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 Sindhupalchowk 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.

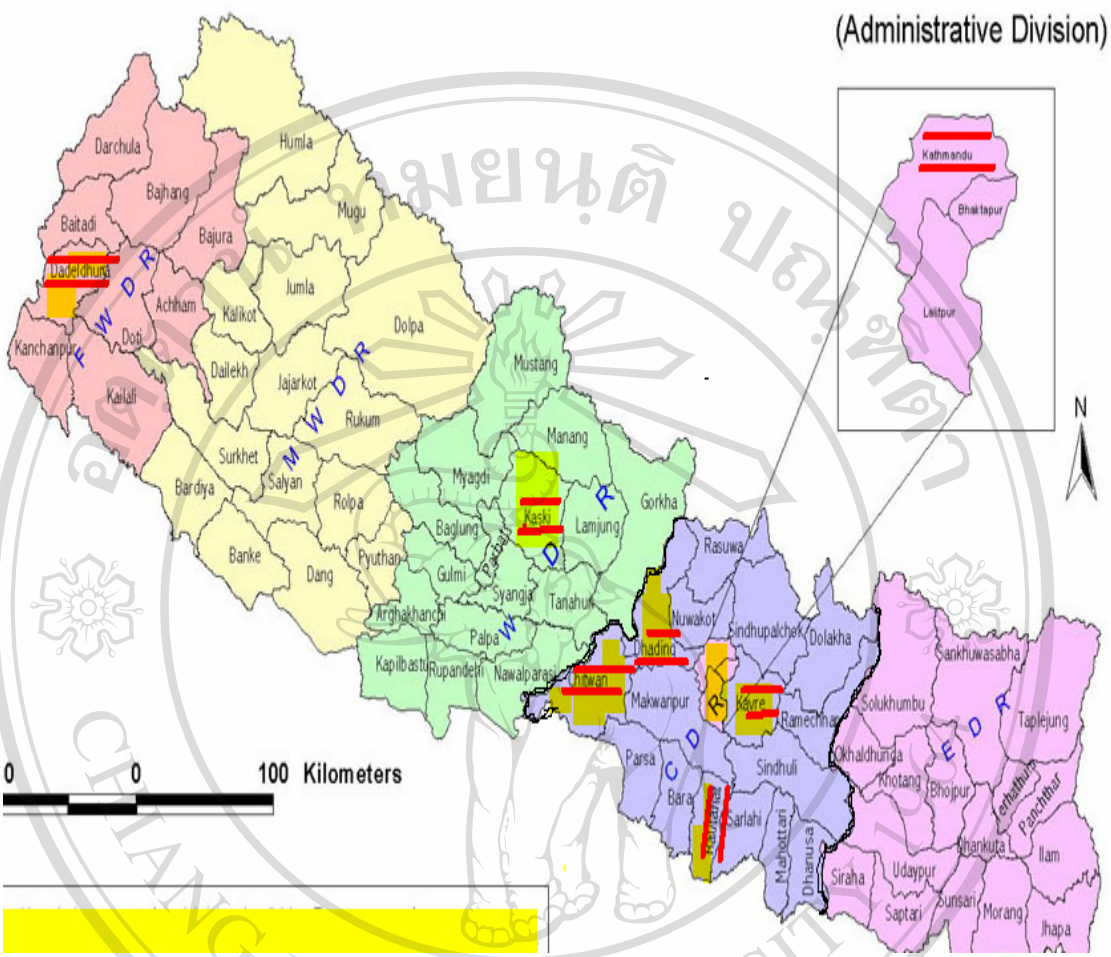

Figure 2: Map of Nepal (study areas are marked)

(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.

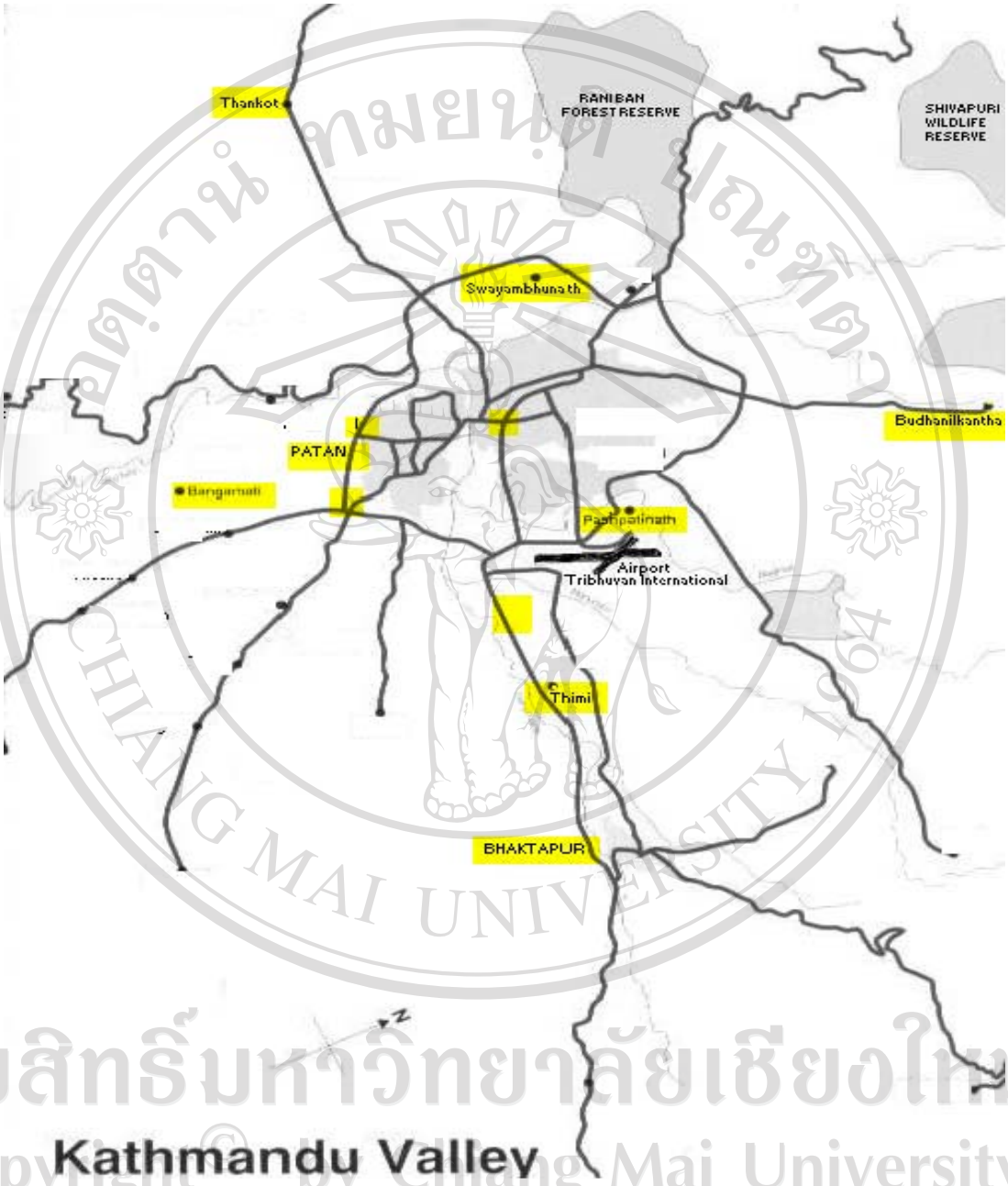

Figure 3: Map of the Kathmandu valley showing the twelve butcheries sites where samples were collected.

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)

$$P_{rg} = (P + Sp - 1) / (Se + Sp - 1)$$

$$= 1.37\%$$

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

$$n = (t * SD / L)^2$$

Where, t = Student's t-value ≅ 1.96 when the desired level of confidence is 95%

$$SD = \text{Standard deviation} = \{P^* (1-P)\}^{1/2}$$

$$= 0.116242$$

$$L = \text{Accepted absolute error} = 1\%$$

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 over night 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 –20ºC. 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 -20^0C. 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^{0}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^{0}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.

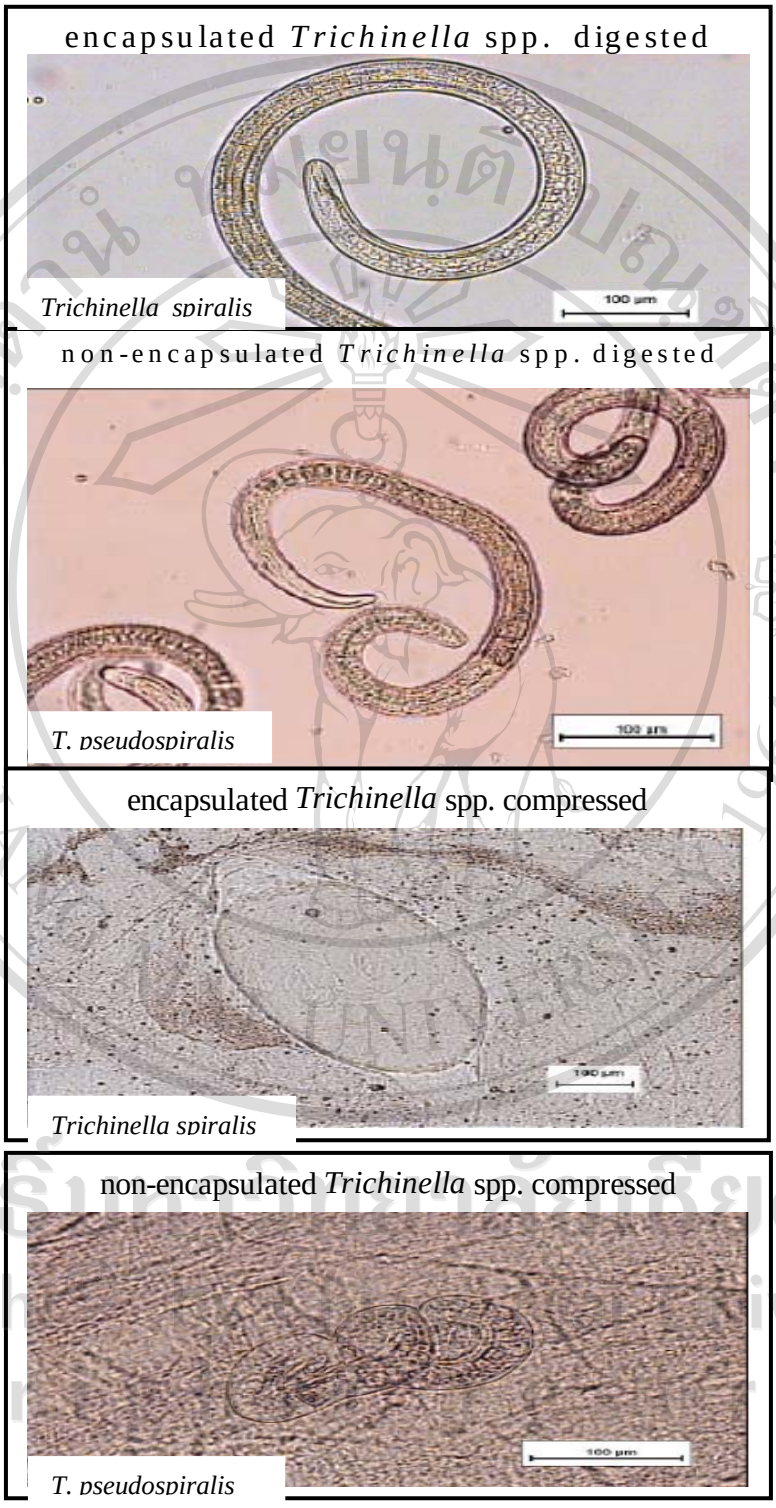

Figure 4: Microscopic illustrations of *Trichinella* spp. larvae in meat samples by different techniques (courtesy: Dr. Karsten Nöckler, BfR. Personnel communication)

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^{0}C
2. *Trichinella*-positive control serum (1 ml, lyophilized), storage at –20^{0}C
3. *Trichinella*-negative control serum (0.5 ml, lyophilized), storage at –20^{0}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^{0}C (SIGMA, product No. A5670)
6. ABTS {2,2'-azino-his-(3-ethylbenzothiazoline sulfonate)} buffer, dry matter from Boehringer, storage at 4-8^{0}C
7. Tablets chromogen ABTS, storage at 4-8^{0}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

KH_2PO_4	0.4 g
Na_2HPO_4 * 12 H_2O	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

	1	2	3	4	5	6	7	8	9	10	11	12
A	Nc1	FS1	FS5	FS9	FS13	FS17	FS21	FS25	FS29	FS33	FS37	FS41
B	Nc1	FS1	FS5	FS9	FS13	FS17	FS21	FS25	FS29	FS33	FS37	FS41
C	Nc1	FS2	FS6	FS10	FS14	FS18	FS22	FS26	FS30	FS34	FS38	FS42
D	Nc1	FS2	FS6	FS10	FS14	FS18	FS22	FS26	FS30	FS34	FS38	FS42
E	Pc1	FS3	FS7	FS11	FS15	FS19	FS23	FS27	FS31	FS35	FS39	FS43
F	Pc1	FS3	FS7	FS11	FS15	FS19	FS23	FS27	FS31	FS35	FS39	FS43
G	Pc1	FS4	FS8	FS12	FS16	FS20	FS24	FS28	FS32	FS36	FS40	PBST
H	Pc1	FS4	FS8	FS12	FS16	FS20	FS24	FS28	FS32	FS36	FS40	PBST

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 37^0C. 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 37^0C, 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 4–8^0C 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

   $NE = OD - OD_{blank}$
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%.

   ELISA-index (%) = $(mNE_{sample}) / (mNE_{pos}) * 100\%$
4. Evaluation of test results

"*Trichinella*- positive"	(+)	ELISA-index (%) ≥ 18
"*Trichinella*-negative"	(-)	ELISA-index (%) < 12
"*Trichinella*-questionable"	(?)	$12 \leq$ 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 37^0C, 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 37^0C. 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

“End-point titration- positive” (+)	ELISA-index (%) ≥ 70 (1:10) and border of titer ≥ 1:80
“End-point titration- negative” (-)	ELISA-index (%) < 40 (1:10) and border of titer < 1:80

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, referrence number LA 163-1/BfR). This technique is genus specific.

Specific band	Molecular weight (kDa)	Range of kDa
I	43.0	42.7-43.2
II	46.9	46.7-47.2
III	62.0	61.5-62.5
IV	66.1	65.4-66.9
V	102.0	101.5-102.4

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.