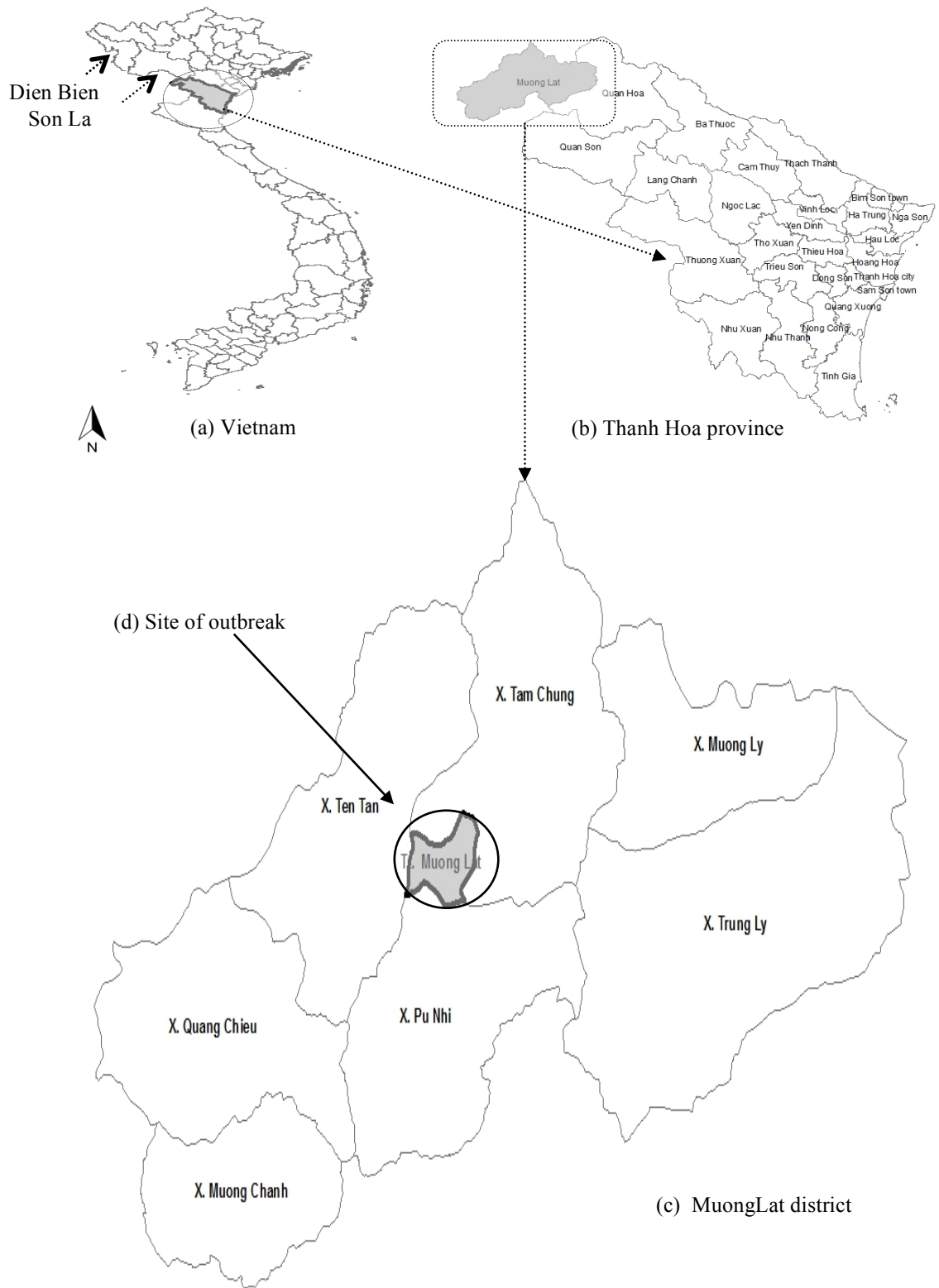


Figure 4.4. Map of Vietnam (a), Thanh Hoa province (b), Muong Lat district (c), with site of the outbreak (d).

4.2.2. Materials and methods

4.2.2.1. Study area and sampling

The mountainous Muong Lat district (850 km²; 35959 inhabitants; 1 town and 7 communes) is located in Thanh Hoa province in the North Central Coast region of Vietnam, bordering Son La province, where an outbreak of human trichinellosis occurred in 2008, and Lao PDR (Figure 4.4). Muong Lat town, where the patients originated from, has a population of 3007 inhabitants, mainly of Kinh ethnicity, distributed over 543 households. All inhabitants of Muong Lat town were informed by the Muong Lat Medicine Centre about the study purpose before collecting samples. Inclusion criteria were (1) consumption of traditional dishes containing raw (lap) or undercooked (nemchua) meat prepared from a hunted wild boar during the previous Tet celebration and (2) presenting at least one of the symptoms that can be associated with trichinellosis (fever, facial oedema, myalgia, diarrhoea, etc.) as proposed by Dupouy-Camet et al (2002). Individuals who met those criteria were blood sampled after giving informed consent. Structured questionnaires were used to collect information on the type of consumed meat, incubation period, and demographic information such as age, gender, and ethnicity. Giemsa stained films prepared from human blood samples were preserved in boxes at room temperature until microscopic examination. Serum was collected from the blood samples by centrifugation and stored at -70 °C until analysis.

4.2.2.2. Laboratory procedures and data analysis

Peripheral blood cell counts were performed by microscopy on Giemsa stained films. A suspected case was defined as a patient with moderate (1000-3000 cells/ μ L) to high (>3000 cells/ μ L) eosinophilia (NIMPE, 2012). Excretory/secretory (E/S) antigen of *T. spiralis* first stage larvae, used in ELISA and Western Blot (WB), was produced using the protocol described by Gómez Morales et al (2012). Serum samples were first tested for the presence of anti-*Trichinella* IgG by ELISA using E/S antigen (Gomez-Morales et al., 2008; Gomez-Morales et al., 2009). The cutoff of each plate was calculated based on the optical densities (OD) of 8 negative samples using a Student' t-test at a probability of $P < 0.001$. Positive controls were obtained from patients with confirmed trichinellosis, and negative controls consisted of serum samples from donors known to be *Trichinella* free. ELISA positive samples were tested by WB for confirmation (Barennes et al., 2008;

Gomez-Morales et al., 2012). A pattern of three bands ranging in size between 48 and 72 kDa was considered to be positive (Gomez-Morales et al., 2012). Univariate and multivariate logistic regression analysis were used to investigate the relation between test seropositivity, age, and gender. Data analysis was performed using STATA/SE 11.0 (Stata Corp., College Station, TX, USA). The significance level was set at $P < 0.05$.

4.2.3. Results

A total of 100 individuals who consumed raw or undercooked hunted wild boar meat were identified as suspected cases of trichinellosis. Among these, the clinical symptoms observed were fever (60%), diarrhoea (41%), abdominal pain (40%), myalgia (37%), facial oedema (24%), and pain (20%). Gender distributions for the sampled individuals were women ($N=60$, 60%) and male ($N=40$, 40%); the median age was 31 years (range: 6-68 years); ethnicity was Kinh ($N=78$, 78%) and minority groups ($N=22$, 22%).

Trichinella antibodies were identified by ELISA in 30 samples of the 100 cases (30%). Of these 30 positive ELISA serum samples, all were also positive in WB. The most common clinical symptoms in these serologically positive patients were fever ($N=27$, 90%), myalgia ($N=26$, 87%), facial oedema ($N=19$, 63%), diarrhea ($N=16$, 53%), pain of the masseter muscles ($N=13$, 43%), and abdominal pain ($N=11$, 37%). The median incubation period was 9 days (range: 4-17 days). A moderate to high eosinophilia was detected in 25/30 individuals (83.3%). The median age was 35 years (range: 6-60 years); ethnicity was Kinh ($N=20$, 67%) and minority groups ($N=10$, 33%). All 30 cases were orally treated with albendazole at a dosage of 15 mg/kg body weight per day for a period of two weeks during which they were clinically monitored by the Muong Lat Medicine Centre. Symptoms resolved in all patients during treatment.

The multivariate analysis showed that the proportion of positive individuals was significantly higher in males (Odds Ratio (OR) = 3.18 (95% CI: 1.27-7.97); $P < 0.05$) and increased with age (Odds Ratio (OR) = 1.04 (95% CI: 1.01-1.08); $P < 0.05$). The univariate analysis indicated the same significant associations.

4.2.4. Discussion

The results suggest that in Muong Lat town, where a trichinellosis outbreak occurred one month before this investigation, another 30 individuals who had eaten raw meat dishes prepared from the same wild boar had trichinellosis. Diagnosis was made based on clinical grounds, eosinophilia, and on ELISA followed by WB; no biopsies could be taken in this study for confirmation. Because anti-*Trichinella* IgG antibodies can persist for many years after infection/exposure to the parasite (Morakote et al., 1992), it cannot be ruled out that the positive serological results were from older infections and not related to the current outbreak. However, the high proportion of seropositive results in the sampled patients strongly suggests an association with this outbreak. Several outbreaks of trichinellosis have been described recently in Northwest Vietnam (Murrell and Pozio, 2011; Taylor et al., 2009; Van et al., 2012). Whether trichinellosis is an emerging infection in Vietnam or the identification of these outbreaks is rather the result of better diagnosis is not known. Trichinellosis is a disease primarily of adults, occurring about equally in both sexes; however, in some countries, among which Vietnam, infection in males occurs more frequently (Murrell and Pozio, 2011), which is consistent with our study.

Previous outbreak studies in Northwest Vietnam and in Lao PDR showed that pigs were the source of infection indicating the presence of a domestic life cycle (Barennes et al., 2008; Thi et al., 2013; Vu et al., 2010). The present study suggests that *Trichinella* spp. are also occurring in wildlife in Vietnam. Until now, the only species identified in the country is *T. spiralis*.

This study demonstrates the weakness of the diagnostic capacity and capability at provincial and district levels. It resulted in the underreporting of the number of patients infected during this outbreak and it highlights the neglected characteristic of the infection, especially in remote rural areas where access to health care is often lacking. This study suggests that passive surveillance based on hospital records is likely to result in underestimating the real burden of trichinellosis due to the underreporting of the cases that did not reach the health care system. The study confirms that traditional dishes including raw lap and undercooked meat nem chao are to be considered as sources of infection. This outbreak emphasizes the need for education on the risks of acquiring this disease and the importance of thoroughly cooking meat. In addition, householders should be encouraged to adopt adequate livestock-breeding practices. Further study is recommended to

investigate the presence of *Trichinella* in people and pigs in Thanh Hoa and neighbouring provinces.

4.2.5. References

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CHAPTER 4

HUMAN TRICHINELLOSIS

Part 3: The predictive value of selected clinical signs and symptoms for trichinellosis diagnosis

Based on the manuscript: **Vu, T.N.**, Pozio, E., Van, D.N., Praet, N., Pezzotti, P., Gabriel, S., Claes, M., Thuy, N.T., Dorny, P., 2014. Anti-*Trichinella* IgG in ethnic minorities living in *Trichinella*-endemic areas in northwest Vietnam: Study of the predictive value of selected clinical signs and symptoms for the diagnosis of trichinellosis. *Acta Trop.* 139C, 93-98.

4.3.1. Introduction

In South-East Asia, trichinellosis outbreaks are usually associated with the consumption of traditional raw or undercooked meat dishes from pork or wild boar during wedding, funeral or New Year parties (Barennes et al., 2008; Khumjui et al., 2008; Kusolsuk et al., 2010; Sayasone et al., 2006; Taylor et al., 2009).

In Vietnam, recently, several outbreaks of trichinellosis were reported in the northwestern part of the country; among which were outbreaks in two villages of the Dien Bien and Son La provinces, in 2004 and 2008, respectively (Pozio, 2007; Taylor et al., 2009; Vu et al., 2013). A seroprevalence study carried out in these provinces, showed the presence of anti-*Trichinella* IgG in domestic pigs in eight out of the 20 districts (Thi et al., 2013).

In countries where human trichinellosis is sporadic, physicians are not familiar with the symptomatology caused by this disease, and the diagnosis is often delayed (Pozio et al., 2001). Clinical symptoms of *Trichinella* infection are non-specific (Madariaga et al., 2007), and often misdiagnosis occurs with other diseases such as, leptospirosis, influenza, salmonellosis, fasciolosis, toxocarosis and typhoid fever (Dupouy-Camet et al., 2002; Taylor et al., 2009). The diagnosis of trichinellosis can be made by the direct demonstration of larvae in a muscle biopsy, which is an invasive and painful method, or by indirect serological methods (Dupouy-Camet et al., 2002). In rural areas of Vietnam, the lack of specific diagnostic tools and of physicians who are familiar with the clinical and laboratory features of this disease may result in under diagnosis of trichinellosis (Vu et al., 2013).

The aim of this study was to investigate the presence of anti-*Trichinella* antibodies and the relationship with clinical signs and symptoms in ethnic communities of northwest Vietnam where trichinellosis outbreaks have been documented.

4.3.2. Materials and methods

4.3.2.1. Study design

The study was performed in the Dien Bien and Son La provinces from September 2010 to May 2012 on two population groups.

The first group consisted of 200 inhabitants from the Quai To and Lang Cheu villages (with a total population of 7622 and 1900 inhabitants, respectively) (Department of statistics of Dien Bien and Son La provinces, 2010), (Figure 4.5), where outbreaks of trichinellosis occurred in September 2004 and August 2008, respectively. The inhabitants of these villages were informed by the local Health Centres on the objectives of the study and individuals in each village were selected based on following inclusion criteria: (1) persons who were not hospitalized and/or treated in the course of the trichinellosis outbreaks; (2) persons with at least one clinical sign or symptom of trichinellosis (namely, fever, facial edema, myalgia, diarrhea, neurological and/or cardiologic signs, conjunctivitis, subungual hemorrhage, cutaneous rash, eosinophilia, increased levels of muscular enzymes) according to Dupouy-Camet and Bruschi (2007); and (3) a history of eating raw or undercooked meat. Information and samples were collected by physicians from the Hanoi Medical University and the Son La and Dien Bien “Prevention Centres of malaria, parasites and arthropods” during two days in November 2010 for each village.

The second population consisted of people from different districts of Dien Bien and Son La provinces who were referred to and hospitalized in the provincial hospitals during the study period and met criteria (2) and (3). Information and samples of this study group were collected in the period between April 2011 and December 2012.

Oral consent to participate in the study was obtained from all participants after explaining them the objectives of the study.

Blood samples were collected and sera were stored in aliquots at -70°C until tested. Giemsa-stained blood smears were stored at room temperature until examination.

A questionnaire was administered to each enrolled person for the collection of clinical and epidemiological data (consumption of raw or undercooked meat, knowledge of trichinellosis, age, gender, ethnicity and employment).

The study was approved by the Institutional Review Board of the Hanoi Medical University of the Vietnamese Ministry of Health (No. 100/HMU IRB).

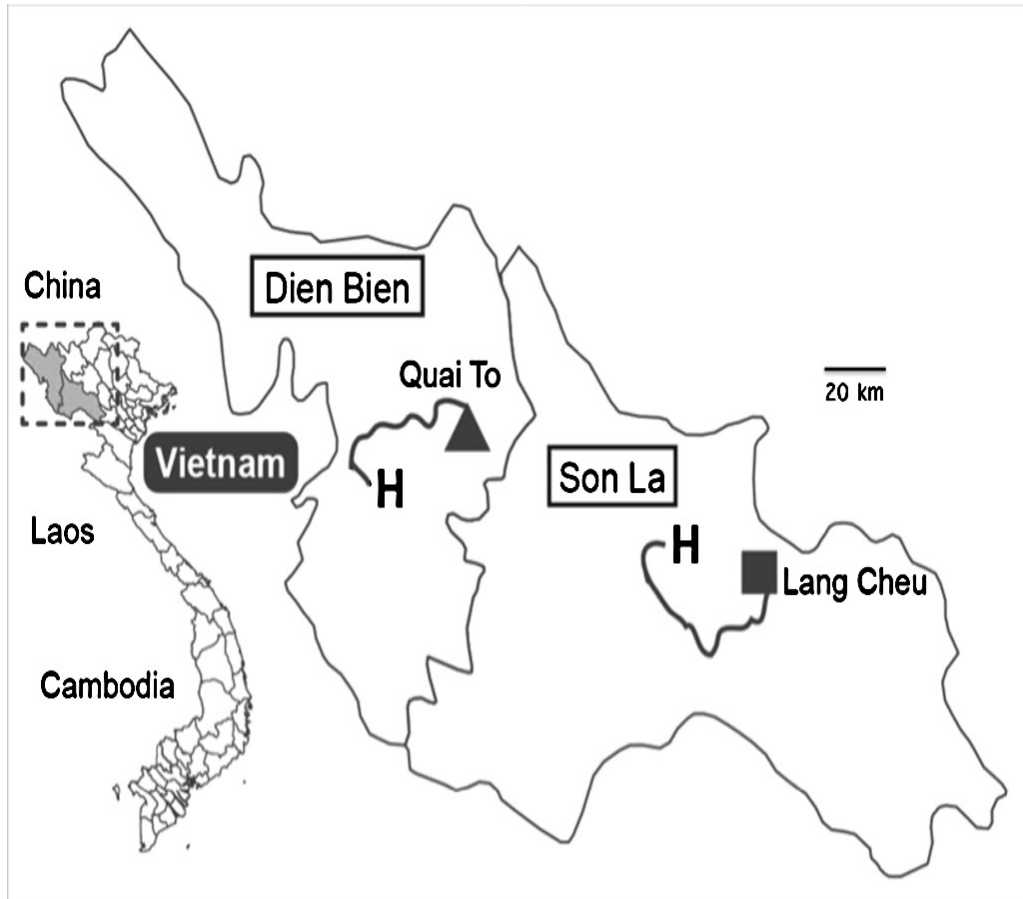


Figure 4.5. Map of Vietnam showing the villages of Quai To and Lang Cheu, and the hospitals (H), where study participants were enrolled. The lines between villages and hospitals show the distance and the winding trail between both.

4.3.2.2. *Laboratory analysis*

Leukocyte and eosinophil counts were done on blood smears of people with a positive serology for *Trichinella* and the slides were stained by Giemsa and read at 1000 \times magnification with immersion oil. A suspected case of trichinellosis was defined as a patient with moderate (1000-3000 cells/ μ l) or high (>3000 cells/ μ l) eosinophilia, and a total leukocyte count of up to 30,000 cells/ μ l (Dupouy-Camet et al., 2002).

Excretory/Secretory (E/S) antigens from *T. spiralis* muscle larvae were produced and used for both ELISA and Western blot (WB) to detect anti-*Trichinella* antibodies in serum samples (Gomez-Morales et al., 2008). The cutoff for each ELISA plate was calculated by the optical density (OD) of eight negative samples by the Student's t-test ($p < 0.001$). Positive controls were from patients with confirmed trichinellosis, and negative controls

consisted of serum samples from donors known to be *Trichinella* free. ELISA positive samples were tested by WB for confirmation. The presence of a three band pattern ranging from 48 to 72 kDa was considered to be diagnostic (Gomez-Morales et al., 2012; Vu et al., 2013).

4.3.2.3. *Statistical analysis*

The association between characteristics of the sampled individuals and the serologic positivity to *Trichinella* infection was initially evaluated by calculating frequency and percentages of positive serology for *Trichinella*, stratified by place of sampling, age, gender, ethnicity and the presence/absence of the signs and symptoms. Chi square (or exact Fisher test when the expected frequency in each cell was <5) was calculated to evaluate the probability that the observed distribution was simply due to chance. The same analyses were repeated stratifying data by villages and hospitals. A test of homogeneity was then performed to establish if the association for each evaluated characteristic was homogeneous in the two sampling places. To quantify the accuracy of each sign and symptom reported in discriminating between serologically positive and negative individuals, ROC curves (AUC) were calculated always stratifying by place of sampling. Multiple exact logistic regression analysis was then performed to establish which characteristics were independently associated with a *Trichinella* positive serology. Logistic models were again stratified by place of sampling. Final models were identified with a backward selection excluding, at each step, the variable (e.g., signs, symptoms, gender and age) with the highest value >0.2 (from the log-likelihood ratio test). When only variables with a p-value <0.2 remained, this was the final model (Hosmer et al., 2013).

Finally, the sensitivity, specificity, the percentage of cases correctly classified, the positive predictive value (PPV) and the negative predictive value (NPV), were calculated on the basis of the presence/absence of the three symptoms with a positive/negative serology for *Trichinella*, which were associated in the multiple exact logistic models by place of sampling. A p-value <0.05 was considered to be significant. Stata software version 11.0 (Stata Cooperation, College Station, TX, USA) was used for the statistical analysis.

4.3.3. Results

Two-hundred people in the Quai To and Lang Cheu villages, all of ethnic minorities, including H'mong and Thai, met the three criteria as described in previous section and were enrolled for the study. Most of them were adults (183, 91.5%, ≥ 18 years old). The mean age was 35 years (range: 7–90; average 34 and 36 years for men and women, respectively). Among these individuals, 96 (48%) were female and 104 (52%) male. Most (166; 83%) of enrolled people were self-employed with limited formal education; the other 34 (17%) people were students. All the enrolled villagers declared consuming raw or undercooked meat. The main clinical signs and symptoms compatible with trichinellosis were, fever (103, 51.5%), abdominal pain (82, 41%), myalgia (81, 40.5%), and diarrhea (35, 17.5%). None of the enrolled people had any knowledge on trichinellosis.

The second group consisted of 445 people who were hospitalized during the study period presenting with clinical signs and symptoms as described under section 4.3.2.1. These people originating from the 20 districts in the two provinces were mainly adults (95.9% adults; 44.1% male and 55.9% female) with an average age of 41 years (43 years for men and 40 years for women; range 6–97 years). Most of them were self-employed (242, 54.3%), servant (142, 31.9%), retired (39, 8.7%) or students (22, 4.9%) and belonged to ethnic minorities including, H'mong, Thai, Ha Nhi and Tay (303, 68.0%). All the enrolled hospitalized persons declared consuming raw or undercooked meat. The main clinical signs and symptoms compatible with trichinellosis were fever (136, 30.5%), diarrhea (130, 29.2%), facial edema (85, 19.1%), myalgia (160, 35.9%) and abdominal pain (57, 12.8%). None of the enrolled people had any knowledge on trichinellosis.

Out of the 645 serum samples tested for anti-*Trichinella* IgG, 14 (2.1%) sera were positive by ELISA. The positivity was confirmed for all the 14 sera by WB. The main clinical signs and symptoms of the 14 positive and 631 negative persons are shown in Table 4.4. Seven positive sera (3.5%; 95% CI: 1.4-7.1) were from people recruited in the two villages, and the other seven positive sera (1.6%; 95% CI: 0.6-3.2) were from hospitalized patients. Eosinophilia was moderate to high in 12 individuals (85.7%), and leukocytosis ($\geq 15,000$) was observed in eight persons (57.1%) out of the 14 persons with a positive serology for *Trichinella*. Overall, the presence of myalgia and facial edema were statistically associated with a positive serology for *Trichinella* sp. ($p < 0.001$ for both symptoms) (data not shown).

When stratifying by place of sampling (Table 4.4), statistically significant associations of myalgia and facial edema with a *Trichinella* positive serology were detected in individuals sampled in the villages. When evaluating individuals sampled in the hospitals, myalgia and abdominal pain were significantly associated with a *Trichinella* positive serology. The association of facial edema with a *Trichinella* positive serology varied significantly in the two places of sampling.

Figure 4.6 shows the estimated ROC curves by the presence/absence of symptoms with the positive/negative serology to *Trichinella* infection stratified by place of sampling. In individuals sampled in the two villages, facial edema, and myalgia were the sign and symptom with the highest accuracy on discriminating positive and negative individuals to *Trichinella* serology; in individuals sampled in the hospitals, myalgia was the symptom with the highest accuracy followed by fever and abdominal pain (Table 4.5).

Finally, Table 4.6 shows the sensitivity, specificity, the percentage of cases correctly classified the positive predictive value (PPV) and the negative predictive value (NPV) calculated, by place of sampling, on the basis of the presence/absence of diarrhea, facial edema and myalgia. The negative predictive value (NPV) is always very close to 100% in persons enrolled from both the villages and hospitals; whereas, the positive predictive value (PPV) is low using any cut-off for the hospital population. In the village population, the simultaneous presence of at least, myalgia and facial edema provides a PPV of 75%.

Table 4.5. Adjusted odds ratios (AOR) of serologically *Trichinella* positive persons by presence of symptoms in villages and hospitals. All the other signs and symptoms were not included in the final models.

Place	Signs and symptom	AOR	95% CI	P-value
Villages	Facial edema	119.1	13.0 to +inf	<0.01
	Myalgia	56.4	4.3 to +inf	<0.01
	Diarrhoea	24.9	1.1 to +inf	0.04
Hospitals	Facial	20.9	2.4 - 167.4	0.01
	Myalgia	56.8	6.9 to + inf	<0.01

Table 4.4. Epidemiological and clinical features of the 645 enrolled people according to the positivity (14 persons) or negativity (631 persons) for anti-*Trichinella* IgG in the serum, stratified by place of sampling.

	Villages			Hospitals			Test of homogeneity p-value
	No. of serologically positive persons (%)	Total	p-value	No. of serologically positive persons (%)	Total	p-value	
<i>Age</i>							
≤18	2 (5.7)	35	0.43	0	18	0.58	0.46
≥18	5 (3.0)	165	0.69	7 (1.6)	427		
<i>Ethnicity</i>							
Kinh	0		0.69	3 (2.1)	145	0.56	0.56
Others	7 (3.5)	200		4 (1.3)	300		
<i>Gender</i>							
Male	3 (2.9)	104	0.62	4 (2.0)	196	0.48	0.40
Female	4 (4.2)			3 (1.2)	249		
<i>Fever</i>							
No	2 (2.1)	97	0.28	3 (1.0)	309	0.12	0.83
Yes	5 (4.9)	103		4 (2.9)	136		
<i>Myalgia</i>							
No	0	119	<0.01	0	285	<0.01	NE
Yes	7 (8.6)	81		7 (4.4)	160		
<i>Facial edema</i>							
No	1 (0.5)	185	<0.01	4 (1.1)	360	0.11	<0.01
Yes	6 (40.0)	15		3 (3.5)	85		
<i>Diarrhoea</i>							
No	4 (2.4)	165	0.07	5 (1.6)	315	0.97	0.23
Yes	3 (8.6)	35		2 (1.5)	130		
<i>Nausea vomiting</i>							
No	7 (3.6)	195	0.67	7 (1.6)	428	0.59	NE
Yes	0	5		0	17		
<i>Abdominal pain</i>							
No	4 (3.4)	118	0.92	4 (1.0)	388	0.02	0.13
Yes	3 (3.7)	82		3 (5.3)	57		
Total	7 (3.5)	200		7 (1.6)	445		

NE = not estimated

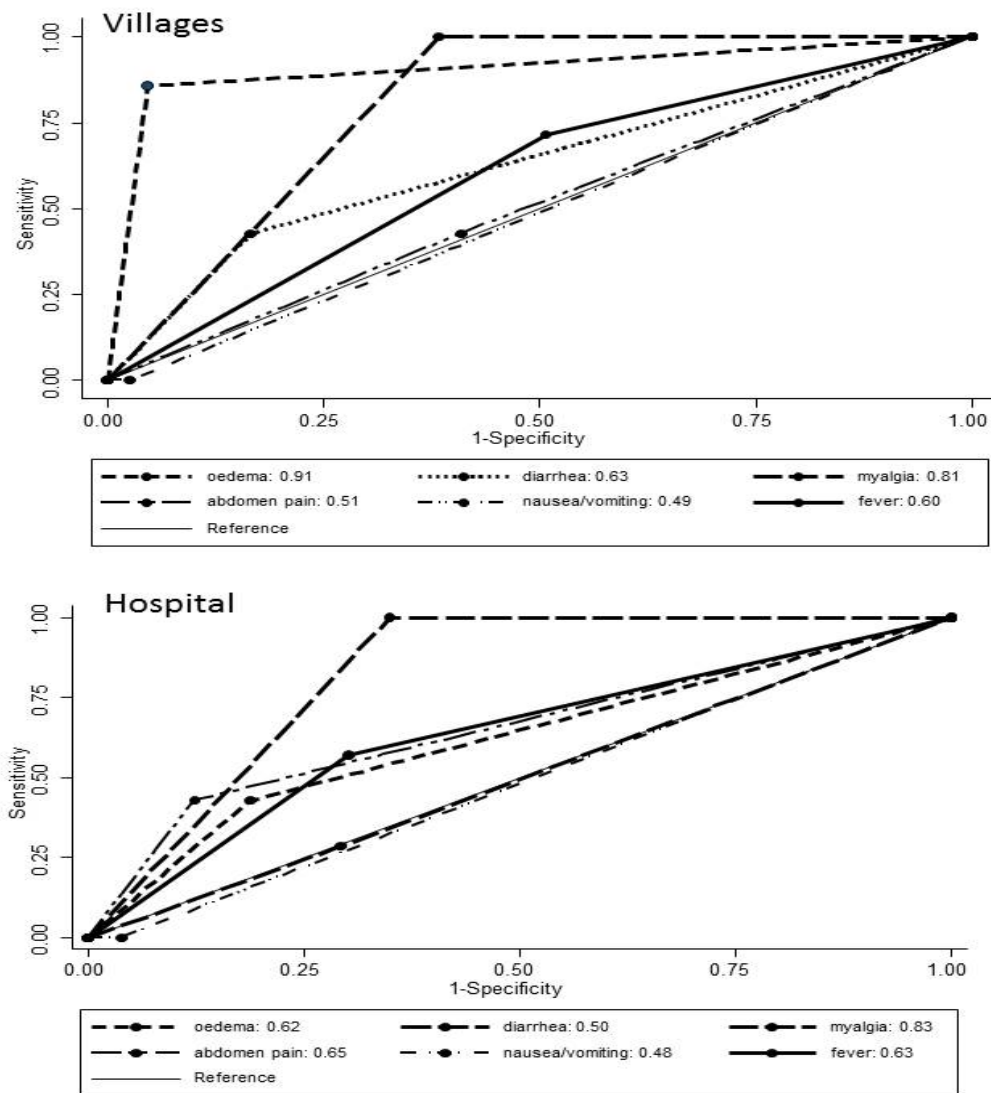


Figure 4.6. The receiver-operator characteristic (ROC) curves of several clinical signs and symptoms on discriminating positive from negative individuals to serologically *Trichinella* positive people. The ROC curves were built by stratifying signs and symptoms by place of sampling (villages or hospitals). Values are the estimated area under the curve for each clinical sign or symptom. Values close to 0.5 and 1 indicate low and high accuracy on discriminating serologically *Trichinella* positive people, respectively.

Table 4.6. Sensitivity (Se), specificity (Sp), percentage of agreement (%agr), positive predictive value (PPV) and negative predictive value (NPV) of diarrhoea and/or facial edema (edema) and/or myalgia with the *Trichinella* serology results. Se, Sp, %agr, PPV and NPV are calculated on each line using the occurrence of the signs and symptoms (ss) (alone or concomitant) as the cut-off.

	Se	Sp	% of agreement ^a	PPV	NPV
VILLAGES					
At least one among diarrhoea, oedema or myalgia	100.0	43.0	45.0	6.4	100.0
Oedema or myalgia alone or two among the three ss ^b	100.0	58.0	59.5	8.0	100.0
Myalgia or two among the three ss ^b	100.0	61.1	62.5	8.5	100.0
At least two concurrent ss	100.0	97.4	97.5	58.3	100.0
Diarrhoea and myalgia or oedema and myalgia	100.0	97.9	98.0	63.6	100.0
At least oedema and myalgia	85.7	99.0	98.5	75.0	99.5
Diarrhoea, oedema and myalgia	28.6	100.0	97.5	100.0	97.5
HOSPITALS					
At least one among diarrhoea, oedema or myalgia	100.0	21.5	22.7	2.0	100.0
Oedema or myalgia alone or two among the three ss ^b	100.0	47.5	48.3	3.0	100.0
Myalgia or two among the three ss ^b	100.0	64.4	64.9	4.3	100.0
At least two concurrent ss	71.4	95.9	95.5	21.7	100.0
Diarrhoea and myalgia or oedema and myalgia	71.4	96.6	96.2	25.0	99.5
At least oedema and myalgia	42.9	98.9	98.0	37.5	99.1
Diarrhoea, oedema and myalgia	0.0	99.8	98.2	0.0	98.4

^a Between the three clinical signs and symptoms and serology.

^b ss = The three clinical signs and symptoms, i.e. diarrhoea, edema and myalgia.

4.3.4. Discussion

Human trichinellosis has emerged as an important zoonosis in Vietnam in the last decade (Pozio, 2007). This study is the first sero-survey in people living in villages of mountainous provinces where human trichinellosis has already been documented (Taylor et al., 2009). The detection of anti-*Trichinella* antibodies in people living in villages where two outbreaks of trichinellosis were documented might indicate that *Trichinella* is still circulating in this area, but can also be the result of persistence of IgG antibodies, as these may be detectable for many years after infection in humans (Harms et al., 1993; Kociecka, 2000; Morakote et al., 1992). In spite of the occurrence of these outbreaks, pigs are not being routinely tested for *Trichinella* larvae at slaughter. A study in neighboring Lao Cai province showed that compared with lowland villages, the sanitation facilities of ethnic groups in the highland are much poorer (Rheinländer et al., 2010). Free roaming of livestock is more commonly practiced in highland villages, and other risk factors for *Trichinella* transmission are present in ethnic minority communities, such as, consumption of dishes based on raw or undercooked pork, the absence of systematic waste collection, the use of pork scraps and offal to feed other pigs, the spread of pork scraps and offal in the environment, and the use of scraps and offal from wild animals as feedstuff for pigs. No veterinary and/or health professionals are working in these mountainous villages where most of the population is illiterate. Since the last outbreaks of trichinellosis in the two study villages, some patients were hospitalized in the provincial hospitals presenting with clinical signs and symptoms compatible with trichinellosis; however, a definitive diagnosis was not reached because of the unavailability of a serological test (personal communication). The present results suggest that trichinellosis occurs not only in people living in villages where these parasites had already been documented in pigs and in synanthropic and wild animals (Thi et al., 2013; Vu et al., 2010), but also in persons living in other villages where *Trichinella* infection in pigs has not been reported yet. *Trichinella* infection was identified in all age groups even if most of the seropositive persons were adults. This can be explained by the eating habits and the long lasting antibody production in humans (Harms et al., 1993). No difference was observed by gender and this is in agreement with previous publications, which showed that both males and females equally acquire trichinellosis (Murrell and Pozio, 2011).

The statistical analysis shows that the concomitant presence of facial edema and myalgia in a person is suggestive of trichinellosis even in the absence of a serological test, assisting community based health workers on the decision to start a specific therapy. This finding is in agreement with the algorithm proposed by Dupouy Camet and Bruschi (2007) who considered facial edema and myalgia among the three most important clinical signs and symptoms of trichinellosis together with fever. The predictive value of facial edema and myalgia in this study is different between persons living in the villages and those from the hospitals, with a PPV of 75% and 37.5%, respectively. This difference could be due to the low overall number of confirmed cases of trichinellosis in this study; but it is more likely that a lower PPV in hospitalized persons is due to the fact that these symptoms are common in other diseases requiring hospital admission. In contrast, the NPV was very similar between the two groups (99.5% vs 99.1%), suggesting that the ratio of the number of patients with trichinellosis confirmed by serology, and the number of healthy persons is equivalent to the prevalence of the disease in the tested populations.

Finally, some study limitations need to be considered. The prevalence of trichinellosis in the Vietnamese rural population under study is likely to be biased due to the selection of the two investigated populations, i.e. persons living in villages where trichinellosis had already been documented and hospitalized persons among which the chance to detect a person suffering from trichinellosis is higher than in the healthy population. Regarding the association with symptoms, we should take into account that, although facial edema and myalgia had significant $OR > 1$, the low prevalence of trichinellosis among the enrolled people affects the 95% CI, which approaches the infinity (Table 4.5).

In light of the present results, trichinellosis can be considered to be a public health issue in the Son La and Dien Bien provinces. This finding has an important implication for developing appropriate intervention strategies for reducing *Trichinella* infection in pigs and supports the need to increase awareness and knowledge on trichinellosis among consumers, farmers, and public health and veterinary services living in the two provinces. Facilities for the diagnosis of trichinellosis in humans should be improved in provincial hospitals because limited access to diagnosis means that most emerging or re-emerging zoonoses remain unrecognized (Macpherson, 2005). Education also plays an important role in establishing political participation in control programs at all levels of the society and for building consensus among stakeholders and professional participants (Schantz et

al., 1983). Health education and scaling up participatory community based hygiene promotion is important for minority people who should be reached and informed to prevent trichinellosis. Public health strategies should be reconsidered in order to fully prevent trichinellosis in humans and eliminate *Trichinella* infections in pigs in these provinces.

4.3.5. References

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CHAPTER 5

Emergence of trichinellosis in Northern Vietnam and the development of a control program

5.1. Introduction

The emergence and re-emergence of trichinellosis in many regions of the world is causing public health hazards, economic problems in animal production systems, and problems related to food safety (Gottstein et al., 2009). Recently, trichinellosis has emerged as a foodborne parasitic infection causing outbreaks in some provinces in the northern part of Vietnam.

In this final chapter, the factors that contribute to the emergence of *Trichinella* infections in the Northern provinces of Vietnam will be discussed; options for appropriate control measures in Vietnam will be formulated as well as recommendations for future research. Finally, general conclusions and future perspectives are presented.

5.2. Emergence of trichinellosis in Northern Vietnam

Since the first report in 1923 on *Trichinella* infection in pigs in Northern Vietnam (Ha Noi city), very little information has been collected in the country on the importance of this parasite as a cause of disease in humans, and on the role of the pig and other animal species in the transmission of *Trichinella* spp. (Blanc et al., 1956; Le Louet and Broudin, 1923; Pozio, 2007). The identification of *Trichinella* infections in domestic, sylvatic and synanthropic animals in this thesis supports the conclusion that this parasite is endemic in Northern Vietnam, and that both the domestic and sylvatic transmission is ongoing. The only *Trichinella* species identified in both humans and animals was *T. spiralis*, which has been described as the dominant species in neighboring countries and the species most associated with human outbreaks overall (Pozio and Zarlenga, 2005).

Five of the six reported outbreaks of human trichinellosis in the country occurred in mountainous provinces of Northern Vietnam, involving 121 infected cases, of which 8 died (NIMPE, 2008; Pozio, 2007). The recently observed increase in the number of human *Trichinella* outbreaks in Northern Vietnam suggests that this parasite is emerging. However, our results, which were obtained by cross-sectional studies, do not provide evidence of spatio-temporal trends. Therefore, it cannot be concluded whether the apparent increase in number of outbreaks is due to an emergent situation or rather to better diagnosis. The population groups affected by the outbreaks mainly consisted of ethnic minorities. These populations are unaware of the risks as well as of basic precautions to

prevent *Trichinella* infection. Moreover, inadequate hygiene and free roaming of animals are still commonly practiced in the rural population of these provinces. Additionally, there is a lack of health care services in Northern provinces of Vietnam. Hence, the deeply rooted habit of eating raw or undercooked pork as well as meat from wild boars, dogs, cats and rats combined with the endemicity of the parasite in this area explains the occurrence of outbreaks of trichinellosis in Northern Vietnam.

5.3. The development of a control program for trichinellosis in Vietnam

The habit of eating raw or undercooked meat is a major risk factor for acquiring a *Trichinella* infection (Dorny et al., 2009; Gottstein et al., 2009). With the fast economic development in the Vietnamese society, the possibility for more Vietnamese people to eat animal proteins has increased over the last few years (Thang and Popkin, 2004). The implementation of a law on food safety in Vietnam has been hampered by the highly decentralized authority (No. 55/QH12/2010; adapted from http://www.vasep.com.vn/Thu-Vien-Van-Ban/71_3226/Luat-so-552010QH12-An-toan-thuc-pham.htm). The regulations of food safety include certification systems for good food production and slaughtering practices. However, not all activities are following the guidelines (Carrique-Mas and Bryant, 2013).

In developed countries, trichinellosis has been declared a disease that must be monitored and suspected cases of trichinellosis must be reported (EC, 2005). In Vietnam, since the first outbreak of human trichinellosis occurred in 1953 (Blanc et al., 1956; Merlea, 1957), a small scale control program of *Trichinella* infection in pigs has been implemented, which has been applied at the epicenter of a human outbreak that occurred in 2008 in Son La province. The Vietnamese Department of Animal health (DAH) attempts to prevent and control the slaughtering of infected pigs, encourages people living in this region to eat cooked meat and to keep pigs enclosed. However, despite efforts towards a control program, free roaming of pigs is still commonly practiced, and pig slaughterhouses are absent in most provinces of Vietnam, therefore, control of *Trichinella* infection has proven not to be very effective. In addition, the guidelines for control of *Trichinella* infection given by the Vietnamese DAH are not adapted to the people with a low income, who do not agree with the destruction of infected animals with a small financial compensation from the Vietnamese Government. Hence, there is an urgent need to develop a legislation

to better control *Trichinella* infections in humans and animals. Developing a control program that is adapted to the Vietnamese situation is complicated by the lack of epidemiological information, budget constraints, and the culinary traditions. Control can be done through reducing the infection in the animal host, constructing slaughterhouses, controlling of slaughtering, and applying meat inspection in slaughterhouses. Therefore, the following recommendations are proposed:

(1) Epidemiological studies

Our studies have shown evidence of *Trichinella* infections in humans and animals in a large number of districts of three Northern Vietnamese provinces. However, the data that we collected in this region are limited and more information on the prevalence of *Trichinella* infections in humans, domestic animals, and wildlife is needed in order to get a full picture of the importance of the problem.

The epidemiology of *Trichinella* infections in other provinces is virtually unknown. Therefore, cross-sectional surveys on both humans and animals should be conducted in target provinces. These include, studies on the infection in different hosts, eating habits, and other risk factors related to *Trichinella* infection, livestock husbandry systems, and veterinary inspection. Moreover, studies should also be conducted on the inspection of *Trichinella* larvae in meat at the slaughterhouses and local markets. Tools for the surveillance of infection and epidemiological investigations in animal populations are the indirect and direct detection methods to detect *Trichinella* larvae and antibodies in meat/serum samples. Molecular approaches should be applied to differentiate the *Trichinella* species.

(2) Changes in livestock production and meat inspection

In our studies, we identified risk factors that are significantly associated with *Trichinella* infection in the Northern provinces of Vietnam. These factors include (1) free roaming of animals, (2) feeding of meat-containing waste, (3) absence of slaughterhouses and meat inspection. Hence, regulations should be strongly enforced by veterinary public health services on all animals at risk for these zoonotic parasites. In addition, livestock owners who breed free roaming pigs should be encouraged to adopt adequate livestock breeding practices. Some key actions for control of *Trichinella* infection in pigs in these provinces can be applied following the guidelines of FAO/WHO/OIE (Dupouy-Camet and Murrell,

2007): (i) architectural and environmental barriers, (ii) feed and feed storage, (iii) rodent control, (iv) farm hygiene including proper disposal of dead animals, and (v) purchase of piglets from farms with controlled housing conditions.

Slaughterhouses are mostly absent in the Northern provinces of Vietnam. Therefore, the Vietnamese Government should construct slaughterhouses and process meat inspection following legislations on food safety. The legislation should cover meat inspection of domestic pigs, horses, cats, dogs, and wild species that are slaughtered for human consumption. An important method for routine inspection is the trichinoscope method. This method is no longer recommended for routine inspection in developed countries but it can still be applied in some regions where laboratory infrastructures are poor, such as in the mountainous provinces of Vietnam. The use of the digestion method, known as a sensitive method and generally considered sufficiently sensitive for ensuring food safety in humans, stopped in Vietnam since 1992; it should become mandatory again in the slaughterhouses as a routine test for *Trichinella* detection.

(3) Strengthening diagnostic capacities and treatment for trichinellosis

The health care services in local health centers should be improved by organizing training courses and by improving the infrastructure and the equipment of the laboratories. The strengthening of capacity in provincial hospitals should be done by training of the medical and laboratory staffs, and by the introduction of diagnostic techniques such as, ELISA for the confirmation of the presumptive diagnosis based on clinical examination and on history of the patients. The problem in Vietnam is that diagnosis is often made several weeks after infection, after referral to a hospital with diagnostic capacity, and that larvae have already encapsulated by that time making curative treatment difficult (Gottstein et al., 2009; Pozio et al., 2001). The concomitant occurrence of facial edema and myalgia has a diagnostic value in outbreak areas (chapter 4); it may guide physicians to a presumptive diagnosis and to a quicker anthelmintic treatment. Moreover, drugs should be provided to the patients free of charge, especially for patients belonging to ethnic minorities who have a low income.

The strategic use of anthelmintics has no potential to provide an effective means of control of *Trichinella* infection in animals (Gottstein et al., 2009). However, administration of anthelmintics can be applied in restricted areas under strict veterinary control in Vietnam.

Experimental results in neighboring China indicated that the use of anthelmintics such as albendazole in animal feeding was an effective method to reduce the prevalence of *Trichinella* infection in pigs (Liu and Boireau, 2002).

The diagnosis and treatment of human trichinellosis can be applied following the guidelines designed by the National Institute of Malariology, Parasitology and Entomology:

(i) Diagnosis:

- Epidemiological factors: patients originating from endemic regions of human trichinellosis or patients who have a history of eating raw or undercooked meat.
- Clinical signs and symptoms.
- White blood cell and eosinophil counts.
- Antibody detecting ELISA and Western Blot to confirm positive ELISA results.

(ii) Treatment:

- Drug: Albendazole 400 mg tablets.
- Dose: 15mg/kg body weight for 7 days.

(4) Health education

Health education has an important role in establishing participation in control programs and for building consensus amongst stakeholders and professional participants (Macpherson, 2005). In Vietnam, health education campaigns have not yet been advocated by the Ministry of Health Portal (MoHP) to control trichinellosis. However, some recommendations to prevent this parasite were documented by NIMPE that carried out serosurveillance on outbreaks of human trichinellosis. NIMPE informed the population on the disease and the risks to acquire the infection by using oral presentations and posters that were displayed in the local health centers. These posters showed that humans can get infected by eating raw or undercooked meat, so cooking the food was recommended to avoid infection; it was also recommended to improve the hygiene situation.

Some public health strategies should be considered in order to fully prevent trichinellosis in humans:

- (i) The population should be informed on the risks of acquiring this disease. Special attention should be given to health education for minority people who should be reached and informed to prevent trichinellosis by thoroughly cooking meat dishes prepared from domestic and wild animals. Those messages can be integrated in general preventive measures for foodborne diseases such as, cysticercosis and toxoplasmosis, and they should be translated into local languages.
- (ii) Meat processing techniques such as drying, smoking and salting are useful ways to inactivate the parasite in meat and meat products if correctly applied (Porto-Fett et al., 2010; Smith et al., 1989; Worley et al., 1986). These methods may be recommended in mountainous regions where electricity and freezing equipments are absent. The local population should be introduced to these processing techniques on locally produced meat.
- (iii) Keeping animals in pens using local materials and avoid free roaming; feed animals with crops feed or cook the kitchen leftovers before feeding to the animals.
- (iv) The population should regularly report to local health centers and local veterinarians in the case of people that are suspected for trichinellosis.
- (v) Suspected trichinellosis patients should have access to health care without charge.

These strategies will aim at reducing transmission of trichinellosis as well as to contribute to controlling/reducing infections with other pathogens such as *Taenia solium*, *Toxoplasma gondii*, bacterial infections etc.

5.4. Suggestions for future research on trichinellosis

The studies in this thesis aimed at contributing to a better understanding of the emergence of trichinellosis in the country. However, there are no official regulations for meat inspection, case management, and control of trichinellosis in Vietnam by the Vietnamese

MoHP and MARD (Ministry of Agriculture and Rural Development). Hence, intersectoral collaboration between the MoHP and MARD should be established and financed for a better understanding of the regional epidemiological situation to improve the veterinary and medical diagnostic capacities, and to establish effective prevention and control programs of trichinellosis in the whole country.

Based on the situation of *Trichinella* infections in humans and animals in Vietnam, we suggest conducting the following studies:

- (i) Investigate the presence of *Trichinella* and determine risk factors in humans and animals in other Vietnamese provinces where the same eating habits and livestock systems prevail.
- (ii) Inspection for *Trichinella* larvae in meat and meat products that are served raw or undercooked in restaurants, local free markets, and markets.
- (iii) Study the genetic polymorphism of *T. spiralis* isolates from different animal species.

5.5. General conclusions

In conclusion, two main epidemiological cycles in domestic and sylvatic animals that are considered as reservoir hosts for *T. spiralis* were found in Northern Vietnam. *T. spiralis* was the only species identified in domestic pigs, wild boars and synanthropic rats, suggesting that this species is endemic in the Northern provinces of Vietnam. To date, only a limited number of studies have been conducted in a specific area, there is no information on the situation in other provinces and no information on *Trichinella* infection in other wildlife species.

These findings make us conclude that there is a need to develop a control program of *Trichinella* infection in Vietnam. The control of this parasite in humans and animals needs a long term and integrated approach that should be based on the improvement of the infrastructure of slaughterhouses, the establishment of specific examination for *Trichinella* during meat inspection, improvement of breeding conditions, education of livestock owners and local populations, and strengthening of the diagnostic and case management capacities of the health care services.

5.6. References

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SUMMARY

Trichinellosis caused by nematodes belonging to the genus *Trichinella* is a meat-borne zoonosis that can cause serious disease and even death in humans. Currently, nine species and three genotypes have been identified and the genus can be divided in two distinct groups, characterized by non-encapsulated and encapsulated species. *Trichinella spiralis* is the species with the widest distribution and it is most commonly associated with disease in humans. Meat from domestic pigs and wildlife that is eaten raw or undercooked is the main source of infection. This parasitic disease is nowadays almost completely controlled in Western countries as a result of systematic meat inspection with specific testing of meat for *Trichinella* larvae, and of the establishment of controlled housing conditions on pig farms. However, trichinellosis still causes a huge economic burden in western countries due to the cost of the rigorous testing. *Trichinella* spp. are continuing to cause outbreaks in many regions of the world where pigs are raised in traditional husbandry systems characterized by free roaming, and feeding with uncooked kitchen leftovers, and where the meat is not properly cooked. On average, yearly 10,000 cases are reported. In Southeast Asia and in China, trichinellosis outbreaks are regularly reported, mainly in ethnic minority communities where the conditions are conducive for transmission. In the last decade, several outbreaks of trichinellosis have been observed in Northern Vietnam, where the disease was previously practically unknown.

In this thesis, we aimed at improving our understanding of the epidemiological situation of *Trichinella* infection in Northern Vietnam that is characterized by mountainous landscapes and the presence of ethnic minorities living in disadvantageous general conditions compared to the general Kinh population. We assessed the seroprevalence of trichinellosis in humans and of *Trichinella* infection in different animal species (pigs, cats, dogs, wild boars, synanthropic rats) in areas where outbreaks had occurred and more generally in the affected provinces, and we identified the *Trichinella* species infecting humans and pigs. We also studied risk factors for infection and assessed the clinical signs and symptoms associated with infection. Finally, we proposed options for control and for further research.

In **the first chapter**, a general review of the literature is given on *Trichinella* spp and trichinellosis, with special focus on the epidemiology, the diagnosis and the situation in Southeast Asia, particularly in Vietnam. This chapter is followed by the **objectives** of the thesis that introduce the research chapters.

The **second chapter** describes our data on *Trichinella* infection in domestic animals. In the first part, we estimated the seroprevalence of anti-*Trichinella* IgG in free-roaming pigs in the Son La province of northwestern Vietnam, where a human outbreak of trichinellosis occurred in June 2008. Serum samples were collected from free-roaming pigs of four communes of the Bac Yen district and tested for *Trichinella* antibodies with a commercial ELISA. From the 1035 pigs from which serum samples were collected, 206 were positive (19.9%). There was a significant difference in the prevalence among communes. Muscle samples from 76 serologically positive pigs were tested by artificial digestion. *Trichinella* larvae were detected in 11 (14.5%) of them. The larvae were identified by multiplex PCR as *T. spiralis*. The results indicate that pigs act as a reservoir and play an important role in the maintenance of the domestic cycle of *T. spiralis* in northwestern Vietnam. In the second part, we determined the seroprevalence of *Trichinella* infection in the domestic lifecycle in two provinces of northwestern Vietnam. Serum samples were obtained from 558 pigs, 125 dogs and 98 cats and tested for *Trichinella* antibodies by ELISA and Western blot, using larval excretory–secretory (E/S) antigens. The overall seroprevalence of antibodies to *Trichinella* was 5.6%, 4% and 0% in pigs, dogs and cats, respectively. In pigs, positive cases were distributed in 8/20 districts of the two provinces. This study suggests that *Trichinella* spp. is circulating in the domestic life cycle in northwestern Vietnam.

In **the third chapter**, we studied the occurrence of *Trichinella* infections in hunted and farm-bred wild boars as well as in synanthropic rats in northwest Vietnam. Evidence of *Trichinella* infection was studied by parasitological, serological and molecular methods. The results showed relatively low prevalence of *T. spiralis* in hunted wild boars (2/62 (3.2%; 95% CI: 0.8– 4.8)) and rats (23/820 (2.8%; 95% CI: 13.7–32.3)). Parasite burdens in the muscle tissues were between 0.1 and 0.03 larvae/g, and 0.1 and 7 larvae/g in wild boars and rats, respectively. Seroprevalence in farm-bred wild boars was negative. *Trichinella*-infected rats were found in 7 of the 20 districts of Dien Bien and Son La provinces. These results indicate that the local population and health centers should be

made aware of the risks of eating raw or undercooked meat dishes prepared from wild animals.

The fourth chapter describes studies on human trichinellosis. In the first part, the 5th outbreak of trichinellosis in Vietnam is described that occurred in a mountainous area in Thanh Hoa province in 2012. The outbreak involved 24 patients who consumed raw meat dishes prepared from a wild boar during the Vietnamese lunar year celebration. Six of these patients visited several hospitals in Hanoi for treatment. Similar clinical signs and symptoms appeared in these patients within 5-8 days after eating infected raw pork, which consisted of fever, muscle pain, difficult moving, edema, difficult swallowing, and difficult breathing. ELISA revealed all (6/6) positive reactions against *T. spiralis* antigen and all cases showed positive biopsy results for *Trichinella* sp. larvae in the muscle. The larvae detected in the patients were identified as *T. spiralis* (Vietnamese strain) by molecular analysis of the mitochondrial cytochrome *c* oxidase subunit III (*cox3*) gene. In the second part, we described a post-outbreak cross-sectional study in the same community. All inhabitants of the community who declared to have eaten undercooked or raw wild boar meat at the celebration and showed at least one clinical sign or symptom compatible with trichinellosis were included in the study and blood sampled. Anti-*Trichinella* IgG were determined by ELISA and Western Blot. Seropositive persons were given albendazole treatment and were followed up. A total of 100 inhabitants met the inclusion criteria. Among these, 30% had antibodies to *Trichinella*. Serologically confirmed cases had fever, myalgia, facial oedema, diarrhoea, and/or pain of the masseter muscles. Clinical symptoms resolved in all patients during albendazole treatment. The results suggest that only a proportion of the trichinellosis cases had sought health care during the outbreak. In the third part of this fourth chapter, we assessed the presence of anti-*Trichinella* IgG in the serum of persons from ethnic minorities from northwest Vietnam with clinical signs and symptoms that are compatible with trichinellosis. A total of 645 persons were enrolled, of which 200 people lived in two villages where outbreaks of human trichinellosis had been documented in 2004 and 2008, and 445 people who were hospitalized in the Dien Bien and Son La provincial hospitals without a definitive diagnosis. Presence of anti-*Trichinella* IgG was demonstrated in serum samples by ELISA and Western blot. Seven (3.5%; 95% CI: 1.4–7.1) persons from the villages and seven (1.6%; 95% CI: 0.6–3.2) hospitalized patients, tested positive by both ELISA and WB.

Fever (N = 13), eosinophilia (N = 12), myalgia (N = 9), facial edema (N = 9) and leukocytosis (N = 8) were the most common clinical signs and symptoms in the serologically positive persons. The concomitant occurrence of facial edema and myalgia among the enrolled persons from the villages, accounted for 75% of the positive predictive value (PPV) and 99.5% of the negative predictive value (NPV), suggesting that they could be used for suspecting trichinellosis when serology is not available.

In the fifth and last chapter, we discuss the findings of our research in the context of the emergence of *Trichinella* infections in Northern Vietnam. The high prevalence (1.6–3.5%) of anti-*Trichinella* IgG in persons from Northern Vietnamese provinces where *T. spiralis* is circulating in pigs and wildlife strongly supports the need to develop control programs to eliminate the infection from pigs and for consumers' education and protection. There is a need to implement surveillance and better diagnosis for trichinellosis in humans and of *Trichinella* infection in slaughter animals and wildlife and to set up educational programs to prevent infection in Northern Vietnam.

SAMENVATTING

Trichinellose, veroorzaakt door nematoden behorende tot het geslacht *Trichinella*, is een vlees-overgedragen zoönose, die ernstige ziekte en zelfs sterfte bij de mens kan veroorzaken. Momenteel zijn er negen *Trichinella* species en drie genotypes gekend en het geslacht kan worden ingedeeld in twee verschillende groepen, gekenmerkt door niet-gekapselde en gekapselde species. *Trichinella spiralis* is het species met de grootste distributie en het wordt ook het meest geassocieerd met ziekte bij de mens. Vlees van gedomesticeerde varkens en wilde dieren, dat rauw of onvoldoende gaar wordt gegeten, is de belangrijkste bron van infectie. Deze parasitaire ziekte is tegenwoordig bijna volledig gecontroleerd in westerse landen als gevolg van de systematische vleeskeuring met specifiek onderzoek van vlees naar *Trichinella* larven, en van de gecontroleerde huisvestingsomstandigheden op varkensbedrijven. Daarentegen veroorzaakt besmetting met *Trichinella* nog steeds een enorme economische last in de westerse landen als gevolg van de kost van het systematisch testen van karkassen. In vele regio's van de wereld blijven deze parasieten echter uitbraken veroorzaken, vooral in gebieden waar varkens in traditionele bedrijven worden gehouden, gekenmerkt door vrije buitenloop, en waar deze worden gevoederd met ongekookte keukenafval, en waar het vlees door de bevolking rauw of niet goed gaar wordt gegeten. Gemiddeld worden jaarlijks 10.000 gevallen van trichinellose gerapporteerd. In Zuidoost-Azië en in China worden uitbraken van trichinellose regelmatig gemeld, vooral in gemeenschappen van etnische minderheden, waar de omstandigheden bevorderlijk zijn voor de transmissie. In de afgelopen tien jaar werden verschillende uitbraken van trichinellose waargenomen in Noord-Vietnam, waar de ziekte voorheen vrijwel onbekend was.

Het doel van dit proefschrift was om een beter inzicht te krijgen in de epidemiologische situatie van *Trichinella* infecties in Noord Vietnam. Dit gebied wordt gekenmerkt door een bergachtig landschap en de aanwezigheid van etnische minderheden die meestal in ongunstigere algemene omstandigheden leven dan de in Vietnam overheersende Kinh bevolking. Wij hebben seroprevalentie studies van *Trichinella* infecties uitgevoerd bij mensen en verschillende diersoorten (varkens, katten, honden, everzwijnen, ratten) in gebieden waar uitbraken werden vastgesteld en meer in het algemeen in de getroffen provincies en we hebben er de *Trichinella* soorten die mensen en varkens besmetten

geïdentificeerd. We hebben ook de risicofactoren van infectie bestudeerd en de klinische tekenen en symptomen onderzocht die geassocieerd worden met infectie. Tot slot, hebben we opties voor de controle van deze parasitaire zoönose in Noord Vietnam voorgesteld, alsook de mogelijkheden voor verder onderzoek.

In **het eerste hoofdstuk**, wordt een algemene literatuuroverzicht gegeven van *Trichinella* spp en trichinellose, met speciale aandacht voor de epidemiologie, de diagnostiek en de situatie in Zuidoost-Azië, met name in Vietnam. Dit hoofdstuk wordt gevolgd door de **doelstellingen** van deze thesis die het eigen onderzoek, beschreven in de volgende hoofdstukken introduceren.

Het tweede hoofdstuk beschrijft het onderzoek dat we uitvoerden op *Trichinella* infecties bij huisdieren. In het eerste deel bepaalden we de seroprevalentie van anti-*Trichinella* IgG in varkens die in traditionele omstandigheden worden gehouden in de provincie Son La in het noordwesten van Vietnam, waar een uitbraak van humane trichinellose werd vastgesteld in juni 2008. Serum monsters werden verzameld van varkens in de vier gemeenten van het district Bac Yen en getest op *Trichinella* antilichamen met een commerciële ELISA. In totaal werd het serum van 1035 varkens verzameld; daarvan waren er 206 positief (19,9%). Er was een significant verschil in de prevalentie tussen de gemeenten. We namen spiermonsters van 76 serologisch positieve varkens die we testten met de kunstmatige verteringsmethode. *Trichinella* larven werden gedetecteerd in 11 (14.5%) van deze monsters. De larven werden als *Trichinella spiralis* geïdentificeerd door middel van de multiplex PCR methode. Deze resultaten tonen aan dat varkens fungeren als reservoir en een belangrijke rol spelen bij de transmissie van *T. spiralis* in het noordwesten van Vietnam. In het tweede deel, hebben we de seroprevalentie van *Trichinella* besmetting bepaald bij gedomesticeerde dieren in twee provincies in het noordwesten van Vietnam. Serummonsters werden verkregen van 558 varkens, 125 honden en 98 katten en getest op *Trichinella*-antilichamen door middel van ELISA en Western blot, met larvale excretie-secretie (E/S) antigenen. De seroprevalentie bedroeg 5,6%, 4% en 0% bij varkens, honden en katten, respectievelijk. Bij varkens werden positieve gevallen in 8/20 districten van deze twee provincies gevonden. Deze studie toont aan dat *Trichinella* spp. aanwezig is in de synantropische levenscyclus in het noordwesten van Vietnam.

In **het derde hoofdstuk**, hebben we de aanwezigheid van trichinen in wilde en gedomesticeerde everzwijnen, en in synantropische ratten geëvalueerd in het noordwesten van Vietnam. *Trichinella* infectie werd gemeten door middel van parasitologische, serologische en moleculaire methoden. De resultaten toonden een relatief lage prevalentie aan van *T. spiralis* in wilde everzwijnen (2-62 (3,2%; 95% CI: 0,8- 4,8)) en ratten (23/820 (2,8%; 95% CI: 1,3-4,8)). De parasitaire load in de spieren bedroeg tussen 0,1 en 0,03 larven/g en 0,1 en 7 larven/g in everzwijnen en ratten, respectievelijk. De seroprevalentie bij gedomesticeerde everzwijnen was negatief. *Trichinella* infectie in ratten werd in 7 van de 20 districten van Dien Bien en Son La provincies aangetoond. Deze resultaten tonen aan dat de lokale bevolking en de gezondheidscentra in deze regio moeten worden ingelicht over de risico's van het eten van gerechten bereid met rauw of onvoldoende verhit vlees van wilde dieren.

Het vierde hoofdstuk beschrijft studies over menselijke trichinellose. In het eerste deel wordt de 5^e uitbraak van trichinellose in Vietnam beschreven, die zich voordeed in een bergachtig gebied in de provincie Thanh Hoa in 2012. Bij deze uitbraak werden 24 patiënten betrokken die tijdens de viering van het Vietnamese maanjaar gerechten hadden gegeten bereid met rauw vlees van een everzwijn. Zes van deze 24 patiënten bezochten diverse ziekenhuizen in Hanoi voor behandeling. De klinische tekenen en symptomen bij deze patiënten, die zich 5 tot 8 dagen na het eten van het besmet vlees voordeden bestonden uit koorts, spierpijn, moeilijk bewegen, oedeem, moeilijk slikken en ademhalingsproblemen. In ELISA waren alle patiënten (6/6) positief en spierbiopsie van alle gevallen was positief op de aanwezigheid van larven van *Trichinella* sp. Deze larven werden geïdentificeerd als *T. spiralis* (Vietnamese stam) met de moleculaire analyse van het mitochondriaal cytochroom c oxidase subeenheid III (cox3) gen. In het tweede deel beschrijven we een cross-sectionele studie vlak na deze uitbraak in dit dorp. Alle inwoners die verklaarden dat ze rauw of onvoldoende verhit vlees van het everzwijn hadden gegeten bij de viering en ten minste één klinisch teken of symptoom compatibel met trichinellose vertoonden werden in de studie opgenomen en er werden bloedmonsters van genomen. Anti-*Trichinella* IgG werden bepaald door middel van ELISA en Western Blot. Seropositieve personen kregen een albendazole behandeling en werden opgevolgd. Een totaal van 100 inwoners voldeden aan de inclusiecriteria. Van deze had 30% antilichamen tegen *Trichinella*. Serologisch bevestigde gevallen hadden koorts, spierpijn, oedeem in het

gezicht, diarree en/of pijn ter hoogte van de kauwspieren. De klinische symptomen verdwenen bij alle patiënten tijdens de albendazole behandeling. Deze resultaten opperen dat slechts een deel van de trichinellose gevallen tijdens de uitbraak gezondheidszorg hadden opgezocht. In het derde deel van dit vierde hoofdstuk onderzochten we de aanwezigheid van anti-*Trichinella* IgG in het serum van personen uit etnische minderheden in het noordwesten van Vietnam met klinische tekenen en symptomen die compatibel zijn met trichinellose. Een totaal van 645 personen werd in de studie opgenomen, waarvan 200 mensen afkomstig van de twee dorpen waar uitbraken van trichinellose voorkwamen in 2004 en 2008, en 445 mensen die werden opgenomen in de Dien Bien en Son La provinciale ziekenhuizen zonder een definitieve diagnose. Aanwezigheid van anti-*Trichinella*-IgG werd aangetoond in serummonsters door middel van ELISA en Western blot. Zeven (3,5%, 95% CI: 1,4-7,1) personen uit de dorpen en zeven (1,6%, 95% CI: 0,6-3,2) gehospitaliseerde patiënten, testten positief in zowel ELISA en WB. Koorts (N = 13), eosinofilie (N = 12), myalgie (N = 9), gezichtsoedeem (N = 9) en leukocytose (N = 8) waren de meest voorkomende klinische tekenen en symptomen in de seropositieve personen. Het gelijktijdig optreden van gezichtsoedeem en spierpijn in de personen uit de dorpen, was goed voor 75% van de positief voorspellende waarde (PPV) en 99,5% van de negatief voorspellende waarde (NPV), wat suggereert dat deze kunnen gebruikt worden voor het stellen van een vermoedelijke diagnose van trichinellose wanneer serologie niet beschikbaar is.

In het **vijfde en laatste hoofdstuk**, bespreken we de resultaten van ons onderzoek in de context van de opkomst van trichinellose in Noord-Vietnam. De hoge prevalentie (1,6-3,5%) van anti-*Trichinella* IgG bij personen uit Noord-Vietnamese provincies waar *T. spiralis* circuleert bij varkens en wilde dieren doet ons besluiten dat programma's moeten ontwikkeld worden om de besmetting van varkens te controleren en voor het informeren en beschermen van de consumenten. Er is behoefte aan een surveillance systeem en aan een verbeterde diagnose van trichinen infecties zowel bij de mens als bij slachtdieren en wilde dieren en het opzetten van educatieve programma's om infectie in Noord-Vietnam te voorkomen.