3. MATERIALS AND METHODS

3.1 Justification for the selection of study locations

Regarding the objectives of the thesis, the first field studies were conducted at 4 slaughterhouses around Phnom Penh city, where slaughter pigs were delivered to slaughterhouses from different areas (provinces) of Cambodia. Based on the district administrative map, slaughterhouses were randomly selected from different regions surrounding Phnom Penh city (Figure 1). At the same time, a visit was made to the selected hospitals to look for data of neurocysticercosis in humans caused by *T. solium* cysticercosis. According to the duration of the research activity, the work schedule was divided into two periods: the first investigation started from November 2004 to February 2005 and the second period started from the end of February to the end of April 2005. Beside places mentioned above, some parts of the laboratory work were conducted at the NAHPIC of DAHP/ Cambodia and another part was performed at the diagnostic laboratory/ CMU (Thailand).

3.2 Slaughterhouse and slaughter pigs

There are two categories of pig slaughter places in Cambodia: slaughter slabs and slaughterhouses. Around Phnom Penh municipality, there are 4 pig slaughter slabs and 4 pig slaughterhouses. The capacity of slaughter pigs at each slaughter slab is 1-50 pigs per day and in each slaughterhouse 100-600 heads per day. Slaughtering was done weekly. The pig inspection was first conducted by tongue examination during the evening and carcass examination in the early morning. The slaughterhouses were:

(a) Slaughterhouse 1 (Beungsalang) is located in the Tuol Kok District, Central part of Phnom Penh city (Figure 1). The slaughterhouse 1 belongs to a private owner, is not registered and consists of the slaughtering of pigs and cattle at the same time,

but in two separate places. The average number of slaughter pigs per day is estimated at about 225 head, and cattle 40-60 head. The environmental condition of this slaughterhouse is very dirty (no hygienic standard).

(b) Slaughterhouse 2 (Domnak Thom I) is located in the Mean Chey District, in the southern part of Phnom Penh city (Figure 1). Slaughterhouse 2 is not registered and belongs to a private owner. The slaughter pigs are on average 550 head per day.

(c) Slaughterhouse 3 (Phreash Phonlear) is located in the Mean Chey District, southeastern part of Phnom Penh municipality (Figure 1). Slaughterhouse 3 is private, not registered. The average number of slaughter pigs is about 275 head per day.

(d) Slaughterhouse 4 (Ruessiekaev) is located in the Ruessiekaev District, in the northern part of the city (Figure 1). The average number of slaughter pigs is estimated around 250 head per day. Moreover, this slaughterhouse is not registered and also belongs to a private company.

3.3 Description of study design

The study design was a cross-sectional study carried out at the pig slaughterhouses to establish the prevalence of cysticercosis as well as sero-prevalence of trichinellosis in the pig slaughtering line. Between November 2004 and April 2005, 432 pigs were examined at the four slaughterhouses in Phnom Penh for cysticercosis and more than 440 serum samples for trichinellosis. The materials presented were selected from all pigs on the days of sampling. Tongue palpation was routinely done before slaughter and meat inspection was conducted after slaughter.

The four slaughterhouses were conveniently selected. The slaughter pigs were separated on the basis of breeds and the study pigs were then randomly selected from each breed. The sampling was done 3-4 times per week.

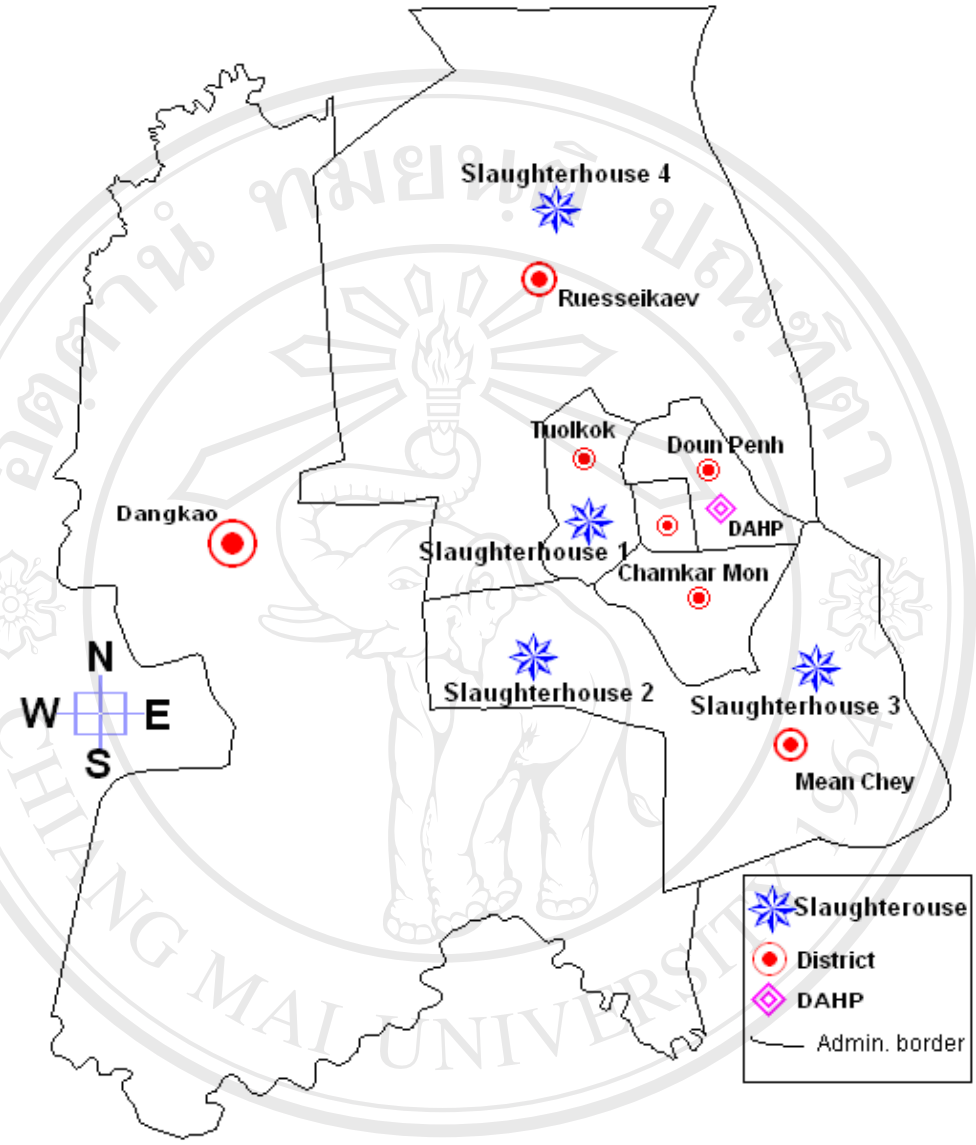

Figure 1. Phnom Penh Map showing slaughterhouses located in different districts around the Phnom Penh Municipality.

3.4 General description of study setting areas

The origin of slaughter pigs was later traced back to farms. Based on a slaughterhouse questionnaire survey and district administrative map, nine provinces, twenty-nine districts and three intensive farms were selected for this study, where pigs have always been delivered to these four slaughterhouses for slaughter. The twenty-nine districts were selected from these nine provinces. These provinces are located in different parts of the country such as central, western, northwestern, southern and southeastern as well as around Phnom Penh (Figure 2). Some provinces were selected such as Takaev (1), Prey Veaeng (2), Svay Rieng (3), Kampong Cham (4), Pousat (5), Kampong Spueu (6), Kandal (7), Kampot (8) and Banteay Mean Chey (9). Out of 432 pigs slaughtered in slaughterhouses during the study, it was of 18.5 %, 7.64 %, 13.42%, 4.62 %, 2.54%, 6.25%, 38.88%, 4.62% and 4.39% from province 1, 2, 3, 4, 5, 6, 7, 8 and 9 respectively. In these provinces, pigs are mainly used for commercial reasons and local consumption. A household keeps one pig to a hundred depending on the production system, which is mostly a fattening system.

3.5 Pig raising and pig breeds in Cambodia

The pig production (farming) system in Cambodia is comprised of 3 categories:

(a) Household raising (as extensive or outdoor): they keep from 1 to10 pigs per household. The pigs are free-range-outdoors all the time.

(b) Semi-intensive system is practiced by small and medium scale (farms): The number of pigs per household ranges from 10 to 200 heads per household or farm. Most of them are free roaming at the daytime and kept in the pens at night time, especially, piglets aged 2-4 months.

(c) Intensive production farms (Indoor): the number of pigs is more than 300 head per farm. The pigs are kept in the pens or piggery all the time (Census, MAFF, 2000).

The pig breeds in Cambodia are divided into 3 types:

(a) Local breeds (native): are slow growers and weigh an average of 60 to 80 kg. These breeds require fewer facilities and easily adapt to local feed and conditions. In addition, their body contains more fats than red muscles.

(b) Exotic breeds: like Landrace, Duroc, Large White, and Petrain. These breeds have been exported from different countries. They are characterized by fast growth and an average weight of 70- 120 kg.

(c) Crossed breeds: characterized by a strong body, aver. weight of 70-150 kg.

3.6 Description of study population

From November 2004 to April 2005, 48 working days were taken to conduct this study in the four slaughterhouses. A total of 62,400 slaughter pigs, 10,800 pigs were slaughtered in slaughterhouse 1, 26,400 pigs in slaughterhouse 2, 13,200 in slaughterhouse 3 and 12,000 in slaughterhouse 4.

3.7 Sampling procedure and biological sample collection

3.7.1 Sample size determination

The sample sizes used in this study were as follows:

(a) Ante mortem inspection 220 (Tongue)

(b) Post mortem inspection: 432 (included 220 tongue inspections)

(c) Blood samples: 440

These sample sizes were calculated using the Win Episcope program as below:

(a) Pig population estimated in Cambodia of 2,000,000.

(b) An estimate of disease prevalence of 50%

(c) An error rate of 5% and 95% confidence.

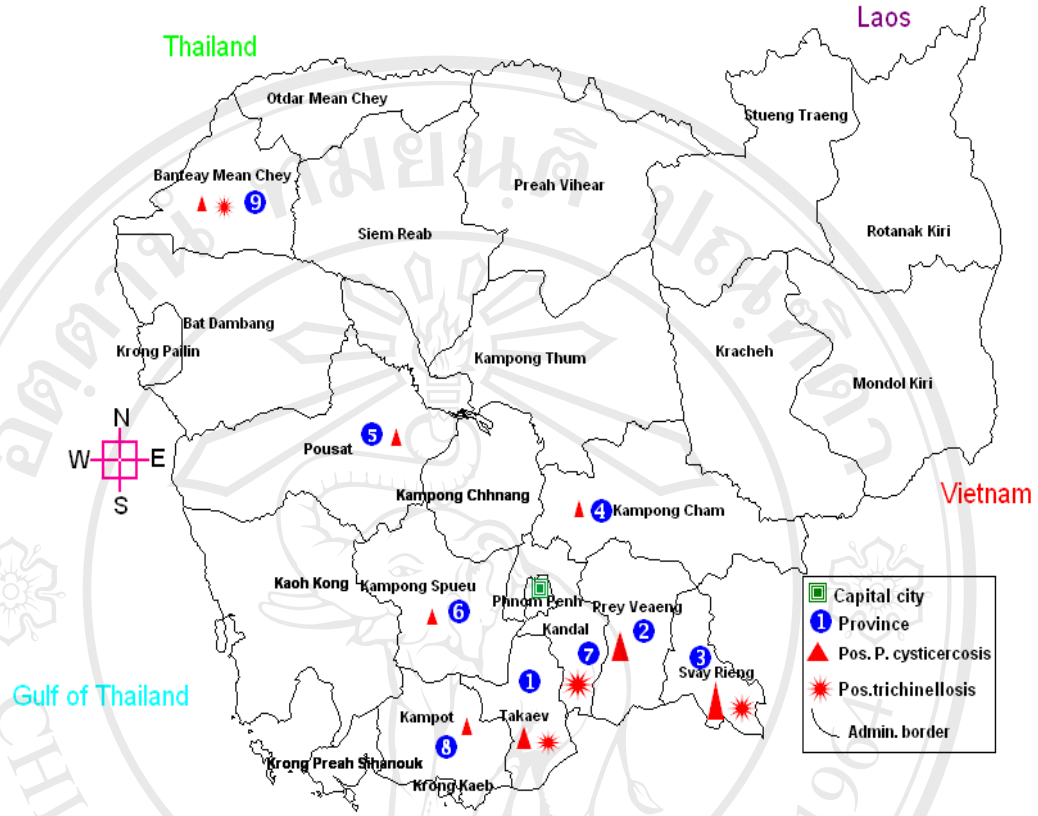

Figure 2. Map of Cambodia showing provinces where slaughter pigs were

3.7.2 Collection of biological samples

3.7.2.1 Cyst collection

In the slaughterhouse, pieces of meat and (or whole) organs with cysts were collected in plastic bags, labeled and transferred to the laboratory. The cysts were harvested, placed in separate plastic bags and kept in deep freeze until required for laboratory analysis.

3.7.2.2 Blood collection and preparation of sera

Blood samples taken at the slaughter process were transferred to the laboratory, kept in the laboratory at room temperature for 6-8 hours and then sera were collected and placed into numbered serum vials and kept in deep freeze for laboratory investigation.

3.8 Ante and post mortem examination for porcine cysticercosis

In the four study slaughterhouses with, a total of 432 pigs, 220 pigs were examined by tongue palpation, and after slaughter, 432 pigs by meat inspection for *T. solium* cysts respectively. The age, breed, sex, weight, and an approximate source of origin of each pig were recorded. The protocols of ante and post mortem examination were as follows:

3.8.1 Ante- mortem examination (Tongue palpation)

It was to conduct examination of the tongue for cysts in the upper and under the base of the tongues of local breed pigs. This was the traditional method for identifying cyst nodules in the tongue muscles. Mature viable *T. solium* larvae are oval (5-8 mm),

fluid-filled, and with a central whitish spot, which is the scolex (Gonzalez, 1994). Briefly the procedure was follows:

A pig was restrained, laterally and recumbent, and the head was stabilized or fixed by the use of a snare. The mouth was opened by the aid of a wooden rod, twisted across the upper and lower jaw and the tongue was gently pulled out using a piece of a cotton cloth. The under-surface of the tongue was thoroughly examined for the presence of cysts of *Taenia solium* larvae (Ngowi *et al*., 2004).

3.8.2 Post-mortem examination (meat inspection) and predilection sites

General provisions for the post-mortem inspection of pig carcasses for cysticerci of *T. solium* were followed which include long and parallel incisions into the external masseter muscles on both sides of the face in an upward direction to completely sever the parotid gland below the ear. Similar incisions were made in the internal masseters. The tongue was detached from the hyoid bone, viewed, palpated and cysts under the surface counted. A deep longitudinal incision covering about three-quarters the thickness of the tongue and covering the whole length of the tongue, diaphragm, esophagus, eyeball, conjunctiva, sexual organs and lymphatic glands was made to examine for cysts. After opening the pericardium, the heart was also visually examined for the presence of cysts. The heart was cut open and a deep (3/4 the thickness of septum) incision into the septum was made to expose any metacestodes. Three equidistant incisions were made in the triceps brachii muscle proximal to the elbow joint. Cysts that were encountered on incisional and intact surfaces were classified and enumerated as either viable (translucent, fluid-filled with invaginated whitish scolices visible) or degenerated (black, sand-like or powdery contents) (Boa *et al.*, 2002). Additionally, the following organs were inspected: lung, kidney, liver, spleen and brain. All organs and muscles with cysts were sliced in such a way that all fully developed cysts could be observed and noted (Dorny, 2004). The Distribution of cysts for those organs and muscle groups where cysts were counted and evaluated criteria as below:

Negative= 0 or no cyst in the carcasses

Low ≤ 1-100 cysts (light infection)

High > 100 cysts (Heavy cases)

3.9 Isolation, identification and characteristics of cysts of porcine cysticercosis

In the laboratory investigation in CMU, microscopic examination and serological test were conducted as indirect non-competitive ELISA test and endpoint titration. The protocols were described as follows:

3.9.1 Morphological examination of *T. solium* cysticerci

A total of 235 cysts were microscopically examined and randomly selected cysts (5 cysts per 1 infected pig) were used to measure the length of the hooks and their morphology.

The procedure of microscopic examination was as follows:

(a) Required specimens were cysts, collected from different sites or organs of infected pigs.

(b) Prepared 10% HCl solution.

(c) The free cysts from muscles put into 10% HCl in a Petri dish for 3-5 minutes

(d) Cyst wall was opened with forceps and scissor

(e) Then the invaginated scoleces were examined by 400 x (ocular 10 x, objective 40 x) magnification, looking for morphology and the presence of rostellum and hooks.

3.9.2 Indirect non-competitive enzyme linked immunosorbent (AB-ELISA)

The total of 440 blood serum samples were first screened by indirect non-competitive AB-ELISA test and doubtful results were re-examined. Test procedure is described as in the instructions below:

The AB-ELISA technique is according to the standard method used in the National Reference Laboratory for trichinellosis, Federal Institute for Risk Assessment (BfR) Berlin Germany. The same institute also provides the ELISA kits. The procedure is the indirect AB-ELISA and serum samples are tested for the specific anti-*Trichinella*-IgG.

An ELISA kit consists of

(a) Microtitre plates coated with *Trichinella* antigen (excretory-secretory antigen of *Trichinella spiralis*) contained 50µl *Trichinella*-E/S- antigen per well, storage at 4-8^{o}C

(b) *Trichinella*- positive control serum (1 ml, lyophilized), storage at –20^{o}C

(c) *Trichinella* negative control serum (1ml, lyophilized), storage at –20^{o}C Additionally, buffers and reagents:

(d) PBS buffer (not included, to be prepared according to protocol)

(e) Anti-pig IgG-peroxidase conjugate pre-diluted 1:10, (1.0 ml), storage at –20^{o}C (SIGMA, product N^{o}. A5670)

(f) ABTS buffer, dry matter from Boehringer, storage at 4-8 ^{o}C

(g) Tablets chromogen ABTS, storage at 4-8 ^{o}C

Test Procedure for AB-ELISA

(a) Preparation of PBS- Tween 20/dilution of prepared PBS- Tween 20 2000 ml of PBS- Tween 20 (pH= 7.2-7.4) consists of:

KH_2PO_4	0.4g
Na_2HPO_4* 12 H_2O	5.8g
NaCl	16.0 g
KCl	0.4 g
Tween 20	1.0 ml
Distilled water	ad 2000 ml

(b) Washing (blocking) of microtiter plate 1 time with aqua dest and 3 times with PBS-T (150µl), (every for 3 min)

(c) Preparation of test and control sera diluted in PBS- Tween 20 (1:100): 1ml (1000µl) PBS, add 10µl test serum samples or control sera, then placed them into the wells (volume 50 µl)

Example: a coated microtiter 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	PBS-T
H	Pc1	FS4	FS8	FS12	FS16	FS20	FS24	FS28	FS32	FS36	FS40	PBS-T

Nc1= negative control serum

Pc1 =positive control serum

FS =samples for field sera

PBS-T= only coated with PBS-Tween 20 (blank of microtiter plate)

(d) Incubation for 30 min at 37 ^{o}C. Then washing as mentioned in the point (b)

(e) Anti-pig IgG- peroxidase-conjugate (pre-diluted 1:10) at final dilution of 1:1200 in PBS Tween 20 (200µl conjugate +24 ml PBS) is added in 50 µl amounts to all wells.

(f) Incubation for 30 min at 37 ^{o}C. Washing and soaking as mentioned in the point (b) and finally, 1 time with aqua dest.

(g) 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

Dilution of 2 tablets ABTS (100 mg) in 100 ml of prepared ABTS buffer. Then, add 50 µl freshly prepared ABTS (substrate indicator system) to all wells. Store the chromogen at 4-8 ^{o}C in the dark (storage is possible for a couple of weeks).

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

(i) Preparation of stop solution for stop reaction of AB-ELISA

Calculation and Evaluation of test results

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

(a) Calculation of netto extinction (NE) of each well:

NE = OD-OD blank

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

(c) 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%

$$\text{ELISA-index (\%)} = \frac{\text{mNE sample}}{\text{MNE pos}} \text{ X } 100\%$$

(d) Evaluation of test results:

"*Trichinella*-negative"	(-)	ELISA- index (%) < 8
"*Trichinella*-questionable"	(+/-)	8 ≤ ELISA- index(%) < 14
"Trichinella-positive"	(+)	ELISA- index (%) > 14 (Nöckler *et al.*, 1995)

3.9.3 Endpoint titration of serum samples positive by single dilution AB-ELISA

Positive and doubtful serum samples were examined using endpoint titration test for confirmation. The test protocol was addressed as below:

(a) The procedure is as indirect non-competitive AB-ELISA, but it is different by a dilution step: 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1: 640 and 1:1280.

(b) Fill 90µl PBS and 10µl test sera into each well of the first row (1:10).

(c) Fill 50µl PBS into each well of second row until the last rows

(d) In the first row (1:10) of microtiter plate suck 3 times and take out 50µl of solution, then move to next row of microtiter plate and do the same way until the last row. Other steps follow AB-ELISA procedure.

(e) Evaluation of test results:

"Endpoint titration-positive" (+)	ELISA- index (%) ≥ 70 (1:10) and border of titer ≥ 1:80
"Endpoint titration- negative" (-)	ELISA- index (%) < 40 (1:10) and border of titer < 1:80 (Nöckler *et al*., 1995)

3.10 Questionnaire surveys

3.10.1 Slaughterhouse and hospital

Questionnaire surveys were done in the four former slaughterhouses and in four-selected hospitals around the Phnom Penh municipality (Hospital data were not available during time of study). 22 questions for slaughterhouses and 13 questions for hospital survey were performed (Appendix 2, I, II).

This was confirmed by examining slaughterhouse records, interviewing pig traders, farm owners and local veterinarians or inspectors and also by the nature of the typical local breeds brought for slaughter.

3.10.2 Pig farm survey ("Trace back" questionnaire survey for pig farm owners)

In total, 132 people answered these questionnaires containing 34 questions at the farm levels of three provinces, where there have been high risks. These areas were selected because most of the pigs brought to slaughterhouses originated here and are more infected (positive) than those of others.

This was administered in an attempt to establish information links of veterinary public health significance between slaughterhouses and farm origins (Appendix2, III). The people interviewed were from different levels and interests within those chosen.

3.11 Data management and analysis

Raw data from the slaughterhouse, laboratory, and questionnaire survey were appropriately coded and entered into a database using the MS. Excel program. Any errors were rechecked also using the MS excel program. Analyses were performed using Win Episcope 2.0, Epical 2000, EpiInfo, PopMap, StataSE 8.0 and SPSS 12.0 programs. Based on the objectives of this study, the analyses generally centered on the following:

(a) Determination of distributions of the various data variables and descriptive statistics of each;

(b) Determination of prevalence and sero-prevalence of porcine cysticercosis and trichinellosis, respectively, by slaughterhouse, farm and province;

(c) Comparison of distribution of prevalence and sero-prevalence of porcine cysticercosis and trichinellosis, respectively, by slaughterhouse, farm, disticts and province. Kruskal Wallis test or Chi-square ($\chi 2$) test were used and the results evaluated using as the criterion.

(d) Assessment of agreement between tongue palpation and meat inspection. Kappa statistic test was used and the results evaluated using the criterion described in Dohoo *et al*. (2003). The criterion is categorized into the following:

<0.2: slight agreement, 0.2-0.4: fair agreement, 0.4-0.6: moderate, 0.6-0.8: substantial and >0.8: almost perfect