A CROSS-SECTIONAL STUDY OF TRICHINELLA SPP. IN PIGS IN THE CENTRAL DEVELOPMENT REGION OF NEPAL USING PEPSIN DIGESTION AND ELISA SEROLOGY

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MASTER OF VETERINARY PUBLIC HEALTH

CHIANG MAI UNIVERSITY AND FREIE UNIVERSITÄT BERLIN
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SANTOSH KUMAR KARN

A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY AND FREIE UNIVERSITÄT BERLIN IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF VETERINARY PUBLIC HEALTH

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THIS THESIS HAS BEEN APPROVED TO BE A PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF VETERINARY PUBLIC HEALTH

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20 September 2007

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Santosh Kumar Karn
Thesis Title | A Cross-sectional Study of *Trichinella* Spp. in Pigs in the Central Development Region of Nepal Using Pepsin Digestion and ELISA Serology

Author | Mr. Santosh Kumar Karn

Degree | Master of Veterinary Public Health

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**ABSTRACT**

This epidemiological cross-sectional study was conducted in five major pig producing districts of the central development region of Nepal from November 2006 to end of April 2007. The study population was slaughtered pigs with an adjusted sample size of 520. The total of 576 pigs were randomly selected and diaphragmatic crus muscles (n = 551), corresponding sera (n = 487) and sera only (n = 25) were collected from them. The meat samples were examined by the 1% Pepsin digestion method to investigate *Trichinella* larvae and sera were investigated by the antibody ELISA to evaluate specific anti-*Trichinella*-IgG. The doubtful and positive sera through antibody ELISA were investigated through the end-point single dilution antibody ELISA and the western blot for confirmatory diagnosis. The descriptive information regarding the slaughtered pigs was collected at the butchery level. In addition to that the questionnaire was surveyed from 40 pig farm owners for assessing the pre-determined risk factors regarding *Trichinella* infection persisting at the farm level.
The Pepsin digestion did not detect *Trichinella* larvae in meat samples. The antibody ELISA primarily showed 2 positive and 14 doubtful results, out of 344 sera analyzed. However, the end-point single dilution antibody ELISA and the western blot had revealed all samples (n = 16) to be truly negative. The slaughtered pigs in this study were local (56.9%), exotic (26.6%) and cross breed (16.5%) and belonged to commercial (26%), semi-commercial (17.2%), scavenging (37.2%) and household (19.6%) raised systems. The questionnaire survey shows that no rodent control (70%), left over feeding practice (65%), garbage dumping and direct outlet of farm waste in the vicinity (82.5%) and the uncooked meat being used as feed (100%) practice existed in farms, which were predetermined risk factors for the *Trichinella* infection. This study suggests that *Trichinella* in this region had a low detection level with a very low prevalence. However, its validity and accuracy could be challenged through increased sample sizes. Furthermore, continuous surveillance and monitoring have to be implemented before concluding that the central development region is free from infection; as virtually no region/area in the world of today can be considered to be *Trichinella* free.
ข้อที่วิทยานิพนธ์
การศึกษาแบบตัดขวางของเชื้อทริคิเนลลาในสุกรในเขตการพัฒนาภาคกลางของเนปาล โดยวิธีย้อมปริชิลและตรวจทางซีรั่มวิทยา ด้วยวิธีอีไลซ์

ผู้เขียน
นาย ซานโตส คูมาร์ คาน

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บทคัดย่อ

ได้ทำการศึกษาทางระบาดวิทยาแบบตัดขวางในพื้นที่ผลิตสุกรสำคัญ 5 แห่งบริเวณเขตการพัฒนาภาคกลางของเนปาล ในช่วงเวลาตั้งแต่เดือนพฤศจิกายน 2549 ถึงสิ้นเดือนมกราคม 2550 ประชากรที่ศึกษาคือสุกรจากโรงฆ่าที่ปรับค่าขนาดตัวอย่างเท่ากับ 520 ตัวอย่างได้สุ่มเลือกจากสุกรทั้งหมด 576 ตัว พร้อมเก็บตัวอย่างเลือดเนื้อ (n = 551) และซาร์ม (n = 487) และมีบางตัวเก็บตัวอย่างเลือด (n = 25) ทำการตรวจด้วยวิธีอีไลซ์ด้วย 1% วิธีย้อมตีปีนปุ๊ป ตรวจทางซีรั่มวิทยาแบบตัวอย่างติดเชื้อทริคิเนลลาและตรวจเซรั่มด้วยวิธีแอนติบอดีอีไลซ์เพื่อหาแอนติ-ทริคิเนลลา อีจีจีจีเพาะ ทำการตรวจเชื้อสายพันธุ์สุกรในตัวอย่างเชื้อที่ให้ผลสงสัย และให้ผลบวก ด้วยวิธีย้อมตีปีนปุ๊ป ตรวจเซรั่มด้วยวิธีแอนติบอดีอีไลซ์และตรวจเซรั่มด้วยวิธีแอนติบอดีอีไลซ์ ได้เก็บข้อมูลเกี่ยวกับตัวอย่างสุกรที่ตรวจและยังได้เก็บข้อมูลตามแบบสอบถามจากเจ้าของสุกร 40 คน เพื่อประเมินปัจจัยเสี่ยงของการติดเชื้อทริคิเนลลาที่ระดับฟาร์ม

การตรวจด้วยวิธีอีไลซ์ไม่พบตัวอย่างของทริคิเนลลาจากตัวอย่างเชื้อที่ตรวจเบื้องต้นด้วยแอนติบอดีอีไลซ์พบตัวอย่าง Rocks 2 ตัวอย่าง และสงสัย 14 ตัวอย่าง จากเชื้อที่มีจำนวน 344 ตัวอย่าง อย่างไรก็ตามการตรวจยืนยันด้วยวิธีย้อมตีปีนปุ๊ป ตรวจเซรั่มด้วยวิธีอีไลซ์และตรวจเซรั่มด้วยวิธีแอนติบอดีอีไลซ์และพบผลลบทั้งหมด 16 ตัวอย่างให้ผลลบ สุภระที่ศึกษาเป็นสุกรพันธุ์พื้นเมืองร้อยละ 56.9
พันธุ์ต่างประเทศ ร้อยละ 26.6 พันธุ์ผสม ร้อยละ 16.5 การเลี้ยงดูเป็นแบบการค้าร้อยละ 26 กิจการค้าร้อยละ 17.2 ปล่อยเสรีตามถนน ร้อยละ 37.2 และเลี้ยงในครัวเรือนร้อยละ 19.6 ผลจากแบบสอบถามพบว่า ไม่มีโปรแกรมควบคุมก้างจัดหนุนร้อยละ 70 ใช้เศษอาหารเลี้ยงร้อยละ 65 อยู่ใกลแหล่งขยะหรือแหล่งพื้นที่ที่เสี่ยงจากการแพร่กระจาย ร้อยละ 82.5 และมีการใช้เศษเนื้อเป็นอาหารสุกรร้อยละ 100 ซึ่งปัจจัยเหล่านี้น่าจะเป็นปัจจัยเสี่ยงต่อการติดเชื้อทริคิเนลล่า ผลการศึกษานี้ชี้ให้เห็นว่าพื้นที่บริเวณนี้จะมีโอกาสตรวจพบการติดเชื้อและความชุกของการติดเชื้ออยู่ในระดับที่ต่ำมาก อย่างไรก็ตามความเข้มงวดได้และความถูกต้องของผลการตรวจควรจะยืนยันด้วยการตรวจจากขนาดตัวอย่างที่สูงขึ้น ยิ่งกว่านั้นควรทำการศึกษาการระบาดอย่างถี่ถ่อมทั้งที่จะระสูรปัญหาพัฒนาการด้านการจัดการกีฏใดที่ไม่เคยปรากฏว่ามีเขตพื้นที่ใดในโลกปัจจุบัน ที่ถูกตันว่าปลอดจากเชื้อทริคิเนลล่า
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<td>AB</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABPSD</td>
<td>Agri-Business Promotion and Statistics Division</td>
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<tr>
<td>ABTS</td>
<td>2, 2'-azino-his-(3-ethylbenzothiazoline sulfonate)</td>
</tr>
<tr>
<td>ACP</td>
<td>Acid phosphatase</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>ALAT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ALDO</td>
<td>Aldolase</td>
</tr>
<tr>
<td>BfR</td>
<td>Bundesinstitut für Risikobewertung</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CBS</td>
<td>Central Bureau of Statistics</td>
</tr>
<tr>
<td>CDR</td>
<td>Central Development Region</td>
</tr>
<tr>
<td>CFT</td>
<td>Compliment Fixation Test</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EEC</td>
<td>European Economic Community</td>
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<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<td>ES</td>
<td>Excretory-secretory</td>
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<td>EST</td>
<td>Esterase</td>
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<td>ESV</td>
<td>Expansion segment five</td>
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<tr>
<td><em>et al.</em></td>
<td>Et alia (and others)</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
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<tr>
<td>FAO</td>
<td>Food and Agricultural Organization</td>
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g    Gram
GDP  Gross Domestic Product
GLDH Glutamate dehydogenase
GOT  Glutamic oxalacetic transaminase
GPD  Glycerol 3-phosphate dehydrogenase
HAT  Haemagglutination Test
ICT  International Commission on Trichinellosis
IFAT Immunofluorescence Antibody Test
IgG  Immunoglobulin G
i.e. Id est (that is)
IHA  Indirect Haemagglutination Test
IIFT Indirect Immunofluorescent Test
km   Kilometre
kDa  Kilodalton
LDH  Lactate dehydrogenase
lpg  Larva per gram
ME   Malic enzyme
ml   Millilitre
mm   Millimetre
Mt   Metric Ton
NE   Netto extinction
NPC  National Planning Commission
OD   Optical density
OIE  Office International des Epizooties
PBS  Phosphate buffer saline
PBST Phosphate buffer saline-tween 20
PCR  Polymerase Chain Reaction
PGD  Phosphogluconate dehydrogenase
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</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
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<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SANCO</td>
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<td>SCVPH</td>
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<td>TLDP</td>
<td>Third Livestock Development Project</td>
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<td>TPI</td>
<td>Triose phosphate isomerase</td>
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<td>TsR1</td>
<td><em>Trichinella spiralis</em> resistance 1</td>
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<tr>
<td>UN-OHRLLS</td>
<td>United Nations office of high representatives for the least developed countries, landlocked developing countries and small islands developing states</td>
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≤ Less than equal to
≥ More than equal to
μl Microlitre
< Less than
> More than
°C Degree Centigrade
5S Large ribosomal subunit
1. INTRODUCTION AND OBJECTIVES

1.1 General overview

Nepal is a landlocked country situated in the lap of the Himalayas bordering with China in the North and India in the South, East and West. Nepal is located between the latitude of 26°22’ to 30°27’ north and the longitude of 80°4’ to 88°12’ east. The elevation ranges from 90 to 8848 metres. The northern range of the country is covered with snow throughout the year where the highest peak of the world, the Mount Everest, stands. The middle range is captured by gorgeous mountains, high peaks, hills and valleys whereas the southern range is characterized by the gigantic plain of alluvial soil which consists of a dense forest area, wildlife reserves and conservation areas. The country exhibits tropical, mesothermal, microthermal, taiga and tundra types of climate. Nepal is divided, administratively, into 5 development regions and 75 districts, whereas geographically in 3 regions; Mountain, Hill and Plain, which accommodate 7.3, 44.3 and 48.4 percentage of the total population, respectively (CBS, 2004). The central development region has an area of 27 410 sq. km and consists of 19 districts.

The total population of the country is 25 million with an annual growth rate of 2.25% (ABPSD, 2006). The population density is 175 persons per sq. km and 65.6% of population are engaged in agriculture (ABPSD, 2006). Nepal is part of the list of the least developed countries where 42% of population lives below the poverty line (UN-OHRLLS, 2006). About 17% of the population in Nepal is undernourished (FAO, 2004). The total health expenditure per capita was US$ 64 in 2003 (WHO, 2006). Agriculture is the major economic sector although it only occupies less than one fifth of the total area of the country (NPC, 2004). It contributes 39.48% to the total GDP (CBS, 2004).
1.2 The livestock sector in Nepal

A distinct pivoted role of the livestock is well established in the farming system of Nepal since it functions as a supplier of manure and high value animal protein (Karki and Ghimire, 2003). The livestock sector contributes 30% of the agricultural GDP (FAO, 2005). About 92% of the rural households benefit from this sector (CBS, 2004). Farmers with a small landholding of between 0.2 and 0.5 hectares land keep almost 25% of the livestock. People who have either no land or own less than 0.2 hectares possess about 11% of the livestock (FAO, 2005). The livestock population of the country, namely cattle, buffalo, goat, pig and fowl are (in million) 7.0, 4.2, 7.4, 0.9 and 23.2 respectively (ABPSD, 2006). The annual growth rate is 1.12% in cattle, 1.93% in buffalo, 2.03% in goat, 4.55% in pig and 4.7% in poultry (Karki and Ghimire, 2003).

1.3 Pig farming and pig breeds in Nepal

Pig rearing is one of the most important means for food security and poverty alleviation in Nepal. The pig production in Nepal concentrates in the hill zones (mostly in the eastern hills) because the resident ethnic groups (Rai, Limbu, Magar, Tamang, Gurung and Tharu) have no reservations to keep and eat pork. In recent years, however, the caste system has become more relaxed and the consumption of pork in the higher caste has increased. That makes a 140% increase in the pig population in two decades (Joshi et al., 2003).

There was a 5.2% annual growth rate in the pig production, which is the highest among the South Asian countries, in-between 1992-2002 (FAO, 2003). The pig population is 58% in the mid hills, 11% in the mountains and 31% in the plain territories (Sharma, 2003). Out of total pig population of 960 827 the administrative regions eastern, central, western, mid-western and far-western proportionate 50%, respectively 18%, 12%, 15% and 5% (ABPSD, 2006). The agriculture households with pig husbandry in the urban and the rural area are 6.4% respectively 12.1% (NPC, 2004).
The exotic breeds Hampshire, Landrace, Tamworth, Saddleback and Fauyen, are introduced in Nepal with a view to upgrade native swine. Bampudke, Chwanche and Hurra are the main indigenous breeds of Nepal (Shaha and Joshi, 2003). Bampudke pigs are found in lower hills, Chwanche in the middle mountains and Hurra pigs are in the plain territories. The pigs are reared here in both the scavenging and the intensive system. In the scavenging system both indigenous and improved pigs are allowed to roam, freely, around road sides and open areas and looking for available food. Therefore it is likely that they suffer from internal parasitic infection and other disease problems. It is estimated that 58% of the pigs are Chwanche, 23% Hurra and 19% are improved breeds. The scavenging rearing system is the preferred husbandry practices (Dhaubhadel, 1992). The common practices of feeding offal and kitchen waste in the backyards to the pigs contributes to the transmission of parasitic diseases in Nepal (Joshi et al., 2005).

1.4 Meat production, inspection, marketing and consumption in Nepal

The meat production in Nepal is largely based on the smallholder subsistence production system except for some recently developed units of commercial poultry and pigs in some few pockets of the country (Joshi and Shaha, 2003). The net meat production amount is 219 205 metric tons and the contribution (’000) of buffalo, goat, pig and chicken is 142, 42, 15 and 15 metric tons, respectively (ABPSD, 2006). However, the meat production has increased in Nepal from the past decade but the average annual growth rate has still fallen behind the neighboring countries, except for pork that was previously at a very low level (TLDP, 2002). In Nepal 52.4% of the total pigs are slaughtered per year. The average carcass weight per pig is 31.8 kg (FAO, 2005).

There are evidences that the existing poor conditions in the slaughter facilities and the meat handling in Nepal contribute to the spread of zoonotic diseases (Joshi et al., 2001). The Department of Livestock Services, the Department of Food and Agriculture Marketing and the Central Food Research Laboratories are indirectly related to the control of the animal slaughtering for the market. Due to a lack of implementation of the ‘Animal Slaughterhouse and Meat Inspection Act-1999’, there
is a reluctant absence of meat inspection in Nepal. Because of this legislative problem the meat from sick or parasite-infected animals is serving as a main source of infection to humans as well as to other animals (Joshi et al., 2003).

The marketing of pork tends to be organized, informally, for the local consumption but it is much more organized for animals destined for urban centers like Kathmandu and Pokhara where the demand for pork is increasing, significantly (TLDP, 2003). The import of livestock products accounts for 1.1% of the total import, the export constitutes of 0.2% of the total export while the net pork import is 0.23% of the total consumption (FAO, 2005).

The actual consumption of meat and meat products in the country is influenced by religious, cultural and economic factors. In Nepal only 2% of the people are vegetarian. Most consume meat with their staple diet of rice and lentils. The imported live animals from India, rather than the local supply, meet much of the urban demands for the consumption. The average per capita daily food intake by the Nepalese population is 2264 kilocalories. 160 kilocalories of that are obtained from different animal products (EarthTrends, 2003). Pork occupies 7% (5626 metric tons, annually) of the total meat consumption in the country. That is the second highest amount after the buffalo meat consumption (ABPSD, 2006). The general meat consumption is highest in-between August-November since it coincides with the Hindu festivals. Pork, however, mostly get consumed during the winter months. In Nepal the consumption of pork through the practice of low heat barbecuing is becoming more popular. That will substantially increase the meat borne parasitic zoonoses problems (Joshi et al., 2005).

1.5 Trichinella and trichinellosis

Roundworms of the genus Trichinella are spread worldwide. They are responsible for one of the most serious helminthic zoonoses. The Trichinella nematode is one of the biggest intracellular parasites (Despommier, 1990). In nature it is maintained by sylvatic and domestic cycles (Pozio, 2000a). The domestic cycle includes domestic pigs which may become infected by the consumption of rodents, by
tail biting, by swills or carcasses of wildlife, foxes etc., by carnivorism and cannibalism (SCVPH, 1998).

The global prevalence of the disease trichinellosis is unknown, but estimates indicate that 11 million peoples might be infected (Wang et al., 2006). The International Commission on Trichinellosis (ICT) has reported more than 10,000 cases of human trichinellosis (1995-1997) and it has been regarded as a re-emerging disease (Dupouy-Camet, 2000). The most common source of human infection worldwide is pork, wild boar and other game meat. However horse, dog and many other animal meats have also served as sources of infection (Dupouy-Camet, 2000). The epidemiological data on *Trichinella* infection in pigs show that this infection is usually confined to small farms with traditional pig rearing practices or grazing in wild areas (Pozio, 2000a).

The trichinellosis is still not under control in many parts of the world, because of the evolution in the food habits of people, new sources of infection other than pork, increase in international trade and tourism (van Knapen, 1997). It is recognized that in many parts of the world none of the existing methods of control for *Trichinella* infection are applied because of the economic problems, erosions of veterinary infrastructures, failure of educational systems, ineffective abattoir control measures and non-awareness of the disease by medicals. These factors are responsible for *Trichinella* infection still in the food chain in large parts of the world (van Knapen, 2000; Murrell and Pozio, 2000).

1.6 Statement of problem

Trichinellosis has been recognized over the past decades in many parts of the world in new hosts and with new epidemiological contexts (Pozio, 2000a, Pozio et al., 2002). The practice of low heat barbecuing is popular among Nepalese. The certain ethnic groups in eastern region are mainly consuming raw pork in their regular dishes. Sporadic suspicions of trichinellosis in humans have been reported by different medical hospitals in Nepal. However there is no confirmation (Joshi et al., 2005). The
pig meat inspection is non-existent in Nepal. So a hypothesis of a high number of human cases is assumed.

Nepal is in the stage of implementing the animal slaughterhouse and meat inspection act. In such aspect, the identification of zoonosis related problems in the country is important and should be brought to the attention of veterinary and public health authorities. The species differentiation of *Trichinella* has not been done yet and the source of *Trichinella* infection either domestic and/or sylvatic in pigs is unknown to the country.

1.7 Scientific justification of the study

Joshi *et al.* (2005) have reported a prevalence of 0.47% with western blot and Sapkota *et al.* (2006) have reported a prevalence of 1% with antibody ELISA on pig’s sera in Kathmandu, Nepal. Both studies suggest serological evidence of trichinellosis.

In pigs high antibody titers against *Trichinella* reveal a recent infection because several months after the infection the antibody level begins to decreases. Therefore doubtful titers are not predictive enough in older animals. So a verification of a realistic prevalence of parasites is required by using a Pepsin digestion method. Concerning the countrywide control of trichinellosis it is important to compare the results of the serology with the Pepsin digestion method. According to the Directives 64/433/EEC and 77/96/EEC a routine slaughter inspection for *Trichinella* by pooled sample digestion is required in all member states of the European Union for the trade between the member countries as well as for pork imported from third world countries (Nöckler *et al.*, 2000).

Preventing *Trichinella* infections in domestic animals requires information about local epidemiological factors. In addition the species of *Trichinella* should be identified. So the relevant economic proposal on its control can be developed as a baseline comparison for future interventions. In this study the *Trichinella* infection in slaughtered pigs of the central development region of Nepal was examined from an epidemiological perspective.
1.8 Objectives of the study

- To investigate the *Trichinella* larval status in meat samples of slaughtered pigs by the Pepsin digestion method

- To compare the results of the Pepsin digestion method with corresponding serum samples by indirect antibody ELISA

- To characterize the species of *Trichinella* larvae by Multiplex PCR
2. LITERATURE REVIEW

2.1 Morphology and development of the genus *Trichinella*

*Trichinella* nematodes belong to the family Trichuridae and are characterized by a length of 1-7 mm, a cylindrical and tapered anterior and posterior ends. The elongated esophagus contains prominent stichocytes and the bacillary bands (Kozek, 2005). Female worms are about twice of the length of males (1.4-1.6 mm) with a similarly located anus. The single uterus is filled with developing eggs in its posterior portion whereas the anterior portion contains fully developed hatching juveniles (Soulsby, 1982). The females are viviparous, laying first larvae and then dying shortly after the completion of the oviposition (Corwin and Stewart, 1999).

In 1835 James Paget has discovered the *Trichinella* parasite from the human muscle and in 1846; Joseph Leidy has first reported it from the swine muscle, referred by Gould (1970a). The German scientists Leuckart, Virchow and Zenker in 1860 have first elucidated the mechanism of the infection and the life cycle as referred by Campbell (1983) and Oivanen (2005). The pivotal events that led to the current taxonomy of *Trichinella* were the experimental attempts to infect laboratory rodents and pigs with isolates from wild animals (Murrell *et al.*, 2000).

According to the zoological classification of the taxonomy of the genus *Trichinella* it belongs to the phylum Nematoda, the class Adenophorea, the order Trichinellida and the super family Trichinelloidea (Noble *et al.*, 1989). But based on results from the ribosomal deoxyribonucleic acid sequences, two classes, Secernentea and Adenophorea, are referred (Blaxter *et al.*, 1998). Today, two main clades are recognized in the genus *Trichinella*, one that encompasses species that encapsulate in the host tissue and a second that does not encapsulate following the muscle cell differentiation. The species and genotypes of the first clad parasitize only in mammals, whereas of the three species that comprise the second clad infects mammals, birds and reptiles (Pozio and Zarlenga, 2005).
2.2 Life cycle

All members of the genus *Trichinella* have a direct life cycle where both adult and larval stages occur in the same host (Kassai, 1999; Bowman *et al.*, 2003), as outlined in figure 1. The infection is passed from host to host through ingestion of infective L1 (first stage) larvae in muscle tissue. The L1 larvae are enclosed in the muscle cells of the previous host. During the digestion process in the recipient host the infective L1 larvae are released into the small intestine. The development of the first to the fifth pre adult larval stages (L1-L5) in the epithelium of the small intestine is rapid and takes only 30 hours (Despommier, 1983; Kociecka *et al.*, 2003). Males die soon after copulation and mature females release about 1500 L1 larvae into the lymph spaces of the small intestine from 5-6 days onward for up to the next two months. The newborn larvae enter the lymphatics, mesenteric veins and proceed to migrate through the arterial circulation for a further 7-8 days as they mature and develop until the larvae are capable of invading the skeletal muscle (Straw *et al.*, 1999). Once in skeletal muscle the larvae enter the muscle cells where they mature further into infective L1 larvae during the first 2-3 weeks (Bowman *et al.*, 2003).

The larva absorbs the nutrients from the muscle cells and increases its length to 1 mm. It finally coils and remains dormant. This alteration in the muscle cell induces to create a ‘nurse cell with or without capsule’ from the original muscle cell. There the larva receives nutrients and remains viable inside the host for many years. The development cycle of a nurse cell after post infection varies depending on the *Trichinella* spp. For *T. spiralis* it is 16 days, for *T. nativa* 20-30 days, for *T. nelsoni* 34 days and for *T. murrelli* 60 days (Näreaho, 2006). The differences in the encapsulation times are probably the result of different maturation and reproduction rates since the capsule becomes apparent around day nine after the arrival of the newborn larvae to the muscles (Li and Ko, 2001). Once in muscle *Trichinella* may survive for several years until ingested by the next host (Kociecka *et al.*, 2003). The living larvae of *Trichinella spiralis* have been found 39 years after the infection in a human (Fröscher *et al.*, 1988) and it has been assumed that even after the death of the
host the parasite remains infective for weeks or even longer (Despommier et al., 1991).

Figure 1: Diagram showing the direct life cycle of *Trichinella* (Adapted from Kassai, 1999)

2.3 Different *Trichinella* species and their epidemiology

From 1835 to the middle of the 20th century it was commonly assumed that all cases of trichinellosis were caused by a single species, *Trichinella spiralis* (Murrell et al., 2000). Today, a total of 11 genotypes of *Trichinella* distributed by climate zones or by host species are identified (Pozio et al., 2002). Eight were recognized at the species level like *T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murrelli*, *T. nelsoni*, *T. papuae* and *T. zimbabwensis* whereas three (T6, T8 and T9) were only defined at an uncertain taxonomic level (Pozio et al., 2001b; Kurdova et al., 2004).
The studies on *Trichinella* have shown that the host range is wider with an extensive geographical distribution (Bolas-Fernandez and Wakelin, 1989; Pozio, 2005). Three classes of vertebrates (mammals, birds and reptiles) are known to act as hosts and even fishes are reported to be paratenic hosts (Moretti *et al.*, 1997). The epidemiology of the *Trichinella* infection varies greatly by the species depending on the diet, the life span, the distribution and the relationship to humans (Pozio, 2005).

*Trichinella spiralis* shows the worldwide distribution. The predominant hosts of this species are domestic and sylvatic swine, horse, brown rat, cat, dog and a broad range of sylvatic carnivores (Pozio, 2001a; Pozio, 2005). *Trichinella* produces more than 90 newborn larvae whereas the other species produce less than 60 newborn larvae per 72 hours (Pozio *et al.*, 1992). *Trichinella spiralis* is specifically characterized by six unique allozyme banding patterns generated by ACP, ALAT, EST, GLDH, PGM and SOD (Murrell *et al.*, 2000). It is the etiological agent of most of the human infections and deaths around the world.

*Trichinella nativa* belongs to the sylvatic cycle. It was documented in the frigid zones of Asia, North America and Europe. This species has limited infectivity for swine and rat but is commonly found in wild canids, bears and walruses. The muscle larvae of this species are high resistant to freezing (Pozio *et al.*, 1994). It was reported that they would survive -18°C temperature for up to 5 years (Dick and Pozio, 2001). *Trichinella nativa* is uniquely characterized by two allozyme markers, ME and 6 PGD (Murrell *et al.*, 2000). Human infections were reported from frigid zones of Canada, Siberia, Greenland and Kamchatka (Nelson *et al.*, 2003).

*Trichinella britovi* belongs to the sylvatic cycle and the larvae of this species are capable of surviving in frozen pig muscles for up to 3 weeks and up to 11 months in carnivore muscles (Dick and Pozio, 2001). It was reported from temperate areas of the polar region and also from the Iberian Peninsula to Kazakhstan, Iran and Turkey (Pozio, 2001a). It is likely that the distribution area encompasses other Asiatic countries (India and China) for which no information is currently available (Pozio and Zarlenga, 2005). The distinctive features of *Trichinella britovi* are its low infectivity
for rats, the greater resistance to freezing, the slow nurse cell development and the low in vitro production of newborn larvae (Murrell et al., 2000). *Trichinella britovi* is identified by a single unique allozyme ACP (La Rosa et al., 1992). It is predominantly reported in wild carnivores and occasionally in pigs and horses (Kapel and Gamble, 2000). Human infection from consumption of free ranging pig, game and horse meat from this species was documented in France, Italy, Spain and Turkey (Pozio et al., 2001a). However the clinical course of disease is benign and no death has been reported yet (Pozio and La Rosa, 2003).

*Trichinella pseudospiralis* is a global species. It does not induce collagen capsule during the muscle phase of the infection. It was recovered from raptorial birds, wild carnivores, omnivores, rats and marsupials in Asia, North America and Europe and from the Australian subcontinent (Pozio, 2005; Pozio and Zarlenga, 2005). *Trichinella pseudospiralis* is identified by 12 unique allozymes ACP, ADA, ALAT, ALDO, GPD, LDH, EST, G6PD, GLDH, GOT, SOD and TPI (La Rosa et al., 1992). The increasing reports of this species in domestic and sylvatic swine in Europe, the USA and in Southeast Asia show that it has a potential to infect humans.

*Trichinella murrelli* has been detected throughout the continental USA and Canada (Pozio et al., 2001b). The infection of this species was documented in sylvatic carnivores living in the temperated areas of the Neoarctic region. This species has a low resistance to freezing and it is weak in its nurse cell development as well as in the newborn larvae production (Malakauskas and Kapel, 2003). It has recovered from wildlife and occasionally from horses but it has a very low infectivity for domestic pigs. Mostly humans get infected from game meat but horses are also identified as a source for a large outbreak in humans (Ancelle, 1998).

*Trichinella nelsoni* is the etiological agent of infection of sylvatic carnivores living in eastern Africa from Kenya to South Africa (Pozio and Zarlenga, 2005). In comparison to other species it has a greater resistance to elevated temperature. *Trichinella nelsoni* has a low infectivity for domestic pigs and rats but it has detected in bush pigs and warthogs (Pozio et al., 1997; Kapel, 2001). This species is identified
by three unique allozymes ADA, GLDH and TPI (La Rosa et al., 1992). The muscle larvae of this species may survive up to 56°C for 60 minutes while the larvae of other species will die within 10 minutes at this temperature (Murrell et al., 2000). This species shows low infectivity in humans. Only a single death was reported due to a high (>4000 larvae/g) larvae load of *T. nelsoni* (Bura and Willett, 1997).

*Trichinella papuae* is a non-encapsulating species of *Trichinella*. The length of *T. papuae* larvae is one third greater than that of *T. pseudospiralis*. It is very resistant to freezing (Murrell et al., 2000) and can survive minus 5°C storage temperature for 4 weeks (Webster et al., 2002). It was detected only in Papua New Guinea and is able to infect both mammals and reptiles (Pozio and Zarlenga, 2005). The wild pigs are the most important reservoir of this species (Owen et al., 2000) and serve as route of transmission to saltwater crocodiles and humans (Owen et al., 2005).

*Trichinella zimbabwensis* is a non-encapsulating species of *Trichinella* and was detected in the farmed crocodiles of Zimbabwe and Ethiopia (Pozio et al., 2002) as well as in sylvatic crocodiles of Mozambique (Pozio, 2005). Under laboratory conditions this species can infect domestic pigs, monkeys, rats, mice and foxes. Therefore it can be concluded that mammals are also a suitable host for this species. However, no naturally infected mammals are found yet (Pozio and Zarlenga, 2005).

### 2.4 Pathogenesis and clinical symptoms referring to *Trichinella spiralis* in the human

*Trichinella* infection of meat from food animals and games is important because of the risk of trichinellosis in humans who eat raw or undercooked meat (OIE, 2004). The larvae of *Trichinella* are present in the voluntary skeletal muscles of their hosts and the ingestion of these tissues shall transmit infections to a susceptible individual. It is estimated that a minimum infective dose of *Trichinella spiralis* for a human is 70-150 ingested larvae (EFSA, 2005). If ≥ 500 larvae are ingested a life-threatening condition will be reached (Murrell, 1985; Battelli et al., 1994; Oivanen, 2005). The transplacental transmission of larvae has occurred in mouse and human,
but not in pig (Bowman et al., 2003). The person-to-person spread of the disease has not been reported yet (Urquhart et al., 1996).

Trichinellosis continues to be a public health concern throughout the world. The experience from the past decade has shown a consistent increase of human cases (Murrell and Pozio, 2000). *Trichinella spiralis* is the etiological agent of most of the human infections and deaths around the world. That is because of its higher pathogenicity and a stronger immune reaction in humans (Gomez Morales et al., 2002). The pathogenesis and the associated clinical symptoms in human beings from *Trichinella spiralis* infection can be described as follows:

**Enteral phase:** The enteral phase starts after consuming the contaminated meat. Most of the time people are asymptomatic. During the first week of the enteral phase moderate to severe symptoms like malaise, upper abdominal pain, mild transient diarrhea, nausea, vomiting and sub febrile temperature can develop in some patients (Hermanowska-Spakowicz et al., 1993). Diarrhea is more persistent than vomiting and lasts up to 3 months, which causes severe dehydration. Together with enteritis this might be an occasional cause of death (Bruschi and Murrell, 2002).

**Parenteral phase:** The parenteral phase begins just after the larvae entered the circulation with the invasion of all organs lasting up to 3 to 4 weeks. The ocular signs like the edema of eyelids, chemosis, conjunctivitis, conjunctival hemorrhage, disturbed vision and blindness may develop in this phase. The periorbital edema is peculiar and ranges in 17-100% of the trichinellosis patients (Tassi et al., 1991). Cardiovascular disturbances and myocarditis are observed in 5-20% of all infected people (Dupouy-Camet et al., 2002).

**Muscle phase:** The muscle phase starts with the entering of the larval in the striated skeletal muscles not before the fourth week. At this time the muscles (extra ocular, masseter, glossal, laryngeal, diaphragmatic, neck and intercostal) of the body usually become painful. The pain may be so severe that it limits the function of arms and legs, inhibits walking, speaking, moving the tongue, breathing and swallowing (Bruschi and Murrell, 2002). The muscles become edematous and the patient feels
weakness with prolonged progressive muscular hypertrophy (Chotmongkol et al., 2005). This is associated with the invasion of the muscles by the migrating larvae which can damage muscle cells, directly or indirectly, with stimulating the infiltration of the inflammatory cells (Bruschi and Murrell, 2002). This causes an increase in the levels of muscle enzymes like creatine phosphokinase, lactate dehydrogenase, aldolase and occasionally asparatate aminotransferase and liver enzyme (Capo and Despommier, 1996; Jonwutiwes et al., 1998; SCVPH, 2001).

Eosinophilia (1400-8700/cubic mm) is present in every case with leukocytosis (12500-18000/cubic mm) (Capo and Despommier, 1996; Dupouy-Camet et al., 2002). Eosinophilia is significantly higher in patients with neurological complications (Fourestie et al., 1993) and correlated with the degree of myalgia (Ferraccioli et al., 1988; Kociecka, 2000). In this phase the central nervous system, the lungs, the kidneys and the skin might be affected (Wang et al., 2006).

The physicians only advance diagnosing the patient as being suspected of trichinellosis if the patient starts to show the trichinellotic syndrome, that is characterized by facial edema, muscle pain and swelling, weakness, fever (39-40°C), anorexia, headache, conjunctivitis and urticaria (Pawlowski, 1983). However, the major complications of this infection are myocarditis and encephalitis, which are both life threatening and often present, simultaneously (Fourestie et al., 1993). The cardiovascular complication symptoms include pain in the heart region, tachycardia and an abnormal electrocardiogram. The cardiovascular complications like thromboembolic disease, specifically deep thrombophlebitis, intraventricular thrombi and/or pulmonary embolism can lead to death (Pratesi et al., 2006).

Previously it was assumed that the mechanical damage induced by the migrating larvae leads to cardiac symptoms (Gould, 1970a). The recent studies, however, have identified organ specific autoantibodies in trichinellosis patient sera (Pratesi et al., 2006). Neurological complications were reported in 3-46% of the cases (Dupouy-Camet et al., 2002). Such patients show unconsciousness, somnolence and apathy and some of them show signs of meningitis and encephalopathy (Feydy et al., 1996).
2.5 Diagnostic measures in animals

The *Trichinella* infection in pigs and other species can be detected, directly or indirectly (OIE 3.5.3 Article; European Union 77/96/EEC Directive, European Council Regulation 807/2003). The direct demonstration is based on the detection of the parasitic larval stages in muscle tissues preferentially from the predilection sites, like diaphragm and tongue in swine (Serrano *et al.*, 1999; Kapel *et al.*, 2005), diaphragm, masseter and tongue in horse (Pozio *et al.*, 1999b; Theodoropoulos *et al.*, 2000; Kapel *et al.*, 2005) and lower limb, tongue and diaphragm in fox (Nöckler and Voigt, 1997). The indirect demonstration of the parasite can be done through serological and molecular techniques. The serological techniques include conventional serology and the enzyme linked immunosorbent assay (ELISA) where molecular techniques are biochemical and molecular means of studies. The International Commission on Trichinellosis (ICT) has been recommending since 1993 that the *Trichinella* isolate has to be characterized by genetic means rather than by other methods (Lichtenfels *et al.*, 1994).

2.5.1 Direct demonstration of the parasite in tissue samples and digests

ICT ([http://monsite.wanadoo.fr/intcomtrichinellosis/](http://monsite.wanadoo.fr/intcomtrichinellosis/)) has recommended that no serological method should be allowed for the testing of individual carcasses of food animals at slaughter. It has also recommended that meat inspected directly through trichinoscopy should only be allowed as a transitional measure and such meat should be clearly marked. Furthermore, such meat has to be limited for the selling on the national market. It is also clearly unacceptable for products where the production process does not kill *Trichinella*. Previously, in the EU legislation (Directive 77/96/EEC) for the direct demonstration of parasites, seven methods were accepted: six digestion methods and trichinoscopy. But in the present EU legislation (SANCO/1900/2002 Rev. 8 draft, in forced 01-2006) the number of inspection methods are reduced to four with the magnetic stirrer digestion as the reference method (Webster *et al.*, 2006), as outlined in table 1.
Table 1: The use of different methods for the direct demonstration of the *Trichinella* larvae in pork and horse meat

<table>
<thead>
<tr>
<th>Method</th>
<th>Description of technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichinoscopy/Compressorium</td>
<td>It is a rapid method for only few samples. It is laborious and has low sensitivity (detection limit 3-5 lpg). This method does not detect the non-encapsulating <em>Trichinella</em>. The sensitivity is less than 43.4% in low larval burden (Beck <em>et al.</em>, 2005).</td>
</tr>
<tr>
<td>Stomacher (mechanical treatment) and sedimentation or filtration</td>
<td>It needs only a short duration (25 min) but the larvae found adhere to plastic bags. It has lower sensitivity than stated in the EU Directive (1-3 lpg). It gives more false negative results. 1 g and 5 g meat of pig/wild boar and horse, respectively, need to be examined according to this method.</td>
</tr>
<tr>
<td>Trichomatic 35 blender</td>
<td>In this method 35 samples can be pooled at the same time. It is easy to manage and needs a very short digestion time (5-8 min). The filter, however, requires extra washing procedures, which is a main demerit of the technique (van Knappen <em>et al.</em>, 1996). The detection limit is 1-3 lpg.</td>
</tr>
<tr>
<td>Magnetic stirrer (constant mechanical treatment)</td>
<td>It needs a short duration time (30 min/100 g). But the filter size needs adjustment for correct sensitivity of 1-3 lpg. The recovery of the larval improves if the filter size changes from 177 μm to 355 μm (Gamble, 1999). 1 g and 5 g of meat need to be examined according to the EU directive.</td>
</tr>
</tbody>
</table>
2.5.2 Indirect demonstration of parasites through serological and molecular techniques

Sero-diagnostic methods are used for the ante mortem as well as for the post mortem examination of the serum samples for *Trichinella* specific antibodies. Under some conditions these techniques may have a higher sensitivity than that of direct detection (Nöckler *et al.*, 2000). It is a useful technique for herd surveillance, for establishing *Trichinella* free areas and for reducing restrictions in international animal trade (SCVPH, 2001). However, the serology will fail to detect both early (<3-4 weeks) infection (Gamble, 1996; Kapel, 2001) and the chronic (>22-23 weeks) infection (Oivanen, 2005).

The serological response in horse is different than in pig (Voigt *et al.*, 1997). In horse the antibody declines within a few months following the infection. This coincides with a decline in the muscle larvae. It makes the serological evaluation of *Trichinella* in horses not significant since even animal harboring hundreds lpg can show a negative serology (OIE, 2004). Another alternative matrix is the use of meat juice in ELISA for detecting *Trichinella* antibodies in animals like previously slaughtered pigs, hunted animals, dead animals etc. which cannot provide blood sera any more. In these days this has increased in popularity (Kapel *et al.*, 1998; Nöckler *et al.*, 2004; Möller *et al.*, 2005).

2.5.2.1 Serological methods

There are several serological diagnostic techniques like the western blot (WB), the complement fixation test (CFT), the haemagglutination test (HAT), the immunofluorescence antibody test (IFAT), the enzyme immunohistochemical technique (EIH) etc. So while choosing any technique the greater attention has to be paid to the technique that has a high validation and quality assurance (Gamble *et al.*, 2004). IFAT and HAT have achieved a higher degree of sensitivity than CFT (Nöckler *et al.*, 2000). However, the many reference laboratories use the enzyme linked immunosorbent assay (ELISA) as a method of choice (Nöckler *et al.*, 2000).
Enzyme linked immunosorbent assay (ELISA)

ELISA is the most common used method for detecting the *Trichinella* infection because it is economical, reliable, readily standardized and provides an acceptable balance of sensitivity and specificity. The ELISA test, using the excretory-secretory (ES) larval antigen or the tyvelose antigen, is useful for the surveillance systems. Several studies have demonstrated that both ES and tyvelose antigens reduce cross-reactions which, however, occur if a crude *Trichinella* antigen is used (Gamble *et al.*, 1997). However, the quality of the ES larval antigen depends on the adherence to proper methods for the cultivation of muscle larvae and the purification of its antigen (Gamble *et al.*, 1988). In pigs the sensitivity and specificity of ELISA using the ES antigen has been reported to range from 93.1-99.2% and from 90.6-99.4% respectively. That means that sufficient time of the infection elapses for the infected animals to develop an antibody response (van der Leck *et al.*, 1992). An infection as few as 1 lpg of tissue can be detected by ELISA (Kapel and Gamble, 2000). A synthetic glycan antigen, which has a high sensitivity and specificity, is also used for the serological testing (Gamble *et al.*, 1997). The recent application of ELISA in the *Trichinella* diagnostic is the immunochromatographic strip (Zhang *et al.*, 2006) for the rapid detection of analytes. It is easy to use and provides a visual end-point. In the strip the ES larval antigen is labeled with colloidal gold as a detector. The Staphylococcal Protein A and goat anti-ES-antibody are blotted on the nitrocellulose membrane for test and control lines, respectively.

Western blot (WB)

The western blot technique can also be applied to detect antibodies in a serum or to define antigens with the aid of immune sera. With this technique the antigens are recognized by their molecular weight (Robert *et al.*, 1996). Industrially produced western blot strips, which seem to work without major cross-reactions, are available for human diagnostics (Yera *et al.*, 2003). However, differences exist in western blot patterns observed between human and animal sera infected with the same *Trichinella*.
species (Dupouy-Camet et al., 1988). Therefore caution should be taken when extrapolating methods to animal diagnostics.

2.5.2.2 Molecular techniques

Biochemical differentiation

The biochemical differentiation of the *Trichinella* genotype via the isoenzyme analysis with starch gel electrophoresis is a very powerful tool for genetic studies at the level of the species and the population. It is possible to infer variability at the DNA level among parasite genotypes via electrophoretic mobility of the enzymatic proteins as markers (Zarlenga and La Rosa, 2000). This technique is able to analyze diverse portions of the genome that have evolved independently of each other. Curran et al. (1985) exploit firstly the repetitive and polymorphic nature of the genomes within this parasite group for differentiation. The fluorescence staining is sufficient to demonstrate differences in the organization of the *Trichinella spiralis* and *Trichinella pseudospiralis* genome, simply through digesting the genomic DNA with the restriction enzyme and then separating the segment by the use of agarose gel (Zarlenga and La Rosa, 2000).

Dame et al. (1987) were the first to use the restriction fragment length polymorphism (RFLP) as a tool for the investigation of the epidemiology and dissemination of *Trichinella* genotypes in sylvatic hosts. The RFLP technique provides large numbers of bands on agarose gel representing genetic heterogeneity within the genus (Zarlenga and La Rosa, 2000). Chamber et al. (1986) have used the DNA probe technology by radio labeling and generated characteristic fingerprints using a genus specific probe TsR1. This technique is used for screening southern blots of the restriction enzyme digested DNA from the same group of parasites. Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting technique. It shows high-resolution power with *Trichinella nativa* (with species specific bands) and is a promising method for detecting population variation (Mikkonen et al., 2005).
All those tests have been used, successfully, to differentiate various *Trichinella* genotypes. They are, however, are either labor intensive, require large sample sizes or use hazardous materials (radioisotopes) and need to work at more sensitive level (Zarlenga *et al*., 1999). This has led to seek alternative approaches like the adaptation of PCR technology.

**Polymerase chain reaction (PCR)**

In general, the PCR methods can be divided into those involving random or arbitrary primers and those involving DNA sequence derived genotype specific primers. Welsh and Mc Clelland (1990) and Williams *et al*. (1990) have described an approach using PCR amplification to generate genetic markers from genomes in the absence of any specific sequence information. This method requires a single, randomly designed primer usually 10 base pair in length. It is used under non-specific amplification conditions to generate a population of amplification products in an agarose gel-banding pattern, which is characteristic of a genotype. The fingerprint pattern obtained with this method is randomly amplified polymorphic DNA (RAPD) and this is supposed to be a simple and effective tool for quickly generating and detecting genetic markers (Bandi *et al*., 1993). It can be demonstrated at a larger set of *Trichinella* species with a single primer by combining RAPD and DNA hybridization (Chacon *et al*., 1994; Dupouy-Camet *et al*., 1994).

The strengths of the RAPD-PCR are a quick result with high sensitivity. The poor reproducibility either from variation in the sample DNA quality or contamination is the most prominent weakness (Zarlenaga and La Rosa, 2000). Another weakness is the necessity of re-optimizing when moving from one laboratory to another (Oivanen, 2005). Single strand conformational polymorphism (SSCP) can be used to characterize and differentiate seven distinct genotypes of *Trichinella* (Gasser *et al*., 1998). SSCP utilizes heat denaturation to induce strand separation in the PCR amplified products which are then subjected to the non-denaturing gel electrophoresis. This technique shows a great promise for defining both inter and intra specific variations in the expansion of segment five (ESV). Several investigations
have been performed using genotype specific primers in association with variations in the length of the ESV sequence to differentiate *Trichinella* genotypes (Zarlenga and Dame, 1992; Arriaga et al., 1995; Zarlenga et al., 1996; Appleyard et al., 1999). However, the unilateral application of SSCP for rapidly delineating genotypes is not significant since radioisotope markers are needed for the final visualization (Zarlenga and La Rosa, 2000).

The Multiplex PCR test can unequivocally distinguish all currently recognized species of *Trichinella* through the amplification of ESV sequences. It is not only used for differentiating species but also as an internal control for the PCR integrity (Zarlenga et al., 1999). The single or two ribosomal DNA fragments are sufficient for characterizing each genotype via the Multiplex PCR technique. Those genotypes are *T. spiralis* (173bp), *T. nelsoni* (155bp, 404bp), *T. nativa* (127bp), *T. britovi* (127bp, 252bp), *T. murrelli* (127bp, 316bp), *T. pseudospiralis* (300bp, 360bp) and *T. papuae* (240bp) fragments (Murrell et al., 2000). The Multiplex PCR is able to perform on individual larva. So it is easy to use for epidemiological investigations where only some few larvae are available (Zarlenga et al., 1999). If the sample, however, stems from the pooled larval DNA, where the possibility of concurrent infection with several species exists, the result may be ambiguous (Zarlenga and Higgins, 2001).

The reverse line blot hybridization (RLBH) is based on variation in the DNA sequence between the 5S ribosomal DNA genes within the genus *Trichinella*. The amplified 5S ribosomal RNA region is analyzed using a cleavage fragment length polymorphism assay (Oivanen, 2005). This method could identify single larvae and it has 10 fold more sensitive than the agarose gel analysis (Rombout et al., 2001). Recently, molecular characterization by inter-simple sequence repeat (ISSR) PCR is described as new molecular technique (Fonseca-Salamanca et al., 2006).
2.6 Diagnostic measures in the human

2.6.1 Parasitological diagnosis

For the identification of *Trichinella* parasites in suspected patients during the rheumatoid phase a muscle biopsy must be collected. In the human, deltoid muscle is preferred because it is more accessible (Gould, 1970b). The trichinoscopy is of great use for identifying the parasites at species level (Zarlenga and La Rosa, 2000). It is also useful for sporadic and doubtful cases, for diagnosing and retrospective analysis (Dupouy-Camet *et al.*, 2002). The histopathological examination method is another technique. It has a higher sensitivity than the trichinoscopy at the early stage of the muscle invasion when the larvae are still very small and not easy to differentiate from the muscle fibers (Wranicz *et al.*, 1998). Even if the larvae are not seen by the histopathology the infected muscle cells undergo basophilic changes once newborn larvae will penetrate it (Capo and Despommier, 1996; Kozek, 2005). The artificial digestion is a more sensitive technique than the direct microscopic observation of tissue (Zarlenga and La Rosa, 2000). To get such sensitivity the digestion has to be carried out not before the days 17-21 after the infection. It is because the larvae of *Trichinella* spp. before that period are not resistant to digestion (Despommier, 1986). The complications for the patient after performing the muscular biopsy are the main obstacle to perform direct demonstration of *Trichinella* in humans (Mahannop *et al.*, 1995).

2.6.2 Sero-diagnosis

The objectives of the sero-diagnosis in humans are an early recognizing of the acute infection for prompt treatments and for a retrospective diagnosis to gather the epidemiological information (Ljungström, 1983). Only from the antibody level in serum it cannot correlate with the severity of a clinical course (Bruschi and Murrell, 2002) because the antibody can be detected even after 19 years of its acute phase (Kozar and Kozar, 1968).
In humans the most common serological tests are the indirect haemagglutination, the bentonite flocculation, the indirect immunofluorescence, the counterimmunoelectrophoresis, the latex agglutination and the ELISA, the last is the most sensitive test (Despommier, 1986; Murrell, 1994; Kociecka et al., 2001). In the ELISA method excretory-secretory larval antigens are preferred, particularly in tropical regions where the cross-reactions with other helminthes Ascaris, Trichuris and Filariae could give false positive result (Au et al., 1983).

A recent study among the Spanish people has reported that the capture ELISA using crude Trichinella spiralis larva group 1 (cTSL-1) antigen is the most effective method for the sero-diagnosis of human trichinellosis since it has 100% of both sensitivity and specificity (Escalante et al., 2004). SCVPH (2001) has suggested that the combination of two techniques i.e. ELISA and IIFT would provide the most reliable results and in case of a rapid (<1 hour) result, the latex agglutination is recommended.

Immunoblotting is considered to be a confirmatory test in some instances (Bruschi and Murrell, 2002). It is useful for follow up studies (Andrews et al., 1995). However, it cannot determine the species of Trichinella responsible for infection (Ranque et al., 2000). Western blotting is a useful tool for investigating ELISA and IIFT cross-reactions (De-La-Rosa et al., 1995). However, possible cross-reactions with cases of anisakiasis (Yera et al., 2003) and schistosomiasis (Gamble et al., 2004) are reported.

2.7 Therapy

In animal models benzimidazole is effective against newborn larvae and for young adults that are on either lymphatic, blood vessels or in intestine. Flubendazole has shown to have high efficiency on gastro-intestinal larvae of Trichinella, but very little efficacy on muscular larvae in pigs (Marinculic et al., 2001). However, in practice, pharmaceutical treatment is only used with human patients not with animals, since symptoms in animals have not been clinically noticed.
In humans the diagnosis of trichinellosis is often late due to a delayed onset of non-specific symptoms after an infected meal (Nunez et al., 2003). After proper diagnosis the proper use of benzimidazole anthelmintics (thiabendazole, mebendazole and albendazole) and corticosteriods are recommended for trichinellosis (Dupouy-Camet et al., 2002; Schellenberg et al., 2003). These drugs are effective in limiting the severity and duration of the disease. Mebendazole (200 mg/day for 5 days) or albendazole (400 mg/day for 3 days) is considered to be a first line drug and should be given to adults (except pregnant woman) as well as to children (5 mg/kg body weight for 4 days) (Hermanowska-Spakowicz et al., 1993; Kociecka, 1993). Any of these doses have to be administered along with prednisolone (30-60 mg/day) in multiple doses for 10-14 days (Dupouy-Camet et al., 2002).

Pyrantel (10 mg/kg for 1-3 days) has been used to treat pregnant woman and young children. This is because pyrantel is not absorbed from the intestinal lumen and acts by paralyzing mainly intestinal parasites (Kociecka, 2000). Mebendazole has shown to prevent occurrence of trichinellosis when given to people 48 hours after consumption of meat which was heavily infected with Trichinella (Kociecka et al., 1996). But if the muscle larvae are already encapsulated then the mebendazole is not sufficient to control the infection (Pozio et al., 2001c). If the infection is due to non-encapsulated species then the albendazole (800 mg/day in 4 doses) is particularly efficient (Jonwutiwes et al., 1998). Emodepside (cyclo-octa-depsi-peptides) is fully effective against benzimidazole resistant nematodes and was found to be most active against Trichinella spiralis (Harder et al., 2003).

2.8 The control of Trichinella in domestic animals

The existing control methods of Trichinella are not infallible. So uniform control measures at farms, slaughters and in the processed meat level are needed (van Knapen, 2000). Since there is such a need the recommendations provided by the International Commission on Trichinellosis (ICT) are based on the best scientific information available (Gamble et al., 2000).
The controls at the farm level include requirements for *Trichinella* free pig production through architectural and environmental barriers, proper feed and feed storage, rodent control and farm hygiene. In addition to that good management practices at the farm and periodic serological testing are also necessary (Murrel and Pozio, 2000). The animals that have access to the environment and/or to potentially contaminated feed (swill, carcass etc.) will always constitute a potential public health threat with regard to trichinellosis. Consequently, such animals must undergo proper inspection at the individual level before they are declared to be suitable for human consumption (van Knapen, 2000). In the US, a pilot program for certifying a pig herd was conducted (Pyburn *et al.*, 2005). However, candidate vaccine through utilizing antigens of the newborn larval was developed (Marti *et al.*, 1987). This approach to eradicate *Trichinella* in herds where the management strategies had not been completely successful is not posing real value. This is also because the costs of the production and application are unacceptably high (Murrel and Pozio, 2000).

Slaughter inspection methods are designed to prevent clinical trichinellosis in the human (Gamble *et al.*, 2000). Therefore carcass infected with *Trichinella* should be condemned (Herenda *et al.*, 2000). The pooled digestion method, using a minimum of 1 g of sample is considered to be sufficient for detecting parasites at the slaughterhouse level. The development and use of proper quality assurance systems including training and education of personnel, test validation and control of critical control points are the prerequisites for minimizing the risks at the slaughterhouse level (Forbes and Gajadhar, 1999).

ICT has recommended that meat from animals that might contain *Trichinella* and are not tested by acceptable methods must be treated with a processing (cooking, freezing and eradication) method. The processing methods have proven to inactivate *Trichinella* for human consumption (Gamble *et al.*, 2000). Freezing meat below the critical temperature of $-30^\circ$C will kill *Trichinella* larvae in an appreciable period of time (Smith, 1975). It depends on the thickness of the muscle cut. 15 cm thick muscle cuts need to be kept for 3 weeks and 69 cm for 4 weeks at $\leq$ minus$15^\circ$C (Gamble *et al.*, 2000). ICT has also recommended cooking at an internal temperature of $71^\circ$C.
(160°F) for totally safe consumption. The change in the meat color from pink to grey and likewise the change in texture (muscle fibers are easily separated from each other) are the indicators which show that the meat has been rendered and safe for the consumption (Gamble et al., 2000). The methods of preparation of meat in the microwave cooking, curing, drying, smoking etc. are not considered to be secure (Gamble et al., 2000).

The motile larvae of *Trichinella nativa* was found in the muscle tissue of a fox which was frozen for 1 year at –18°C (Kapel et al., 1999). The study conducted by Worley et al. (1986) in frozen skeletal musculature of wild carnivores has also revealed that 50-60% of the larvae in grizzly bear meat are alive even after having been stored for 27 months at –6.5°C to –20°C. This is because the antifreeze protein molecule which is common in most wild animals protects the worms in their muscle tissue from getting ice crystallized (Dick, 1983).

### 2.9 Economic impacts

As a result of the mandatory inspection the European Union spends, annually, US$ 570 million for controlling *Trichinella* infection from slaughtered pigs (Pozio, 1998), and around US$ 5 million a year for controlling the infection from horse carcasses (Pozio, 2000a). The annual economic cost of swine and human trichinellosis in the United States has reached over US$ 1 billion for health regulatory activities to prevent the infection which includes the utility costs for treating pork through freezing/heating/curing (Roberts and Murrell, 1993; Roberts et al., 1994; Bruschi and Murrell, 1999). The cost for each human infection is estimated to amount around US$ 6000 (Robert et al., 1994) in the United States and around US$ 3000 in France (Pozio, 2000a). Such direct and indirect costs have turned trichinellosis into being one of the most costly parasitic zoonosis (Murrell and Pozio, 2000). One example for the economic loss is a Chinese abattoir which (due to high prevalence of *Trichinella* infection in the slaughtered pigs) lost in-between 1975-1985 US$ 550 000 (Wu, 1995).
2.10 Trichinellosis in animals and humans in the global context

Over the past decades trichinellosis has been recognized in many parts of the world, in new hosts and with new epidemiological contexts, except from the desert zones (Pozio et al., 2002). From a public health point of view the situation is worrisome in Argentina, Croatia, Yugoslavia, Russia, Romania, Latvia, Lithuania and China. The most reported sources of the origin of *Trichinella* infection in human being are from pork and horse meat. The other unusual meat of animals are also potential sources for the outbreak of the disease in humans such as bears in Greenland, Canada, USA, Japan, Eastern Europe and China (Soule, 1991), walrus in Canada and Alaska (Morgolis et al., 1979), cougar in USA (Dworkin et al., 1996), fox in Italy (Dupouy-Camet, 2000), mutton in China (Wang et al., 1998), warthog in Africa (Kefenie and Bero, 1992) and dog in Slovakia and Thailand (Dubinsky et al., 1999; Srikitjakarn et al., 1981). Irrespective of that Wang et al. (2007) has documented *Trichinella* infection in cattle, muntjak, bamboo rat, weasel, shrew and also in moles. Recently, *Trichinella* infection in farmed fur animals was also reported in Estonia (Miller et al., 2006).

In the USA, pigs are not tested, regularly, for *Trichinella* parasites. In a survey of 180 (n = 4078 pigs) farms in the northeastern states 0.37% prevalence and 6.4% herd prevalence were reported (Gamble et al., 1999). The most common source of infections is pork but infections from wild game are also increasing from 27% to 42% (Moorhead et al., 1999). The number of human cases with trichinellosis, however, has been decreasing since 1947. In-between 1991- 1995, 230 human cases were reported and out of those 3 patients have died (Oivanen, 2005).

In Canada, *Trichinella* infection in pigs was reported only in the Nova Scotia region and sporadically in farmed wild boars. A national serologic survey (1996/97) in pigs with using ELISA technique has shown no positive result (Gajadhar et al., 1997; Appleyard et al., 2002). Human outbreaks have occurred due to the consumption of wild game and raw walrus meat (Dick et al., 1986; Schellenberg et al., 2003). In-between 1991-1997 about 3 to 49 human cases, annually, were reported,
mainly from the northwest-territories and from the Quebec region (Murrel and Pozio, 2000).

Trichinellosis is still endemic in most countries of the EU. Denmark and The Netherlands are free from domestic trichinellosis but sylvatic trichinellosis in low prevalence is still reported in these countries (Pozio, 1998). According to the annual reports of zoonotic agents in the EU, domestic trichinellosis was detected in Italy, France, Finland and Spain, whereas sylvatic trichinellosis was found in Austria, France, Finland, Germany, Ireland, Italy, the Netherlands, Spain and Sweden in-between 1996-2003. At the British Isles, however, there has been no report of this parasite. Recently, T. britovi infection in a sow was reported from Sardinia and Corsica which were previously seen as Trichinella free Mediterranean islands (Pozio et al., 2006).

In Germany the domestic pig and horse population are considered to be free from Trichinella (Nöckler et al., 2006). However in-between 1991-2003 about 156 wild boars were found being infected. Concerning the species in polar bears T. nativa, in wild boars and raccoon dogs T. spiralis and in red foxes both T. spiralis and T. britovi were detected (ITRC, 2005; Pozio et al., 2000). The mixed T. spiralis and T. pseudospiralis infection was detected in a wild boar from Mecklenburg, Germany (Nöckler et al., 2006).

Outbreaks of trichinellosis appear to increase in the EU member states. But this trend is difficult to appreciate. Pozio (2000b) has calculated over 3300 human cases of trichinellosis in France and Italy (1975-2000) from the consumption of imported horse meat. No human trichinellosis from the consumption of local domestic or wild animals (except from Sweden, where about 2600 cases were reported at that time) was reported from Austria, Belgium, Britain, Denmark, Finland, Ireland, Luxembourg, the Netherlands and Portugal in-between 1978-1998 (Pozio, 1998). However, in 1999 the Netherlands, Germany, Spain, France, the UK and Austria have reported all together 49 human trichinellosis cases with an increasing tendency of 88 cases in the year 2000 (SCVPH, 2001). The total human infection, acquired in the EU in-between 1999-2003, ranged from 48 to 67 per year. Recently in 2007, an outbreak
of 21 cases of *Trichinella britovi* infection has reported among persons of Spain and Sweden related to Spanish wild boar sausage (http://www.promedmail.org).

Human infections were reported, annually, in all of the new EU member states Bulgaria, Byelorussia, Poland, Romania, Serbia and the Ukraine (Pozio, 2001a). In Romania the incidence of infection have increased 17 times since 1983 (Olteanu, 1997). In 2007, an outbreak of trichinellosis in Zachodniopomorskie voivodeship (West Pomerania) Poland has affected 201 people (Golab *et al.*, 2007). Later on 4 imported cases from victim of that outbreak are also reported in Hamburg, Germany (http://www.promedmail.org). In Eastern Europe, Slovakia and Kazakhstan there is an emergence of non-pork sources like dogs, wild games and wild boars as source of infection (Murrell and Pozio, 2000; Pozio, 2001a). In the Tvier and Smolensk region of Russia the prevalence of *Trichinella* in wolf, fox and domestic dog is as high as 97.3%, 48% and 7.7%, respectively. The carcasses of these animals seem to maintain the infection in the population (Casulli *et al.*, 2001).

Trichinellosis is also a major public health problem in South America. In Mexico, *Trichinella* infections are reported in pig, rat, cat and dog in several states. About 758 human trichinellosis cases were found in between 1952-1997 (Ortega-pierres *et al.*, 2000). In Argentina during 1974-1983, 894 human cases were reported from 15 provinces (Ortega-pierres *et al.*, 2000). During 1990-1999 an unexpectedly high number of 5217 cases was documented (Murrell and Pozio, 2000; Bolpe and Bofi, 2001). In Chile at least 26 outbreaks (where 1300 peoples were infected) happened in between 1981-1995 (Ortega-pierres *et al.*, 2000).

In Sub-Saharan Africa the sylvatic trichinellosis was confined to wildlife living in natural parks and protected areas where the spotted hyena is the main reservoir with a prevalence of 43-85% (Pozio *et al.*, 1997). Trichinellosis in human is derived from the sylvatic cycle with ≤100 human infections reported from Ethiopia, Kenya, Senegal and Tanzania. The low level of the infection in sylvatic suidae relates to the practice of eating only cooked meat and the religious laws that forbid pork consumption (Murrell and Pozio, 2000).
In Australian region, *Trichinella pseudospiralis* is widespread in the Tasmanian wildlife (Obendorf et al., 1990; Obendorf and Clarke, 1992). In Papua New Guinea *T. papuae* was identified in domestic and sylvatic swine (Pozio et al, 1999a). The first human case of *T. pseudospiralis* infection was noticed in New Zealand (Ainsworth et al., 1994).

*Trichinella* found in Asia and the Pacific Rim includes both encapsulated species and non-encapsulated species. It is assumed that numerous outbreaks of trichinellosis have happened in these regions but insufficient epidemiological data are available for many countries (Takahashi et al., 2000). In Japan the first case in raccoon dogs was reported in 1957 and after that several investigators have isolated *Trichinella* from black bears that were identified as T9 (Nagano et al., 1999). It is likely that trichinellosis has occurred at a low level in Japan for many years but the outbreak of 1980 that has infected 60 peoples has increased the intention of Japanese physicians to this disease (Takahashi et al., 2000). In Central Asia *Trichinella* (*T. nativa, T. britovi*) in wolf, red fox and golden jackal was documented (Shaikenov and Boev, 1983).

In Southeast Asia *T. spiralis, T. pseudospiralis* and *T. papuae* were detected in sylvatic and domestic animals and in humans (Pozio, 2001b). Trichinellosis has occurred in all parts of Thailand but is more abundant in the northern areas where 11.4% of hill tribe pigs and 0.02% of the commercial pigs are positive (Takahashi et al., 2000). The source of infection has acquired through consumption of local dishes called labh and nham. The source of human infection in the country stems mainly from the hill tribe pigs’, jackals’, black bears’ and rats’ meat (Suriyanon and Khunklin, 1972). An examination of a dog’s diaphragm in the Sakon Nakorn province where dog meat is used for human consumption has revealed that 1.6% (n = 421) of the dogs were infected with trichinellosis (Srikitjakarn et al., 1981). It was reported that in-between 1962-1991 there were about 118 outbreaks in Thailand which infected 5400 people and out of that 95 have died (Khamboonruang, 1991). In Indonesia, Laos, Malaysia and Myanmar, the *Trichinella* infection was also reported in domestic animals and/or humans, referred by Pozio et al.(2001b). A sero-prevalence of 1.4% was reported in Cambodia (Sovyra, 2005). Chomel et al. (1993) have reported a high
sero-positive rate (19.5%) of trichinellosis among children and teenagers in Bali, Indonesia. Recently, an outbreak investigation has confirmed 22 trichinellosis cases through western blot in Laos (Sayasone et al., 2006).

In China, the greatest spreading of the infection was documented from Hubai, Henan, Yunnan, and the Guangxi provinces with two epidemiological cycles, *Trichinella spiralis* in pigs and *Trichinella nativa* in dogs (Liu and Boireau, 2002). In these provinces 5% prevalence were reported in pigs (Cui et al., 2006) whereas in the northern, southern and coastal regions 7.5% prevalence were found (Chan and Ko, 1992). The prevalence of *Trichinella* is 1.2% (2/163) in cattle, 1.4% (3/215) in sheep, 2.1% (1/47) in beef cattle and 16.2% (5654/34983) in dog (Wang et al., 2007). This infection in herbivorous is probably associated with ingesting either hay or forage mixed with raw swill containing pork scraps or grazing in contaminated pastures with infected rodent carcasses. The highest number of human cases (with a mortality of 0.95%) was reported in China (Takahashi et al., 2000). In-between 1964-1999 there were 548 outbreaks of human Trichinellosis. 525 of them were associated with eating pork and the remaining 23 outbreaks were related to the consumption of goat, dog and game meat (Wang et al., 2007). In that period 25 161 cases were reported with 240 deaths (Lui and Boireau, 2002). The occurrences of human trichinellosis (1992-1996) in the Henan province have shown that the peak of the infection rate is found in winter; a decrease in spring and a lower number in summer and autumn (Wang et al., 1998). In-between 2000-2003 there were 17 outbreaks of human trichinellosis with 828 cases and 11 deaths reported (Wang et al., 2006).

For the Indian subcontinent there are only some very few old reports that describe the presence of *Trichinella* in synanthropic and sylvatic animals (Murrell and Pozio, 2000; Mohan et al., 2002). The first human case in India has been reported, recently (Mohan et al., 2002; Handa, 2003). In Nepal, however, no human case has been documented yet. A study conducted in Hong Kong on the sera of 18 Nepalese soldiers with clinical manifestations of acute trichinellosis has found 94% positive from IHA, RIA and ELISA and 56% positive by muscle biopsies. That has revealed the presence of the infection among the population (Au et al., 1983).
2.11 Veterinary public health regulatory measures on trichinellosis control

*Trichinella* inspection for every slaughtered pig is mandatory in many of the EU member countries whereas in some countries it is required only for pork trading to other member countries (Nöckler *et al.*, 2000). The EU is currently preparing a legislation to set aside the testing on *Trichinella* free farms and/or areas. A similar intent has also emerged in North America (Oivannen, 2005). In the USA meat inspection for *Trichinella* is not mandatory. Other methods for controlling infections in animals and humans are used like the consumers are advised to cook or freeze pork properly at home (Bruschi and Murrell, 2002) and good farm management practices including rodent control and avoidance of feeding waste to pigs are implemented at the farm level (Gajadhar and Gamble, 2000). However, the increasing interest in bio-organic pig farming may bring drawbacks and such an interest needs to be tackled with new aspects of control for ceasing *Trichinella* infection (Pozio, 2001a; Oivannen, 2005).

The quality assurance requirements in the laboratory analysis will eventually impact *Trichinella* diagnostics at meat inspection worldwide. The methods that have used in each laboratory must be validated (Gamble *et al.*, 2000), so that the test results generated from the laboratory are reliable and scientifically defensible according to the defined parameters of the tests (Gajadhar and Forbes, 2002). In Canada and in some other countries, an obligatory proficiency testing of laboratories is already in place (Gajadhar and Forbes, 2002; Pozio and Christensson, 2004). In the areas where irregular outbreaks of trichinellosis are reported either as small family outbreaks or large-scale urban type outbreaks it is obvious that continued meat inspection at the abattoir is necessary (van Knapen, 2000). In such endemic areas an individual carcass should be examined by a method with high sensitivity (Pepsin digestion method) but not with serological testing (van Knapen, 2000).

A decade ago the Chinese food hygiene regulation (FHR) had tolerated that carcasses with light infection could be consumed after high temperature treatment (Lui and Boireau, 2002). After 1996 a new FHR has implemented. It is recommended that the infected carcass must be destroyed even if there is only one larva found in
carcass. To increase sensitivity the pooled sample digestion technique was implemented in slaughterhouses (Lui and Boireau, 2002). In Nepal there is a lack of reliable epidemiological information on zoonotic diseases of public health importance (Gongal, 2003). However, in 2001 the slaughterhouse and meat inspection regulation was passed by the government (FAOLEX, 2001) but it has not been implemented yet (Gongal, 2003). There is an urgent need for harmonizing the national food safety standard with the international standards and a sanitary phyto-sanitary measure.

In addition to improved control methods there is a need to accurately record and communicate information regarding the occurrence of *Trichinella* in man and animals (Kim, 1991; Dupouy-Camet, 2000). The computerized database for recording animal and human occurrences is integral for assessing the risk of trichinellosis in food animals from around the world (Polley *et al.*, 2000). To accomplish control in all parts of the world and to overcome problems associated with global commerce it is necessary for international organizations like ITC, OIE, FAO to play key roles in standardizing, accrediting and ensuring equivalency of protocols for the global control of *Trichinella* infection (Gajadhar and Gamble, 2000).
2 MATERIAL AND METHODS

3.1 Study location

In this study the samples were collected from butcheries of seven different districts. Five districts (the Kathmandu valley, Kavre, Dhading, Rauthat and Chitwan) belong to the CDR and each one of the remaining belongs to the western and far-western region of Nepal, as illustrated in figure 2. These districts have a ≥ 10 000 pig population and are the main pig producing areas. The Kathmandu valley consists of the Kathmandu metropolitan, Lalitpur sub-metropolitan and Bhakatpur municipality which have a total area of 600 sq. km. The valley is located at 1300 metres altitude and is surrounded by mountains ranging from 1500-2800 metres.

The Kavre district is close to the Kathmandu valley and is a passageway to China. In the North, Kavre borders on Sindupalchowk and China (Tibet). About 80% of the Kavre area is hilly and mountainous and people reside up to 3018 metres. Dhading is one of the least developed hill districts in central Nepal with an altitude varying from 430 to 7409 metres. It is situated west of Kathmandu and is closer in terms of distance. But a large part of the district is very remote to access. Rauthat and Chitwan have low plain areas and these districts are situated between middle hills and the Siwalik mountains. The study area in these districts ranges from 124-244 metres and borders in the south on India.

The Kaski district belongs to the western region and is located 200 km west from Kathmandu. It has one sub-metropolitan city, Pokhara, which has a sharp rise in the altitude and the highest precipitation rate (>4000 mm/year). The samples were collected from that area. The samples were also collected from the Dadeldhura district of the far-western region, which is the most remote and developmentally challenged district of Nepal. It borders on India and is the end part of Nepal in west.
Figure 2: Map of Nepal (study areas are marked)

(Courtesy: WWF, Nepal)
3.2 Butcheries

Most of the slaughtering is practiced in the country either in small butcheries, street sides, riversides, open pasturelands or country yards in urban as well as in rural areas. The butcheries usually dispose their wastes on streets, in the municipality drainage system or at the riverside. Only some negligible personnel of butcheries had used solid waste disposable containers. The slaughtering of pigs was performed without hygienic control because of unsatisfactory slaughtering procedures and infrastructures. Animals were dragged on their way to slaughter and were allowed direct contact and seeing other animals being slaughtered. Butcheries had mostly slaughtered pigs without stunning and perfect bleeding and almost all have floor-dressing practice with limited access of water. Although, the Kathmandu valley is the biggest market for pork consumption it does not exist any slaughterhouse for pigs and the butcheries’ hygienic states are below the standard. However, in Talchikhel (Patan) of the Kathmandu valley there are four butcheries constructed by the Third Livestock Development Project of the Ministry of Agriculture and Co-operatives, which produce comparatively hygienic meat. But the slaughtering is very minimal around 5-6 pigs/day /butchery.

The capacity of pigs slaughtered in Chitwan in a syndicate system ranges between 7-10 pigs/day. Most butchers prefer to slaughter specific breeds. The slaughter slabs of Kathmandu prefer Pakharibas black cross, the butcheries of the plain territory prefer exotic and local breeds of pig. Most of the butcheries have their own shop for the retailing. The selling of porks there takes place under hygienic conditions. A total of 38 butcheries from different districts were included in this study. In the Kathmandu valley 12 butcheries were visited for the sample collection from once to eleven times. In other districts samples were collected from 3-6 butcheries/district from once to nine times. The sites of the Kathmandu valley where samples were collected are shown in figure 3.
Figure 3: Map of the Kathmandu valley showing the twelve butcheries sites where samples were collected.
3.3 Study design and study population

This study was based on a cross-sectional observational study design. It was carried out at the butcheries, which were intended for pigs slaughtering. They were located in the central development region and in two outside districts of Nepal. The sampling unit was the individual pig. The study was carried out from November 2006 to April 2007.

The study population was pigs (local, exotic, cross breed, wild), which were brought to butcheries in that districts for the purpose of slaughter. The demographic factors like age, sex, husbandry practices and rearing system were recorded for individual pig. Muscle samples and/or serum samples were collected for Trichinella investigation. Those pigs were reared either commercially, semi-commercially, scavenging or household way.

3.4 Sample size determination

Sapkota et al. (2006) has reported 1% seroprevalence of Trichinella in slaughtered pigs of the Kathmandu valley, Nepal. ELISA used for such a study has a diagnostic sensitivity (Se) of 72.9% and a specificity (Sp) of 99.6% (Nöckler et al., 2004). So the true prevalence calculation using Se and Sp (Salman, 2003)

\[
P_{tr} = \frac{P + Sp - 1}{Se + Sp - 1}
\]

\[= 1.37\%
\]

The adjusted sample size was calculated by using formula based on win Episcope 2.0 (EPIDECON),

\[n = \left(\frac{t \times SD}{L}\right)^2\]

Where, \(t = \) Student’s t-value \(\approx 1.96\) when the desired level of confidence is 95%

\[SD = \text{Standard deviation} = \sqrt{P \times (1-P)}\]

\[= 0.116242\]

\[L = \text{Accepted absolute error} = 1\%\]

The adjusted sample size was 520 pigs. By this sample the results could estimate a percentage at 95% CI with 1% of allowable error.
3.5 Definition of samples and sampling strategies

A total of 576 pigs were investigated in the different districts. From each of the slaughtered pigs 25-30 g of diaphragmatic crus muscle together with 10 ml blood by heart puncture was taken. Meat materials were stored in cooling boxes and blood samples were centrifuged after standing overnight at room temperature for separating serum at 3000 rotations per minute for 10 minutes. The criteria like age, sex, breed, origin, farm status and rearing system of the investigated pigs were recorded.

The sample collection and the questionnaire survey were overall strategies for this study. The sample allocations to each district were calculated according to the probability proportional sampling. The deficient samples of the Dhading district were fulfilled from the Kaski district of western and Dadeldhura district of the far-western region. The butcheries were selected by convenient sampling, whereas the slaughter pigs carcass were selected by simple random sampling with sampling fraction of 0.33.

2.6 Laboratory analysis

Batches of 10 pooled muscle samples (5 g each) were digested using the Pepsin digestion method of the Federal Institute for Risk Assessment (BfR) Berlin, Germany. Later on a single probe was investigated by Trichinoscopy with the compressorium to identify the infected animal. The serum samples were marked as positive or negative serum compared with the results of the Pepsin digestion and were stored at –20°C. Later on 68% of all collected sera were selected randomly for further analysis. Serum samples were investigated by ELISA using ES-Trichinella larval antigen, according to the standard of the operating procedures of the National Reference Laboratory for Trichinellosis, BfR, Germany. With the help of the indirect ELISA it was possible to test serum samples for the specific anti-Trichinella-IgG in a relatively short time (about 3 to 4 hours). The confirmatory diagnosis in the serum by the western blot technique was done in a reference laboratory at BfR. The larvae was collected from the positive pigs (if any) by Pepsin digestion method and stored in 60% ethanol at –20°C. The larval materials (if any) that were separated by the
digestion method could be used for species differentiation through the Multiplex PCR techniques (Zarlenga et al., 1999). The overall laboratory diagnosis of *Trichinella* spp. is shown in the flow diagram in appendix A.

3.6.1 Artificial digestion method

A total of 50 g minced muscle sample (each 10 g or 5 g per pig) was digested using an artificial digestive fluid consisting of 1 litre of tap water (44-46°C), 8 ml of 25% HCL and 5 g of Pepsin (30 000 IE/g). The digest was stirred for 30 minutes at a temperature of 44-46°C in a two-litre glass beaker using a hot plate magnetic stirrer. During the process the larvae were released from the muscle. The digestion fluid was then poured through a metallic sieve (0.18 mm) into the glass funnel closed by a rubber hose with a clamp. The larvae were allowed to settle for 30 minutes and then 40 ml of sample fluid were quickly released into the 50 ml tube. After further 10 minutes it allowed sedimentation to clarify the suspension. Then 30 ml of supernatant was drawn off and the remaining 10 ml of sediment were poured into the petri dish. Then the sample in the petri dish was examined for at least 8 minutes by a stereomicroscope (15-40x magnification) for the visualization of *Trichinella* larvae. In the case of a positive result the batches were subdivided in 5 samples each and the procedure had to be repeated to determine which sample was positive.

3.6.2 Compressorium technique

The muscle samples were cut into 28 pieces each along the muscle fibers about lentil like in sizes. Then the muscles were compressed between two glass plates (compressorium) until they become translucent. After that they were examined, individually, for *Trichinella* larvae, using a stereomicroscope at 15-40x magnifications.

The larvae visualization for encapsulated and non-encapsulated *Trichinella* spp. has a different morphology. It also varies in shape based on the technique applied, as shown in figure 4.
Figure 4: Microscopic illustrations of *Trichinella* spp. larvae in meat samples by different techniques (courtesy: Dr. Karsten Nöckler, BfR. Personnel communication)
3.6.3 Indirect, non-competitive enzyme linked immunosorbent assay (AB-ELISA)

All the test kits were supplied on request from the Bundesinstitut für Risikobewertung (Dr. Karsten Nöckler). The detailed protocol developed by the BfR for the serological examination of field sera for anti-*Trichinella*-IgG in pig is as follows:

The ELISA-Kit consisted of

1. Microtitre plates coated with *Trichinella* antigen (excretory-secretory antigen of *Trichinella spiralis*); 80 μl *Trichinella*–ES-antigen per well, storage at 4-8°C
2. *Trichinella*-positive control serum (1 ml, lyophilized), storage at –20°C
3. *Trichinella*-negative control serum (0.5 ml, lyophilized), storage at –20°C
4. PBS buffer (Phosphate Buffered Saline) (to be prepared according to the protocol)
5. Anti-pig IgG-peroxidase conjugate pre-diluted 1:10, (1.0 ml), storage at –20°C
   (SIGMA, product No. A5670)
6. ABTS {2,2’-azino-his-(3-ethylbenzothiazoline sulfonate)} buffer, dry matter from Boehringer, storage at 4-8°C
7. Tablets chromogen ABTS, storage at 4-8°C.

Test procedure for ELISA

1. The BfR has supplied already mixed reagents and only Tween 20 was needed to add for the preparation of PBS-T.

2000 ml of PBS–Tween 20 (pH = 7.2-7.4) consists of

- KH₂PO₄: 0.4 g
- Na₂HPO₄ * 12 H₂O: 5.8 g
- NaCl: 16.0 g
- KCl: 0.4 g
- Tween 20: 1.0 ml
- distilled water: ad 2000 ml

2. Washing (blocking) of the microtiter plate 1 time with aqua dest and 3 times with 150 μl PBS–T (each for 3 min).
3. Preparation of test and control sera diluted in PBS-T (1:100)

1 ml (1000 μl) PBS-T was placed in eppendorf vials and in that 10 μl of the test serum samples or control (positive, negative) sera were added, separately. These were mixed, properly, so the diluted test and control is in 1:100 with PBS-Tween 20. Then 50 μl of the diluted control sera were placed in the control column and 50 μl of the diluted test sera in 86 wells and the remaining two had only PBS-T as blank in the microtitre plate, as shown in example.

Example of a coated micro titer plate with serum samples

<table>
<thead>
<tr>
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<th>1</th>
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<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>A</td>
<td>Nc1</td>
<td>FS1</td>
<td>FS5</td>
<td>FS9</td>
<td>FS13</td>
<td>FS17</td>
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<tr>
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<td>FS1</td>
<td>FS5</td>
<td>FS9</td>
<td>FS13</td>
<td>FS17</td>
<td>FS21</td>
<td>FS25</td>
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<td>FS33</td>
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<tr>
<td>C</td>
<td>Nc1</td>
<td>FS2</td>
<td>FS6</td>
<td>FS10</td>
<td>FS14</td>
<td>FS18</td>
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<td>FS26</td>
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<td>FS28</td>
<td>FS32</td>
<td>FS36</td>
<td>FS40</td>
<td>PBST</td>
</tr>
</tbody>
</table>

Nc1  =  negative control serum  
Pc1  =  positive control serum  
FS_  =  samples of field sera  
PBST = only coated with PBS-Tween 20 (blanks of micro titer plate)

4. Incubate for 30 minute at 37°C. Then the micro titer plate was washed off 1 time with aqua dest and 3 times with 150 μl PBS–T (each for 3 minute).

5. The anti-pig IgG–Peroxidase-conjugate was pre-diluted 1:10 and it was converted into the final working solution which was at 1:1200. This was done through putting 10 μl conjugate and 12 ml PBS-T in conical flask and then a 50 μl amount was added to all wells.
6. Incubation for 30 minute at 37°C, then washing and soaking 1 time with aqua dest and 3 times with 150 μl PBS–T (each for 3 minute) and finally 1 time with aqua dest.

7. Preparation of ABTS buffer

Separate dilution of prepared citric phosphate buffer (pH = 3.4-3.6)

ABTS buffer: dry matter 1.67 g
Distilled water ad 100 ml

Dissolving of 2 tablets ABTS (100 mg) in 100 ml of prepared ABTS buffer, adding of 50 μl of freshly prepared ABTS (substrate indicator system) to all wells.
Store the chromogen at 4–8°C in the dark (storage is possible for a couple of weeks).

8. Measurement of the extinction of all wells with the reader at 405 nm if the positive control serum has an extinction value (OD) of 1.300-1.400. To reach this OD value an incubation period of about 20-40 minutes at room temperature is needed.

Calculation and evaluation of test results

The results are calculated according to the ‘reference standard methods’, i.e. OD values of samples which are related to those of the positive control in % as ELISA-index in the following way:

1. Calculation of netto extinction (NE) of each well
   \[ \text{NE} = \text{OD} - \text{OD}_{\text{blank}} \]

2. Calculation of mean netto extinction (mNE) of positive and negative control and samples

3. Calculation of ELISA-index. The mean extinction of the sample (mNE_{sample}) is related to the mean extinction of the positive control (mNE_{pos}). The positive control has an ELISA-index of 100%.
   \[ \text{ELISA-index (\%)} = \frac{\text{mNE}_{\text{sample}}}{\text{mNE}_{\text{pos}}} \times 100\% \]

4. Evaluation of test results
   
   “Trichinella- positive” (+)  \quad \text{ELISA-index (\%)} \geq 18
   
   “Trichinella-negative” (-)  \quad \text{ELISA-index (\%)} < 12
   
   “Trichinella-questionable” (?)  \quad 12 \leq \text{ELISA-index (\%)} < 18
3.6.4 End-point titration by the single dilution AB-ELISA

The positive and the questionable samples were examined using the end-point titration test for the confirmation according to the procedure described by Nöckler et al. (1995).

The ELISA plate of the test kit is washed with PBS-T buffer and blot dry. In the first 4 wells of first column 10 μl of negative control (diluted in PBS-T 1:100) are added and in the remaining 4 wells the positive control is added with the same dilution and amount. The positive and doubtful samples found in the screening test are diluted as 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280 in PBS-T. Dilution steps are made as follows.

In the first row of the ELISA plate except the first well, mix 10 μl of sample and 90 μl of PBS-T and in wells of the remaining rows except the first put 50 μl of PBS-T in each. Then mix sample and PBS-T thoroughly in the first row and draw out 50 μl from there and put it into the second row, mix it well in the second row and again draw 50 μl from there and put it into the third row. In the same way proceed up to the last row and discard 50 μl solutions. Incubate the plate for half an hour at 37°C, take it out of the incubator and wash it by buffer three times and blot dry. Add the conjugate and incubate again for half an hour at 37°C. Wash and soak once with aqua dest and 3 times with 150 μl PBS–T (each for 3 minute) and finally once with aqua dest. Then add ABTS and read.

Evaluation of test results

<table>
<thead>
<tr>
<th>“End-point titration- positive” (+)</th>
<th>ELISA-index (%) ≥ 70 (1:10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>and border of titer ≥ 1:80</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>“End-point titration- negative” (-)</th>
<th>ELISA-index (%) &lt; 40 (1:10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>and border of titer &lt; 1:80</td>
</tr>
</tbody>
</table>
3.6.5 Western blot technique

The samples that were doubtful and positive through the AB-ELISA were sent to the National Reference Laboratory for Trichinellosis (the Bundesinstitut für Risikobewertung) Berlin, Germany for a confirmatory by the western blot. Western blotting was performed according to the standard methods (OE Mikrobiologie, reference number LA 163-1/BfR). This technique is genus specific.

<table>
<thead>
<tr>
<th>Specific band</th>
<th>Molecular weight (kDa)</th>
<th>Range of kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>43.0</td>
<td>42.7-43.2</td>
</tr>
<tr>
<td>II</td>
<td>46.9</td>
<td>46.7-47.2</td>
</tr>
<tr>
<td>III</td>
<td>62.0</td>
<td>61.5-62.5</td>
</tr>
<tr>
<td>IV</td>
<td>66.1</td>
<td>65.4-66.9</td>
</tr>
<tr>
<td>V</td>
<td>102.0</td>
<td>101.5-102.4</td>
</tr>
</tbody>
</table>

Evaluation of test result
Positive: Test serum shows at least two types of specific band.

3.6.6 Multiplex PCR

Prior to the molecular analysis, the larvae should be rehydrated in a decreasing series of ethanol. Larval DNA for species detection would be isolated from single or pooled samples of larvae. Based on the observation of the variation in the expansion segment V (ESV) region in ribosomal DNA, a Multiplex PCR should develop. It is capable of distinguishing eight Trichinella species or genotypes. By combining five primers in a single PCR reaction each genotype can be recognized by a specific amplification profile.

2.7 Questionnaire survey

The questionnaire was surveyed among 40 pig farm owners. These farms were located in the studied districts and their selection was based on convenient sampling.
These farms may or may not supply their pigs for slaughter to the butcheries which were selected in this study design. The survey was conducted, sporadically, based on the accessibility from January 2007 to March 2007. The questionnaire as shown in appendix B reveals minimum standards for hygiene at the farm level based on the pre-determined risk factors for *Trichinella* control. It was administered to the owners of the farms and an interview was done face to face. These questionnaires were administered for gathering information regarding the farm management practices which exist in the area and to assess the precautionary measures at the farmers’ level.

### 3.8 Data management and analysis

The spreadsheet of data entry for an individual sample was done and then the statistical analyses were performed using Microsoft Excel, the statistical package for social sciences (SPSS) 11.0 Windows and win Episcope 2.0 (EPIDECON). The prevalence was estimated as the number of infected and/or exposed individuals from the total was analyzed. The ELISA indices were calculated according to the protocol of the BfR. The slaughtered pig status and the questionnaires survey data were processed through SPSS. The reproducibility of the test was assessed by coefficient of variation based on Mahannop *et al.* (1995). The possible maximum prevalence was calculated based on the formula of the win Episcope program manual.
4. RESULTS

4.1 Characteristics of the slaughtered pigs

A total of 576 pigs were selected. Meat and serum samples were collected from 487 of those pigs whereas from 64 of those pigs only meat and from 25 of those pigs only blood samples were collected. The highest number of samples was collected from the Kathmandu valley (184), followed by Rauthat (98), Chitwan (92), Kavre (85), Pokhara (42) Dhading (40), and far-western (35). Based on the geographical location, 45.31% (261/576) were collected from the mountain region (Kathmandu, Pokhara and far-western), whereas 32.98% (190/576) were collected from the plain region (Rauthat and Chitwan) and 21.70% (125/576) from the hilly region (Kavre and Dhading) of Nepal.

Pigs selected for this study had characteristics of 4 different states of the farms: 26% of the pigs were from commercial farms, 17.2% from semi-commercial farms, 37.2% from scavenging and 19.6% from household raised pigs. The pigs from all three types i.e. indoor (44.4%), outdoor (37.8%) and mixed (17.7%) system of rearing were recorded. The breed characteristics of the sampled pigs were local 56.9% (Hurra, Bampudake, Wild), exotic 26.6% (Landrace, Yorkshire, Hampshire) and Pakharibas cross 16.5% (Dharane kalo). Regarding gender the pigs were 59% males and 41% females. Regarding the age, 37.8% samples were from pigs below 1 year of age, 56.1% pigs were between 1-2 years of age and 6.1% pigs were more than 2 years of age. It was found that both immature (<5 month) and senile (>3 years) pigs were slaughtered.

4.2 Trichinella investigation through Pepsin digestion

The meat samples from a total of 551 pigs were analyzed through the Pepsin digestion technique. Out of that 90 individual meat samples were also analyzed
through compressorium. *Trichinella* larvae were not found in any of those pork samples.

4.3 *Trichinella* investigation through indirect ELISA serology

A total of 344 randomly selected sera were tested for antibodies against *Trichinella spiralis*. Out of 344 sera, only 320 sera had corresponding meat samples that were already analyzed through the Pepsin digestion which was negative. All sera were tested by AB-ELISA. The ELISA OD indices of the 344 sera were shown graphically in appendix C. It was found that 14 samples were doubtful (12 ≤ ELISA-index <18) and 2 samples were positive (≥ 18 ELISA index). The detailed information regarding such doubtful and positive sera is presented in table 2. The clusture column comparing ELISA OD values with ELISA indices (appendix C), it was found that ELISA OD value of serum samples had positive correlation with ELISA index. The trend of the serum samples showed that if ELISA OD value of serum sample was more than 0.23 then the ELISA index can be expected arithmatically more than 12 which means either it was doubtful or positive through AB-ELISA.

These doubtful and positive samples were tested again for end-point titer single dilution AB-ELISA; from that all samples had ELISA index less than 70%, and border of titre less than 1:80. Based on the test evaluation criteria all serum samples failed to show antibodies against *Trichinella spiralis*, as summarized in table 3.

The precision of the ELISA-test was determined by testing at different times the same positive or negative control sera. The percentage of the coefficient of variation (%CV) was calculated which was used for assessing the reproducibility of the test according to Mahannop *et al.* (1995). The result showed that coefficient of variation of method increased with concentration of the sample. It means that if the test sera have higher concentration of the antibodies against *Trichinella spiralis* then the variation is higher in result output. The reproducibility of positive and negative control sera used for AB-ELISA test is shown in table 4.
Table 2: Results of the AB-ELISA of serum samples from pigs of different regions, breeds and management systems of Nepal

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Site of collection</th>
<th>Status of pig</th>
<th>ELISA index (%)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>82</td>
<td>Kavre</td>
<td>Black Cross</td>
<td>3 Year Female</td>
<td>14.05</td>
</tr>
<tr>
<td>92</td>
<td>Kathmandu</td>
<td>Local</td>
<td>2 Year Male</td>
<td>16.84</td>
</tr>
<tr>
<td>178</td>
<td>Chitwan</td>
<td>Exotic</td>
<td>1 Year Male</td>
<td>19.43</td>
</tr>
<tr>
<td>191</td>
<td>Chitwan</td>
<td>Exotic</td>
<td>3 Year Female</td>
<td>13.44</td>
</tr>
<tr>
<td>202</td>
<td>Chitwan</td>
<td>Exotic</td>
<td>16 month Female</td>
<td>13.79</td>
</tr>
<tr>
<td>213</td>
<td>Rauthat</td>
<td>Local</td>
<td>14 month Female</td>
<td>17.75</td>
</tr>
<tr>
<td>264</td>
<td>Kathmandu</td>
<td>Exotic</td>
<td>1 Year Female</td>
<td>15.60</td>
</tr>
<tr>
<td>269</td>
<td>Kathmandu</td>
<td>Black Cross</td>
<td>18 month Female</td>
<td>17.15</td>
</tr>
<tr>
<td>376</td>
<td>Kavre</td>
<td>Black Cross</td>
<td>3 Year Female</td>
<td>13.96</td>
</tr>
<tr>
<td>378</td>
<td>Kathmandu</td>
<td>Local</td>
<td>11 month Female</td>
<td>12.58</td>
</tr>
<tr>
<td>443</td>
<td>Pokhara</td>
<td>Local</td>
<td>3 Year Female</td>
<td>15.34</td>
</tr>
<tr>
<td>535</td>
<td>Far-western</td>
<td>Local</td>
<td>5 month Male</td>
<td>17.13</td>
</tr>
<tr>
<td>540</td>
<td>Far-western</td>
<td>Local</td>
<td>1 Year Male</td>
<td>17.20</td>
</tr>
<tr>
<td>542</td>
<td>Far-western</td>
<td>Local</td>
<td>1 Year Male</td>
<td>12.58</td>
</tr>
<tr>
<td>551</td>
<td>Far-western</td>
<td>Local</td>
<td>5 month Female</td>
<td>12.50</td>
</tr>
<tr>
<td>565</td>
<td>Far-western</td>
<td>Local</td>
<td>2 year Male</td>
<td>18.78</td>
</tr>
</tbody>
</table>

Semi-comm = Semi-commercial
Table 3: ELISA indices and titration observations of serum samples from pigs of different regions, breeds and management systems of Nepal that were positive and doubtful through AB-ELISA

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>IgG 1:10 ELISA %</th>
<th>Border of titer</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>82</td>
<td>19.71</td>
<td>1:10</td>
<td>Negative</td>
</tr>
<tr>
<td>92</td>
<td>21.10</td>
<td>1:10</td>
<td>Negative</td>
</tr>
<tr>
<td>178</td>
<td>34.61</td>
<td>1:20</td>
<td>Negative</td>
</tr>
<tr>
<td>191</td>
<td>27.17</td>
<td>1:10</td>
<td>Negative</td>
</tr>
<tr>
<td>202</td>
<td>22.34</td>
<td>1:10</td>
<td>Negative</td>
</tr>
<tr>
<td>213</td>
<td>24.11</td>
<td>1:10</td>
<td>Negative</td>
</tr>
<tr>
<td>264</td>
<td>49.97</td>
<td>1:40</td>
<td>Negative</td>
</tr>
<tr>
<td>269</td>
<td>48.15</td>
<td>1:40</td>
<td>Negative</td>
</tr>
<tr>
<td>376</td>
<td>39.26</td>
<td>1:40</td>
<td>Negative</td>
</tr>
<tr>
<td>378</td>
<td>32.59</td>
<td>1:20</td>
<td>Negative</td>
</tr>
<tr>
<td>443</td>
<td>54.57</td>
<td>1:40</td>
<td>Negative</td>
</tr>
<tr>
<td>535</td>
<td>34.02</td>
<td>1:20</td>
<td>Negative</td>
</tr>
<tr>
<td>540</td>
<td>46.64</td>
<td>1:40</td>
<td>Negative</td>
</tr>
<tr>
<td>542</td>
<td>21.96</td>
<td>1:10</td>
<td>Negative</td>
</tr>
<tr>
<td>551</td>
<td>35.45</td>
<td>1:20</td>
<td>Negative</td>
</tr>
<tr>
<td>565</td>
<td>47.19</td>
<td>1:40</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Table 4: The reproducibility test of the *Trichinella* of control sera used for AB-ELISA

<table>
<thead>
<tr>
<th>Control sera</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
<th>Mean (x)</th>
<th>S.D</th>
<th>%CV = (S.D/x)*100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>1.127</td>
<td>2.203</td>
<td>1.253</td>
<td>1.537</td>
<td>1.921</td>
<td>1.6145</td>
<td>0.477066</td>
<td>29.54%</td>
</tr>
<tr>
<td></td>
<td>0.945</td>
<td>2.295</td>
<td>1.255</td>
<td>1.415</td>
<td>2.074</td>
<td>2.026</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.942</td>
<td>2.214</td>
<td>1.202</td>
<td>1.449</td>
<td>1.974</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>0.106</td>
<td>0.103</td>
<td>0.115</td>
<td>0.101</td>
<td>0.072</td>
<td>0.09736</td>
<td>0.014143</td>
<td>14.52%</td>
</tr>
<tr>
<td></td>
<td>0.096</td>
<td>0.096</td>
<td>0.087</td>
<td>0.083</td>
<td>0.117</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.109</td>
<td>0.111</td>
<td>0.083</td>
<td>0.084</td>
<td>0.124</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.091</td>
<td>0.103</td>
<td>0.086</td>
<td>0.083</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S.D = Standard deviation

4.4 *Trichinella* investigation through western blot

16 serum samples were sent to the BfR for confirmatory diagnosis of trichinellosis. The molecular sizes of the bands were evaluated by a comparison with a molecular size ladder. The ladder used for comparison had well-recognized molecular weight patterns of 25, 37, 50, 75, 100, 150 and 250 kDa. Sera from animals with parasitic infections other than trichinellosis would produce very different patterns of molecular weight. The tested sera had not shown specific band on its ladder, as shown in figure 5.

The comprehensive details of each sample specific and non-specific bands patterns were summarized in appendix D. Based on the evaluation criteria, it had found that none of the sample had shown two types of specific bands on its ladder. This means all the tested sera samples were negative for *Trichinella* genotype through western blot. Thus it was concluded that all serum samples which were doubtful and positive through AB-ELISA were actually true negative samples.
PC = Positive control
NC = Negative control
kDa = Molecular weight of ladder in kilodalton

Figure 5: Western blot analyses of sera from pigs that were positive and doubtful through AB-ELISA
4.5 Possible maximum prevalence of *Trichinella*

It was found that from the all tested meat samples of pig that there was no positive result for *Trichinella* by the Pepsin digestion method. However firstly through AB-ELISA of randomly selected sera, 2 samples were positive and 14 were doubtful, but the confirmation of these samples through end-point titration single dilution ELISA and western blot had revealed that all samples were true negative for *Trichinella* spp. In that aspect the following mentioned formula was used based on win Episcope 2.0 to estimate the maximum number of possible positive animals in the central development region of Nepal.

\[
D = \left[1 - (1 - CL)^{1/n}\right] \times \left[N - \frac{n-1}{2}\right]
\]

Where,
- \(D\) = The maximum number of *Trichinella* positive animals
- \(CL\) = Confidence limit as a fraction
- \(n\) = Samples size that showed negative
- \(N\) = Total number of pigs in central development region

\[
D = \left[1 - (1 - 0.95)^{1/576}\right] \times \left[172949 - \frac{576-1}{2}\right]
\]

= 896 Pigs

Possible maximum prevalence = \(\frac{896}{172949}\) * 100 = 0.52% at 95% confidence interval and with sampling fraction of 0.33%.

4.6 Pig producer questionnaire survey

52.5% of the respondents had a farm in the urban area and were under 40 years of age. Most of the farms (60%) have no employee *i.e.* the owner and his/her family are involved in the pig husbandry. The surveyed farmers have low pig holding capacity (62.5%) and more precisely 77.5%, 97.5% and 52.5% have kept only 1-20 sows, boars and piglets, respectively. This questionnaire surveys had found that at the farms the *Trichinella* control measures were very negligible, as shown in table 5.
Table 5: Status of pig farms with hygienic measures for *Trichinella* control in Nepal (n = 40)

<table>
<thead>
<tr>
<th>No.</th>
<th>Factors to be considered</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rodent control program exists in the farm</td>
<td>12</td>
<td>30%</td>
</tr>
<tr>
<td>2</td>
<td>Architectural barrier in the farm for wild life</td>
<td>29</td>
<td>72.5%</td>
</tr>
<tr>
<td>3</td>
<td>Feed containing leftovers (hotel, home)</td>
<td>26</td>
<td>65%</td>
</tr>
<tr>
<td>4</td>
<td>Waste food containing meat products was cooked to inactivate <em>Trichinella</em></td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>5</td>
<td>Garbage dumping in the vicinity</td>
<td>33</td>
<td>82.5%</td>
</tr>
<tr>
<td>6</td>
<td>Bird access from dumping area to the farm</td>
<td>17</td>
<td>42.5%</td>
</tr>
<tr>
<td>7</td>
<td>Direct outlet of farm wastes due to lack of facility</td>
<td>33</td>
<td>82.5%</td>
</tr>
<tr>
<td>8</td>
<td>Information about pork diseases</td>
<td>18</td>
<td>45%</td>
</tr>
<tr>
<td>9</td>
<td>Awareness regarding <em>Trichinella</em> control</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>
5. DISCUSSIONS AND CONCLUSION

5.1 Discussions

This study was the first study comparing the methods for the detection of 
*Trichinella* larvae in diaphragm muscles and specific antibodies in blood sera of pigs in the central development region of Nepal. The pig farm’s management practices in the central development region were investigated with particular relevance to the transmission of trichinellosis. The Pepsin digestion method had failed to detect *Trichinella* larvae from meat samples although 5-10 g muscle per pig was used. However firstly through antibody ELISA of randomly selected 344 sera, 2 samples were positive and 14 were doubtful. The confirmation of these samples through end-point titration single dilution ELISA at central laboratory, Faculty of Veterinary Medicine (CMU) had failed to detect antibodies against *Trichinella spiralis*. Further confirmation of these 16 sera at the Bundesinstitut für Risikobewertung, Berlin through end-point single dilution ELISA and the western blot had also revealed that all tested serum samples were true negative for *Trichinella* genotype. This could be due to the testing and sampling process or it may be indicative of a very low prevalence of *Trichinella* spp. in surveyed areas of Nepal.

The total pork production in the central development region in 2005/2006 was 3428 metric tons. This amount was supplied 6% from the mountain districts, 31% from the hill districts and 63% from the plain districts (ABPSD, 2006). From the districts where more than 10 000 pigs were raised, the necessary amount of pigs was selected through probability proportional sampling. Through these approach different geographical areas which was the major pig producing areas of the central development region were included in the study. The sampling strategies and the collection were sufficient enough at 1% allowable error to an estimate percentage at 95% confidence interval. Therefore this study design was reliable to represent the prevalence of slaughtered pigs in the CDR.
The Pepsin digestion test has a detection limit of 1-3 larvae/g according to the directive 77/96/EEC. A minimum of 1 g of muscle tissue is sufficient to detect *Trichinella* where the aim is to prevent clinical trichinellosis (Gamble *et al.*, 2000). In practice this is true for high larval densities but in case of low infection rates the larval are not distributed, homogenously. Using 1 g muscle samples from pigs, experimentally infected with *Trichinella spiralis*, the sensitivity of the pooled sample method of artificial digestion was between 3-5 lpg whereas a 5 g sample had an increased sensitivity of this method to approx 1 lpg (Nöckler *et al.*, 2000). Since there was no significant difference between the 3 and 5 g samples in detecting the infection in pork containing 1.0-1.9 lpg, 3 g samples might be considered to be the minimum size (Forbes *et al.*, 1998; Forbes and Gajadhar, 1999). Given that the average amount of diaphragmatic crus muscle collected in this study was around 25-30 g and no sample was smaller than 5-10 g it is very likely that the Pepsin digestion would have detected a positive result, if there had been one. However, this test did not have a direct impact on the reduction of the disease in pigs. The human error and accidents would always allow for some probability of infection from tested meat (van Knapen, 2000; Gajadhar and Forbes, 2002).

Many authors had reported on a successful use of *Trichinella* excretory-secretory antigens in the indirect ELISA for the detection of specific antibodies in various animal species (Murrel *et al.*, 1986; Smith, 1987; Smith and Snowdon, 1989; Nöckler *et al.*, 1995; Gamble *et al.*, 1996). The test antigen was considered to be an important factor for the identification of specific antibodies which is essential for the specificity of the ELISA result (Nöckler *et al.*, 2000). In this study the ES antigen was used which had more specificity than somatic antigens. Here only 67% (344/512) sera were tested randomly but with the specific consideration to select sera that were not hemolyzed and of good quality. It also increased the sensitivity and specificity of ELISA (Dedek, 1992). However, 37.8% of the slaughtered pigs were less than one year of age where *Trichinella* investigation was less reported. But in this study the slaughtered pig had a mode value of 1-2 years of age. So it was unlikely that the result of the serological testing of such animals were influenced by false negative results due to declining antibody titer. It is also well known that a serum titer is detectable up
to 130 weeks (Nöckler et al., 2000). Therefore if the infection would have persisted in the observed population it might have been detected.

The sera tested by western blot had also shown negative finding. It showed that none of the tested serum had two specific bands in its ladder that is essential according to BfR evaluating procedure to be concluded as positive. So it is concluded that tested sera were true negative for Trichinella spp.

It was well documented that the management factors which are necessary for Trichinella control in farms were not practiced in Nepal. The pig farming was not commercialized and the bio-security measures were not implemented (Dhaubhadel, 1992). However, this study revealed no positive results through Pepsin digestion and ELISA serology. It was interesting to see that under similar farm conditions with similar study designs in different parts of world similar results were found. In the studies carried out in pigs or some sylvatic animals from Brazil, Colombia, Venezuela, Paraguay and Bolivia were also found no positive results through Pepsin digestion (Acha and Szyfres, 1986). Several studies were carried out in Colombia and Guatemala (Schenone, 1984), but there was no evidence of Trichinella found.

However, all randomly selected serum samples were negative but, initially, when the sera were tested through AB-ELISA, two samples were positive and 14 samples were doubtful. This may be due to the ES antigen, since it will increase the sensitivity and specificity of the test. Gamble et al. (1983) and van Knapen et al. (1984) had suggested the use of ES antigens allowing for the detection of natural infections even those with low parasitic burdens. Similarly, a survey conducted in Ecuador (Chavez-Larrea et al., 2005) in 2331 pigs at slaughterhouse and 646 pigs of free roaming demonstrated the presence of specific antibodies in 44 samples but failed to confirm the presence of parasites. Since in that study ES antigen was used like in our study the results may not comment, unambiguously.

Since the risk factors were abundant for Trichinella its initial prevalence was low. Epidemiological calculation was done to estimate the maximum probable
prevalence of *Trichinella* in CDR which was 0.52%. The similar prevalence rates of 0.47% and 1% were estimated, respectively, by Joshi *et al.* (2005) and Sapkota *et al.* (2006). However, in their studies only sera were tested so the reliability of the true prevalence is questioned.

Feeding of offal and kitchen wastes were a very common practice in pig farming in Nepal (Joshi *et al.*, 2005). Questionnaire surveys showed that 65% of the farm pigs were allowed to scavenge for feed or they were being tethered during the day along roadsides and were fed with household refuse. These husbandry conditions result in a high mortality and might lead to cannibalism amongst pigs (Yepez-Mulia *et al.*, 1994). 82.5% of the farm owners of pig farms which had responded to the interview had their farm access to garbage. It was a well-documented source of *Trichinella* outbreak (Acha and Szyfres, 2003). This may indicated that either there was no reservoir in Nepal or rats that were get assess to the dead carcass did not reach up to the pig farms. This issue, however, went beyond the scope of this study.

The low prevalence of *Trichinella* in the pig population of the central development region of Nepal in comparison to the high prevalence in the pig population of China could be related to the differences in the ecosystems. It may be due to a natural mountainous barrier of two neighboring countries and also due to the fact that there is no import export of food items between these geographically close nations. In China the prevalence of the *Trichinella* infection in pig was 5% (n = 40 000) and in similar epidemiological studies (n = 2000) in the northern, southern and coastal region a 7.5% positive rate was indicated (Chan and Ko, 1992). However, there were no pig isolates from Tibet (Xizang province of west China) that is the bordering province to Nepal. Also it was expected that the occurring species would be most probably *Trichinella britovi* and *Trichinella pseudospiralis*, because of the temperate climate in that area (Wang *et al.*, 2007). But it was well known that the first outbreak of human trichinellosis in China came from Tibet and in-between 1964-1998 there were 5 outbreaks with a 1/117 mortality rate (Takahashi *et al.*, 2000). From 2000-2003 there were 3 outbreaks with a mortality rate of 4/50 (Wang *et al.*, 2006). Sampling from 2000-2003 in the adjacent province to Tibet and Qinghai indicated a
3.2% prevalence of infected pork. Concerning this aspect the emergence of *Trichinella* in Nepal in near future cannot be ignored.

However, this preliminary survey to estimate the prevalence of *Trichinella* in Nepal did not reflected that the central development region is free from *Trichinella*. The recent finding that *Trichinella* infection was documented in animals of 3 islands (Ireland, Croatia, Sardinia) previously considered to be *Trichinella* free, strongly supports the concept that there is neither a region nor an area nor a country that could be considered to be *Trichinella* free, totally (EFSA, 2005; Pozio and Zarlenga, 2005). This was well explained through the outbreak in Sardinia (the Mediterranean island) that was supposed to be *Trichinella* (Pozio *et al.*, 2006) free after negative results from an examination of 4427 sera from domestic pigs, 668 wild boars and 8 red foxes and meat from 2036 wild boar and 32 red foxes (Pintore *et al.*, 1996). But the outbreak in 2005 (11 persons were infected) raised the questions of the validity of the concept of a *Trichinella* free area.

The role of the rodents as a reservoir in domestic habitat is a topic of debate (Schad *et al.*, 1987). A survey carried out in Croatia had suggested that the brown rat acts as vector or a victim rather than as a reservoir (Stojcevic *et al.*, 2004). However, 70% of the responded farmers of Nepal had no rodent control program in his/her farm. Since the rodent is as confirmed source it might be the major route of disease transmission from farm to farm in Nepal. It was reported that infected rats represent an offshoot of the domestic cycle, being recipient of infection from that cycle (Campbell, 1983).

The finding of this study may postulate there was only a very low prevalence of *Trichinella* in the CDR of Nepal. But the questionnaire survey showed that the farming management system and the epidemiological factors supported the transmission of parasites. It should raise the attention of scientific authorities to search, precisely, with high sample sizes and with high sensitive techniques so that the quality assurance regarding the *Trichinella* status of the region could be developed. It was assumed that in economically less developed conditions pigs
presented at slaughterhouses were those which were raised under improved hygiene standards in opposition to those pigs roaming around in villages, slaughtered in rudimentary conditions and destined in local consumption. In that case the presence and zoonotic importance of *Trichinella* should be confirmed. The decision whether this should bear practical consequences for the routine meat inspection must be evaluated in respect of Nepal.

While the surveillance of domestic pigs, wild animals and games could be improved (Zimmermann, 1983) the current study indicates that the risk for humans contracting *Trichinella* infection from eating pork or pork products from pigs of the surveyed districts of the CDR was exceedingly low in comparison to other food borne disease problems existing in that region. It was necessary to search for the possible species as a reservoir for *Trichinella* in Nepal in the context that there is a wide range of suitable hosts. It was beyond the scope of this study but it was thought that before documenting a possible reservoir it is necessary to know at least two criteria: the area where the host species is detected should be similar in size or larger than the area where *Trichinella* is present; and the host species should maintain the infection for years, independently, of the presence of *Trichinella* in other animals living in the same area (Pozio, 2005).

However, a complicated issue for *Trichinella* is the presence of wildlife reservoir both in terrestrial and marine mammals (EFSA, 2005). Since Nepal is landlocked but has open borders it is not possible to form efficient barriers to prevent the introduction and establishment of *Trichinella* in reservoir animals in a habitat.

### 5.2 Conclusion and recommendations

The negative results through Pepsin digestion, end-point titer single dilution antibody ELISA and western blot during the period of this study conclude that pigs of the central development region of Nepal were not infected or had a very low infection with trichinellosis at the slaughterhouse level. However, negative finding do not guarantee that the CDR is free from *Trichinella* since comprehensive and complete
knowledge on the epidemiological situation of the parasite in domestic pigs and wildlife within country is unknown. Freedom from *Trichinella* infection within a given area is difficult to document. Despite a negative result from hundreds of millions of swine carcasses it is assumed that the infection exists there at a low prevalence (EFSA, 2005). This study is not applicable for species characterization. Based on the findings of this study the following recommendations are made for the detection of *Trichinella* parasites in order to reduce the risk of trichinellosis in the Nepalese population.

1. Surveillance and monitoring for trichinellosis in pigs

It is recommended that intensive national surveillance is essential for trichinellosis control in domestic pigs in Nepal based on the OIE guideline. To achieve this goal in an efficient manner the production practices that prevent trichinae infection have to be documented. A herd’s status using a statistically based sample of finishing animals is to be monitored. Any suspicion of disease is followed at the field level by trace back, quarantine and laboratory testing. Surveillance and monitoring are followed through the official system which means to organize surveys, to collect and collate the data and to operate quality control of routine laboratories. In case that *Trichinella* (after all this effort) will not be investigated within the next 5 years the country can apply for the region free certification.

2. Implementation of the meat inspection act

The meat inspection act should be implemented as soon as possible to insure safe meat control during slaughtering, transportation and distribution. In addition to that safe and controlled elimination of all offal and condemned materials is also recommended. The official meat inspection act of the country should require regulations that implement and support a quality assurance program, proper training in both identification methods for everyone involved in the chain of trichinellosis control. For the development of proficiency programs it is necessary to evaluate the quality of the examiners and the examination process. The establishment of cost
effective laboratory facilities for the diagnosis of trichinellosis should be brought to the attention of the veterinary and public health authorities of Nepal.

3. Awareness regarding trichinellosis

Awareness generating programs should be launched focusing on safe meat consumption habits. The suggested target groups for those programs are butchers, food shop vendors and ethnic communities. In addition the authorities must, systematically, carry out veterinary and sanitary educational programs for both swine breeders and consumers to maximize prevention and control measures. If properly implemented erroneous results in the examination of meat for *Trichinella* can be substantially reduced or eliminated. To achieve these goals, veterinary and human medicines should co-operate in trying to solve any problems of zoonotic origin.


[www.iss.it/site/Trichinella/index.asp](http://www.iss.it/site/Trichinella/index.asp)


Näreaho, A. (2006): Experimental and immunological comparison of *Trichinella spiralis* and *Trichinella nativa*. Finland: University of Helsinki, Faculty of Veterinary Medicine, Dissertation.


http://www.oie.int/eng/normes/mcode/en_chapitre_2.2.9.htm

Oivanen, L. (2005): Endemic trichinellosis-experimental and epidemiological studies. Finland: University of Helsinki, Faculty of Veterinary Medicine, Dissertation. 


Pozio, E. (2005): The broad spectrum of *Trichinella* hosts: From cold to warm blooded animals. *Veterinary Parasitology* 132, 3-11.


APPENDICES

Appendix A: The flow chart of laboratory techniques

576 slaughter pigs

10 ml blood by heart puncture

Serum by centrifugation

25-30 g diaphragmatic crus muscle

Pepsin digestion (10 pooled sample)

Pepsin digestion (5 pooled sample)

Antibody ELISA

Positive

Doubtful

Negative

Positive

Doubtful

Negative

End-point titration antibody ELISA and also by western Blot analysis

Discard

Trichinoscopy/ Compressorium

Larva seen

No larva

Discard

Pepsin digestion (Single sample identification)

Multiplex PCR for species differentiation
Appendix B: Pig producer questionnaire survey

Date of Survey:………………………………………………………………………..
Farmer name: .................. Age of farmer: .......
Farmer’s address: ...................... Telephone: ...........

Farm geographical location:
- Mountain region
- Hills region
- Plain region

Education level:

Date of farm establishment:

Number of people involved in farm:

Pigs stocking density:

Training in pig husbandry Yes ☐ No ☐

Size of pig production:
- Household raising
- Small and Medium
- Intensive

Pig production system:
- Free ranging
- Mixed
- Indoor

Rodent control program: Yes ☐ No ☐

Wildlife access to farm: Yes ☐ No ☐

Possibilities to cannibalism:

Target of pig production:
- Piglet producing
- Fattening purpose
- Mixed
Pigs purchase from where:

- Commercial farm
- Non commercial farm
- Backward farm

Pig breeding for raising:

- Local
- Exotic breeds
- Cross breed
- All types of breeds

Hygiene barriers exist in farm for persons entering the farm:

Cooking feed before providing to pig:

Types of feed:

- Ration feed
- Leftovers (hotel, home)
- Mixing of ration and leftovers
- Traditional

Garbage dumping in vicinity:

Birds access from dumping area to the farm:

Waste management:

- Anaerobic
- Aerobic
- Anaerobic + Aerobic
- Direct outlet to outside
- No facilities

Information about pork borne disease: Yes No

Knowledge about *Trichinella* control program: Yes No

Pork consumption habit:

- Cooked
- Uncooked/Mixed
Appendix C: ELISA reading of serological samples

Clusture column showing ELISA indices

Clusture column comparing ELISA OD values with ELISA indices
Appendix D: Specific and non specific band patterns of tested sera and control sera analyzed through western blot

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NS = Non specific, PC = Positive control, NC = Negative control, kDa = Kilodalton
DECLARATION

I, the under signed, declared that the thesis is my original work and has not been presented for a degree in any university.

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