



Development of a dipstick ELISA for the detection of circulating antigens of *Taenia saginata*/*Taenia solium* cysticercosis

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Table of Contents	<u>Page</u>
Acknowledgements	iii
List of Tables.....	vi
List of Figures	vi
List of Annexes	vii
List of abbreviations and acronyms	viii
Summary	ix
Contribution by others.....	x
Chapter I: INTRODUCTION.....	1
Chapter II: LITERATURE REVIEW	2
2.1. <i>Taenia saginata</i> and <i>Taenia solium</i> life cycle.....	2
2.1.1. <i>Taenia saginata</i>	2
2.1.2. <i>Taenia solium</i>	2
2.2. Clinical symptoms of cysticercosis	3
2.2.1. Cattle	3
2.2.2. Humans.....	3
2.2.3. Pigs.....	3
2.3. Epidemiology of cysticercosis	3
2.3.1. <i>Taenia saginata</i>	3
2.3.2. <i>Taenia solium</i>	4
2.4. Diagnosis of cysticercosis	4
2.4.1. Diagnosis of cysticercosis in cattle	4
2.4.1.1. Carcass inspection	4
2.4.1.2. Enzyme-Linked Immunosorbent Assay	5
2.4.1.2.1. <i>Antibody detection ELISA</i>	5
2.4.1.2.2. <i>Antigen detection ELISA</i>	5
2.4.1.3. Electroimmunotransfer Blot.....	6
2.4.1.4. Dipstick-immunoassay	6
2.4.1.5. Dot-ELISA	6
2.4.2. Diagnosis of cysticercosis in humans.....	7
2.4.2.1. Imaging techniques	7
2.4.2.2. Electroimmunotransfer Blot.....	7
2.4.2.3. Enzyme-Linked Immunosorbent Assay	8
2.4.2.3.1. <i>Antibody detection ELISA</i>	8

2.4.2.3.2. <i>Antigen detection ELISA</i>	8
2.4.2.4. Dot ELISA.....	9
2.4.3. Diagnosis of cysticercosis in pigs	10
2.4.3.1. Carcass inspection	10
2.4.3.2. Tongue palpation.....	10
2.4.3.3. Electroimmunotransfer Blot.....	10
2.4.3.4. Enzyme-Linked Immunosorbent Assay	11
2.4.3.4.1. <i>Antibody detection ELISA</i>	11
2.4.3.4.2. <i>Antigen detection ELISA</i>	11
Chapter III: MATERIALS AND METHODS	12
3.1. Serum samples.....	12
3.2. Trichloroacetic acid (TCA) treatment of serum.....	12
3.3. Microplate Ag-ELISA.....	12
3.4. Dipstick Ag-ELISA.....	13
3.4.1. Initial reference protocol	13
3.4.2. Protocol optimization tests	13
3.5. Adapted protocol for dipstick Ag-ELISA	14
3.5.1. Determination of the detection level	15
3.5.2. Evaluation of the dipstick Ag-ELISA	15
Chapter IV: RESULTS.....	16
4.1. Protocol optimization tests	16
4.2. Determination of the detection level	20
4.3. Evaluation of the dipstick Ag-ELISA	20
Chapter V: DISCUSSION AND CONCLUSION	22
Chapter VI: RECOMMENDATIONS	25
REFERENCES.....	26
Annexes.....	32
Short curriculum vitae.....	41

List of Tables

Table I. Results of the microplate Ag-ELISA and dipstick Ag-ELISA tests on a serial dilution of a <i>T. saginata</i> cysticercosis positive serum sample (P ₂) obtained from artificially infected cattle.	20
Table II. Results of the microplate Ag-ELISA and dipstick ELISA for the detection of ES antigens of <i>T. saginata</i> in serum samples from naturally infected cattle using the adapted protocol.	21
Table III. Results of microplate Ag-ELISA and dipstick Ag-ELISA for the detection of ES antigens of <i>T. solium</i> in serum samples from naturally infected pig using the adapted protocol.	21

List of Figures

Figure I. Life cycle of <i>T. saginata</i> and <i>T. solium</i>	2
Figure II. A photo image showing the results of a dip washing (top) and a stream washing (bottom) methods.....	16
Figure III. A photo image showing the results of 60 min (top), 45 min (middle) and 30 min (bottom) incubation times.....	16
Figure IV. A photo image showing the results of 37°C (top) and 25 ° C (bottom) incubation temperature.	17
Figure V. A photo image showing results of incubation with shaking (top) and incubation without shaking (bottom).....	17
Figure VI. A close up photo image of a positive stick showing the narrow band formed due to shaking during incubation.....	17
Figure VII. A photo image showing the results of PBS-T20 wash (top) and tap water wash (bottom).	18
Figure VIII. A photo image showing the results of substrates: ODN (top) and DAB (bottom).	18
Figure IX. A photo image showing the results of TCA treated serum (top) and TCA non-treated serum (bottom).....	19
Figure X. A photo image showing the results of TCA pre-treated centrifuged serum (top) and TCA pre-treated non-centrifuged serum (bottom).....	19
Figure XI. A photo image showing results of overnight (4°C) coating incubation (top) and 1 h coating incubation (bottom).....	19
Figure XII. A photo image showing the results of the dipstick Ag-ELISA detection level on serial dilutions of a <i>T. saginata</i> cysticercosis positive serum sample (P ₂) obtained from artificially infected cattle.	20

List of Annexes

Annex I. Test procedure for washing under a stream of PBS-T20 from a wash bottle.....	32
Annex II. Test procedure for 30 min incubation time and 45 min incubation time	33
Annex III. Test procedure for 25°C incubation temperature.....	34
Annex IV. Test procedure for incubation without shaking of the sticks	35
Annex V. Test procedure for tap water washing	36
Annex VI. Test procedure for DAB substrate	36
Annex VII. Test procedure for TCA non-treated serum	37
Annex VIII. Test procedure for TCA treated non-centrifuged serum	38
Annex IX. Test procedure for overnight coating and 1 h coating incubation	39
Annex X. A photo image showing the results of a serial dilution of a positive sample (P ₂) at 37°C incubation.....	39
Annex XI. A photo image showing the results of a serial dilution of a positive sample (P ₂) at 25°C incubation.....	40

List of abbreviations and acronyms

Ab-ELISA: Antibody detection ELISA
Ag-ELISA: Antigen detection ELISA
CT: Computer tomography
DAB: Diaminobenzidine
DMSO: Dimethyl sulfoxide
EITB: Electroimmunotransfer blot
ELISA: Enzyme-linked immunosorbent assay
ES: Excretory/secretory
GP: Glycoprotein
IgG: Immunoglobulin G
IgM: Immunoglobulin M
LLGP: Lentil-lectin purified glycoprotein
MoAb: Monoclonal antibody
MRI: Magnetic resonance imaging
NBCS: New born calf serum
NCC: Neurocysticercosis
ODN: o-dianisidine dihydrochloride
OPD: Orthophenylenediamine dihydrochloride
PBS-T20: Phosphate buffered saline + 0.5% Tween 20
RO-DI water: Reverse osmotic deionized water
Streptavidin-HRP: Horseradish peroxidase conjugated streptavidin
TCA: Trichloroacetic acid

Summary

Taeniosis/cysticercosis complex is a major public health problem worldwide. Control of this zoonosis requires a good diagnostic test to identify animals harboring live metacestodes. Visual meat inspection, which is the only public health measure implemented to control human infections, is a poorly sensitive method to detect such animals. The monoclonal antibody (MoAb)-158C11 and 60H8-based enzyme-linked immunosorbent assay (ELISA) for the detection of circulating antigens (Ag-ELISA) is a much more sensitive assay. However, expensive laboratory equipment is needed to carry out this test. Therefore, the aim of the present study was to develop a MoAb-158C11 and 60H8-based dipstick Ag-ELISA using the Nunc-immunoTM stick system (Maxisorp), in order to reduce the need for expensive equipment. Series of tests were done to determine the optimal condition of the dipstick Ag-ELISA using three positive and two negative serum samples. After determination of the optimal conditions, the assay was evaluated on cattle (n=30) and pig (n=15) serum samples. The performance level of the assay was determined on serial dilutions from a positive sample. The standard Ag-ELISA on microplates was used as a reference test. The dipstick Ag-ELISA detected a maximum dilution having 0.348 OD reading as measured on the standard Ag-ELISA. The standard Ag-ELISA detected a dilution with 0.170 OD reading. The dilution test showed that dipstick Ag-ELISA is less sensitive than standard Ag-ELISA. However, the assay detected all standard Ag-ELISA positive sera from cattle (n=11) and pigs (n=7) naturally infected with *Taenia saginata* and *Taenia solium* metacestodes, respectively. There was no false positive result, suggesting the specificity of the test. These findings, especially the result obtained from testing of serum samples from naturally infected cattle and pigs, suggest that the test is helpful for the diagnosis of *T. saginata* and *T. solium* cysticercosis. This assay can be used in settings where the microplate ELISA is impractical.

Keywords: Nunc-immunoTM, MaxiSorp, Dipstick, ELISA, Cysticercosis, *Taenia saginata*, *Taenia solium*, Cattle, Pig

Contribution by others

Serum samples used in this study were contributed by Dorien Vandersnickt and Dr. Nicolas Praet.

Chapter I: INTRODUCTION

Cysticercosis is a larval tapeworm infection acquired from ingestion of embryonated *Taenia saginata* (cattle) or *Taenia solium* (pigs and humans) eggs excreted with faeces from human carriers who harbour the adult tapeworm in the intestines. The hatched embryos migrate throughout the body and develop into cysticerci (Minozzo *et al.*, 2002; Boa *et al.*, 2002). In humans, the invasion of the central nervous system with *T. solium* cysticerci known as neurocysticercosis (NCC) is one of the emerging diseases worldwide, and the major cause of epileptic seizures in areas of endemicity (Cruz *et al.*, 1999; Garcia *et al.*, 1999b). Moreover, cysticercosis is an important cause of economic losses in pig and cattle production due to downgrading and/or condemnation of carcasses as untreated infected meat is an important source of this zoonosis (Carabin *et al.*, 2006; Phiri, 2006). Visual meat inspection, which is the only public health measure implemented to control human infections, is a poorly sensitive method and detects mainly heavily infected animals (Boa *et al.*, 2002; Wanzala *et al.*, 2003). Detection of cysticerci depends on the expertise of the meat inspector as well as on the stage of development of the cysticerci (Minozzo *et al.*, 2002; Wanzala *et al.*, 2003). The method is more sensitive to detect dead, degenerated, or calcified cysticerci than viable cysts, as they have the same pinkish-red color as the meat (Onyango-Abuje *et al.*, 1996; Wanzala *et al.*, 2003). Moreover, the method detects infection only after slaughter when it is too late to make decision on treatment.

The need for diagnostic tests superior to meat inspection have led to the development of serological tests. Antibody detection diagnostic tests indicate exposure to infection and not necessarily the presence of an established, viable infection. But from public health point of view only living metacestodes are important. Contrary to meat inspection and antibody detection methods, the monoclonal antibody (MoAb)-based enzyme-linked immunosorbent assays (ELISAs) for the detection of excretory/secretory (ES) products indicate infection with live metacestodes (Harrison *et al.*, 1989; Brandt *et al.*, 1992). The 158C11 and 60H8 MoAb based antigen detection ELISA (Ag-ELISA) is much more sensitive to identify active infections of *T. saginata* and *T. solium* metacestodes (Brandt *et al.*, 1992; Van Kerckhoven *et al.*, 1998; Dorny *et al.*, 2000). The assay is an important tool to detect individual cases or to screen populations for the presence of viable cysticerci. This information is required by public health services in order to make decisions on treatment or control programs (Erhart *et al.*, 2002; Wanzala *et al.*, 2002; Sikasunge *et al.*, 2008). The assay requires relatively expensive laboratory equipment. The dipstick methods are more applicable in the resource deprived field laboratories of developing countries (Birku *et al.*, 1999). Studies that have used the polystyrene surfaced Nunc-immunoTM dipsticks for the detection of pathogens in food samples demonstrated the usefulness of the sticks for immunological tests (Aldus *et al.*, 2003; Blazkova *et al.*, 2006). Therefore, the aim of this study was to develop a MoAb-158C11 and 60H8-based dipstick ELISA using the Nunc-immunoTM stick system (Maxisorp), in order to reduce the need for expensive equipment.

Chapter II: LITERATURE REVIEW

2.1. *Taenia saginata* and *Taenia solium* life cycle

2.1.1. *Taenia saginata*

Humans, the only final host, acquire *T. saginata* tapeworm infection from ingesting raw or undercooked infected beef. The development of the tapeworm is similar to that of *T. solium*. The tapeworm becomes sexually mature after three months and begins shedding gravid proglottids. When cattle ingest eggs, embryos hatch and activate under the influence of gastric and intestinal juices, and then penetrate the intestinal mucosa to reach the general circulation. Oncospheres develop primarily in skeletal and cardiac muscles and less frequently in fat and visceral organs. Cysticerci become infective to man in about ten weeks (Flisser *et al.*, 2005).

2.1.2. *Taenia solium*

Humans, the only final host, acquire adult *T. solium* infection through ingestion of undercooked pork infected with cysticerci (*Cysticercus cellulosae*). The cysticercus evaginates its scolex and attaches to the mucosa of the small intestines. The tapeworm develops and intermittently sheds gravid proglottids, which are expelled into the stool. Proglottids can be detected in stool samples from 8 to 12 weeks post-infection onwards. The intermediate host, typically the pig, is infected by ingesting parasite eggs or gravid proglottids. The oncosphere escapes from the eggs by the help of bile and digestive enzymes. The activated onchosphere penetrates the intestinal mucosa and is then transported via the vascular or lymphatic system to the skeletal muscles and other tissues of pigs. They evolve into larvae that enlarge and mature into cysticerci in approximately eight weeks. The lifecycle is completed when humans ingest pork contaminated with cysts. After “accidental” ingestion of the eggs from pigs contaminated food, the metacestodes can also develop in humans where they constitute a dead-end stage. Man can also become infected through fecal-oral autoinfection in patients harboring the adult parasite in their intestines, or when living in a contaminated environment (Carpio *et al.*, 1998; Garcia *et al.*, 2003a).

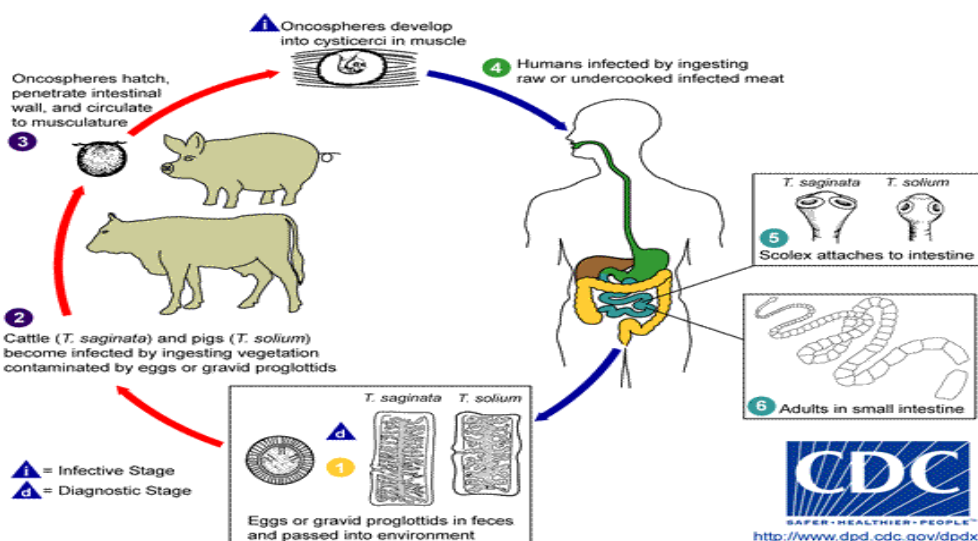


Figure I. Life cycle of *T. saginata* and *T. solium* (

http://www.stanford.edu/class/humbio103/ParaSites2006/Taenia_solium/Life%20Cycle.html)

2.2. Clinical symptoms of cysticercosis

2.2.1. Cattle

T. saginata cysticerci are randomly distributed throughout the skeletal and cardiac muscles, and visceral organs of infected cattle (Kyvsgaard *et al.*, 1990; Minozzo *et al.*, 2002). Mild symptoms such as increased pulse and respiratory rates, as well as a slight increase in body temperature depending on dose of infection were reported at early stages of experimental infections. The gross and microscopic evaluation of the lesions revealed local inflammatory reactions (Oryan *et al.*, 1998).

2.2.2. Humans

T. solium cysticerci can develop in any human tissues; however, the central nervous system (CNS), skeletal muscle, subcutaneous tissue and the eye are predilection sites. The clinical manifestation of human cysticercosis ranges from asymptomatic to deadly disease depending on the location and stage of development of the cysts. Most symptoms develop over a period of a few days, weeks, or months, with periods of remission and relapse, probably due to different evolutionary stages of the parasite. Three evolutionary phases of the parasite are known: active (when the parasite is alive), transitional (when in degenerative phase), and inactive (when with evidence of death) (Carpio *et al.*, 1998). The most severe form of human infection results from the presence of cysticerci in the subarachnoid space at the base of the skull or in the ventricles leading to epilepsy, psychic and demential signs and symptoms, and increased intracranial pressure, the last condition being responsible for the high lethality of the disease (Fleury *et al.*, 2007). *T. solium* cysticercosis is a possible risk factor for human brain tumors and hematological malignancies due to DNA damage induced by the genotoxic factors of the parasite secretions (Herrera *et al.*, 2000; Herrera *et al.*, 2003). Ocular cysticercosis commonly causes impaired vision, swelling of eyelid and subconjunctiva, diplopia and proptosis (Kaliaperumal *et al.*, 2005; Madigubba *et al.*, 2007). Solitary or disseminated forms of nodular swellings of subcutaneous and muscular cysticercosis are also frequently reported with diagnostic dilemma with solitary tumors (Bhigjee & Sanyika, 1999; Ergen *et al.*, 2005; Jankharia *et al.*, 2005).

2.2.3. Pigs

T. solium cysticerci are found in almost all tissues of pigs (Boa *et al.*, 2002), but mostly remain asymptomatic. A study on clinical signs of NCC in naturally infected swine showed excessive salivation, blinking and tearing. All infections with the clinical signs were confirmed by MRI (Prasad *et al.*, 2006).

2.3. Epidemiology of cysticercosis

2.3.1. *Taenia saginata*

T. saginata is distributed globally but the infection is particularly important in Africa, Latin America and Asia as well as Mediterranean countries. The prevalence rates vary from very low (0.03%) in North America and Europe, to very high in Africa and Latin America, 10 to 80% (Murrell, 2005). Eating raw or semi-raw beef is the major risk factor for infection in

humans. Transmission to cattle can occur via contaminated pastures, fodder, or water with *T. saginata* eggs. Infected farm workers and herdsman represent major risk factors in the epidemiology of bovine cysticercosis. Uncontrolled defecation and inadequate destruction of viable taenid eggs in sewage also play an important role in the spreading of *T. saginata* infection (Murrell, 2005; Dorny & Praet, 2007).

2.3.2. *Taenia solium*

T. solium is an important zoonosis in many pork-consuming developing countries of Latin America, Asia and Africa (Geerts, 1995; Rajshekhar *et al.*, 2003; Flisser *et al.*, 2003; Phiri *et al.*, 2003). In these countries consumption of uninspected pork is the major source of human *T. solium* taeniosis, and consequently a major risk factor for human and pig cysticercosis. Human cysticercosis is increasingly diagnosed in some developed countries due to increased immigration of tapeworm carriers from endemic areas (del la Garza *et al.*, 2005). It is reported that among the rising number of cases a proportion appears to be locally acquired due to household contact with tapeworm infected immigrants (Townes *et al.*, 2004; DeGiorgio *et al.*, 2005).

In endemic regions poor sanitary conditions and free roaming of domestic pigs are important factors in the transfer of *T. solium* infection from humans to pigs (Prasad *et al.*, 2007). Largely depending on the above factors the prevalence of porcine cysticercosis greatly varies within and/or between countries. For example, prevalence from 0.03% to 56% are reported in East and Southern Africa (Phiri *et al.*, 2003), similar variability in results have been obtained for other endemic countries (Rajshekhar *et al.*, 2003; Flisser *et al.*, 2003; Phiri *et al.*, 2003). However, most of the surveys were done with tongue palpation and post-mortem examination, which might have underestimated the actual prevalence.

2.4. Diagnosis of cysticercosis

2.4.1. Diagnosis of cysticercosis in cattle

2.4.1.1. Carcass inspection

Meat inspection of cattle is the only public health measure implemented to prevent *T. saginata* transmission to people and is based on the partial incision and inspection of target organs/muscle groups: heart, masseters, tongue, *triceps brachii*, and diaphragm; and careful visual search of *T. saginata* cysts. Although the density is higher in these organs/muscle groups cysticerci can be found in all body parts of cattle. For example, studies on naturally infected calves (n=25) have demonstrated that only 14.1% of the total carcass cysts were found in inspection sites indicated above and that only 5.8% of the total carcass cysts appear on cut surfaces created by meat inspection technique (Wanzala *et al.*, 2003). A higher proportion (34%) of cysts was found in the target organs/muscle groups in experimentally infected calves (n=23) without including *triceps brachii* (Kyvsgaard *et al.*, 1990). Meat inspection procedure detected 50% (n=24) of the carcass dissection positive and 48% (n=25) of Ag-ELISA positive naturally infected calves (Wanzala *et al.*, 2003). Another study found a sensitivity of 38% (n=60) in carcass dissection positive naturally infected calves. They demonstrated that 27% of animals with one to 10 cysts, 42.9% of animals with 11 to 20 cysts, and 77.8% with more than 20 cysts were revealed by the routine procedure (Walther & Koske, 1980). The lack of precision of the visual identification of specific cysts might

overestimate the prevalence through misdiagnosis of other morphological alterations in affected muscles. It is difficult to differentiate between old lesions caused by cysticerci and other lesions. For example, of meat inspection identified *T. saginata* cysticercus lesions only 76.7% (n=335) of them gave positive PCR result: 91% of 78 viable cysts and 70.7% of 239 dead (degenerating and calcified) cysts (Geysen *et al.*, 2007). In another study PCR confirmed only 52.4% of the cysticercus lesions recovered during routine meat inspection: 80% of 25 viable cysts and 49.6% of 242 dead cysts (Abuseir *et al.*, 2006).

2.4.1.2. Enzyme-Linked Immunosorbent Assay

2.4.1.2.1. Antibody detection ELISA

The antibody detection ELISA (Ab-ELISA) for the diagnosis of *T. saginata* cysticercosis detects specific antibodies in serum of infected cattle from three weeks post-infection onwards (Kamanga-Sollo *et al.*, 1987). The efficiency of the assay depends on the quality of the diagnostic antigen used. The diagnostic antigens are either from crude homogenates of *T. saginata* cysticerci, or from cyst fluids and/or crude homogenates of related parasites *T. hydatigena*, *T. crassiceps* and *T. solium* (Craig & Rickard, 1980; Geerts *et al.*, 1981; Kamanga-Sollo *et al.*, 1987; Monteiro *et al.*, 2006). Such crude antigens contain species aspecific isotopes and give cross-reactions with serum from cattle infected with heterologous helminths, such as *Fasciola hepatica*, and *T. hydatigena* (Craig & Rickard, 1980). Since *T. hydatigena* metacestode is not commonly found in cattle, cross reaction may not present a major drawback in practice (Geerts *et al.*, 1981). Ab-ELISA using saline extracted antigens from different structures of *T. solium* and *T. crassiceps* showed a high number of false-negative reactions to the sera of naturally infected animals due to light infections resulting in an immunological response with low antibody production. Such assays are deficient to diagnose lightly infected animals at slaughterhouses (Monteiro *et al.*, 2006). In natural conditions light grade infections are common. For example, an ELISA using hydrosoluble antigens of *T. crassiceps* demonstrated a sensitivity and specificity of 37.5% (n=24) and 95.7% (n=69), respectively (Geerts *et al.*, 1981). The ELISA using a recombinant antigen of *T. saginata* oncosphere adhesion protein (Hp6-Tsag) showed 100% sensitivity and a specificity of 93.2% in experimentally infected cattle (Ferrer *et al.*, 2007).

2.4.1.2.2. Antigen detection ELISA

The level of antibody titer did not correspond with live cysticerci burden in naturally infected animals (Onyango-Abuje *et al.*, 1996). In slaughterhouse cattle antigen assay was found three times as sensitive as meat inspection. Furthermore, the assay detects live cysticerci which are most likely missed, as they have the same pinkish-red color as the meat (Onyango-Abuje *et al.*, 1996; Wanzala *et al.*, 2003). Two MoAb-based ELISA systems, HP10 and (158C11 and 60H8), were developed for the detection of *T. saginata* cysticercosis (Harrison *et al.*, 1989; Brandt *et al.*, 1992; Van Kerckhoven *et al.*, 1998). Both assays recognized the circulating glycoprotein antigens secreted by the viable metacestodes from four weeks after infection onwards (Harrison *et al.*, 1989; Onyango-Abuje *et al.*, 1996). A mouse monoclonal antibody IgM coded HP10, developed against glycoproteins from surface enriched extract of *T. saginata* cysticerci, was used for the development of a diagnostic ELISA to detect these glycoproteins in the serum of *T. saginata* infected cattle. The assay performed best when homologous HP10 MoAbs are used. With the exception of the one useful cross-reaction with *T. solium*, the assay exhibited acceptable specificity. However, only cattle harboring 200 live 8 to 16 weeks-old cysticerci were consistently detected (Harrison *et al.*, 1989). In a recent

study conducted in Kenya the assay detected 75% (n=20) of naturally infected cattle harboring one or more live cyst(s) at carcass dissection. All animals with five and more cysts were Ag-ELISA positive (Wanzala *et al.*, 2007). Another study found a sensitivity of 83% (n=6) for animals with ≥ 30 live cysticerci, which dropped to 22% (n=23) for animals with 1-29 live cysts (Onyango-Abuje *et al.*, 1996).

The other MoAb-based antigen detection ELISA system was developed by Brandt *et al.* (1992) and modified by Van Kerckhoven *et al.* (1998) and Dorny *et al.* (2000). Two MoAbs (158C11 and 60H8) of the IgG1 isotype were produced against the secretion and excretion products (ES) of *T. saginata* cysticerci. The assay yielded a sensitivity of 92% and a specificity of 98.7% in heat treated sera from cattle harbouring more than 50 viable cysts. Only 12.8% (n=47) of animals carrying less than 50 viable cysts were detected (Van Kerckhoven *et al.*, 1998). The low sensitivity of both Ag-ELISA systems indicate that the assays likely fail to detect animals with light infections, which is very common condition (Brandt *et al.*, 1992).

2.4.1.3. Electroimmunotransfer Blot

There is limited information on the use of the electroimmunotransfer blot (EITB) for the diagnosis *T. saginata* cysticercosis. A hydrophobic fraction, 10 to 18 kDa, isolated from cyst fluid of *T. hydatigena* metacestodes, collected from naturally infected goats, was evaluated in immunoblot and dot blot procedures and detected *T. saginata* infection in 14 and 17 out of 21 calves, respectively (Bogh *et al.*, 1995). To our knowledge there is no study describing the use of an EITB that employs purified glycoprotein diagnostic antigens (Tsang *et al.*, 1989; Ito *et al.*, 1998) for the detection of *T. saginata* cysticercosis.

2.4.1.4. Dipstick-immunoassay

Although dipstick immunoassays are more users friendly as compared to traditional Ag-ELISA and EITB, there have been limited researches done towards the development and standardization of such assays. Hayunga *et al.* (1991a) developed a dipstick antibody detection ELISA for the diagnosis of *T. saginata* and *T. solium* cysticercosis using ammonium sulphate-soluble fractions of *T. hydatigena* cyst fluid antigen (ThFAS) adsorbed on Immobilon P membrane dipsticks. The assay detected 6 out of 7 (85.7%) cysticercotic cattle three weeks after experimental infection (Hayunga *et al.*, 1991b).

2.4.1.5. Dot-ELISA

Although considerable progress has been made to develop simple dot-immunoassays for *T. saginata* cysticercosis, the techniques are not standardized (Jiang *et al.*, 1990; Draelants *et al.*, 1995b; Biswas *et al.*, 2004; Agudelo *et al.*, 2005). Draelants *et al.* (1995b) developed an antigen detection dot-ELISA using MoAbs (2H8 and 12G5) of IgM isotype and nitrocellulose membrane as described by Brandt *et al.* (1992). The assay gave 87.5% and 93.5% sensitivity and specificity, respectively in cattle with more than 100 viable cysts. The values are very similar to those obtained with a sandwich ELISA.

2.4.2. Diagnosis of cysticercosis in humans

2.4.2.1. Imaging techniques

Imaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound permit visualization and identification of the parasite in the brain, eye, and other organs (Carpio *et al.*, 1998; Jankharia *et al.*, 2005; Kalra *et al.*, 2006). The pathological findings depend on the stages of evolution of the parasite. The oncospheres that pass into the brain parenchyma evolve through vesicular, colloidal, granular-nodular, and calcified phases. CT scans and MRIs are the most sensitive and specific imaging tools that can identify these four phases. MRI is more useful in detecting non-calcified parenchymal, subarachnoid, or ventricular cysts and the presence of associated inflammation, whereas the CT scan is referred to for the detection of parenchymal calcifications (Carpio *et al.*, 1998). The techniques are also useful to judge the need for medical or surgical treatments, and assess the effectiveness of interventions (Nash *et al.*, 2005). In some cases the lesion might mimic lesions from other infectious or noninfectious diseases and correct diagnosis might require the use of additional immunodiagnostic tests (Garcia *et al.*, 2000; Suzuki *et al.*, 2007). The major drawback of the neuroimaging techniques are their high cost and restricted availability in developing countries (Garcia & Del Brutto, 2003). Ultrasound is commonly used for the diagnosis of extraneural cysticercosis (Sundaram *et al.*, 2004; Jankharia *et al.*, 2005).

2.4.2.2. Electroimmunotransfer Blot

The EITB that utilizes lentil-lectin purified glycoproteins (LL-GP) had a profound impact on the diagnosis of *T. solium* cysticercosis based on antibody detection. To produce the assay, cysts from naturally infected pigs are homogenized and solubilised in urea. The resultant extract is eventually purified using lentil-lectin affinity chromatography. The LL-GPs are separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to nitrocellulose membranes. The GPs contain seven major bands designated as Gp50, Gp42-39, Gp24, Gp21, Gp18, Gp14, and Gp13. The EITB detects antibodies against any one of the seven LL-GP antigens. The original description and evaluation of the EITB was performed using sera and/or cerebrospinal fluid from parasitologically confirmed cases. This assay was very sensitive (98%) and specific (100%). No sera from *Echinococcus granulosus*/*E. multilocularis* (n=34) and other heterologous infections (n=376) recognized the GP bands (Tsang *et al.*, 1989). In pathologically and CT and/or MRI confirmed population with two or more lesions the EITB demonstrated 94% (n=32) sensitivity. However, the sensitivity was only 28% (n=18) in patients with a single lesion (Wilson *et al.*, 1991). The LL-GP antigen mixtures show non-specific reaction, particularly GP bands higher than LL-GP region and can therefore not be used in ELISA format (Tsang *et al.*, 1989). The assay has been used for epidemiological surveys of human cysticercosis (Garcia *et al.*, 1991; Garcia *et al.*, 1999a; Rodriguez-Hidalgo *et al.*, 2006).

A simple method to purify GPs using preparative isoelectric-focusing electrophoresis (pH 9.2-9.6) from cyst fluid and/or intact cyst of *T. solium* has been described by Ito *et al.* (1998). The purified component contained at least three major bands ranging from 10 to 26 kDa, when separated using SDS-PAGE. The GPs were evaluated using human sera from cysticercosis patients (n=53), echinococcosis (n=100) and other diseases (n=107) in the EITB format. Sera from *T. saginata* and *T. s. asiatica* infected people were not recognized by the GPs. The EITB showed 100% specificity and sensitivity. These GP antigens are also applicable in ELISA format.

The EITB that utilizes a 10 kDa native antigen, a subunit component of a 150 kDa complex, purified from fluid of *T. solium* cyst showed a sensitivity of 85% (n=247) and specificity of 90% in detecting cysticercosis in human serum (Yang *et al.*, 1998). Its recombinant form gave 97% (n=190) sensitivity and 98% (n=180) specificity in sera from patients with active NCC (Chung *et al.*, 1999). Synthetic proteins of 8 kDa from LLGPs were also used in EITB format. The assay demonstrated sensitivity of 82-94% in sera (n=107) from patients with two or more viable cysts and specificity of 76-100% (Scheel *et al.*, 2005).

2.4.2.3. Enzyme-Linked Immunosorbent Assay

2.4.2.3.1. Antibody detection ELISA

The diagnostic efficiency of antibody detection immunological tests relies on the quality of antigens used (Pardini *et al.*, 2002). Antigens used in most Ab-ELISAs for the diagnosis of *T. solium* cysticercosis are produced either from cyst fluids, crude homogenates of the cysticerci or from the related parasite *T. crassiceps*. ELISAs using antigens derived from cyst fluid produce relatively more specific results compared to those using antigens derived from crude extract of whole parasite or cyst membranes (Bueno *et al.*, 2001; Arruda *et al.*, 2005; Ishida *et al.*, 2006). Excretory/secretory products of the parasite produced *in vitro* were also used in the ELISA format (Ko & Ng, 1998). These unpurified antigens have shown cross reactivity with non-specific infections such as *T. hydatigena*, *E. granulosus*, *Hymenolepis nana*, and *Ascaris suum* (Montenegro *et al.*, 1994; Ko & Ng, 1998; Pinto *et al.*, 2000). Such ELISAs coated with crude or partially purified parasite products are better for screening than for confirmatory diagnosis (Bueno *et al.*, 2001).

Improved antigen purification techniques have lead to much more specific results (Parkhouse & Harrison, 1987; Montenegro *et al.*, 1994; Ito *et al.*, 1998; Machado *et al.*, 2007). The ELISA using the isoelectric-focusing purified glycoprotein antigen from cyst fluid showed a sensitivity and specificity of 100% in sera (n=53) of confirmed NCC patients (Ito *et al.*, 1998). Recombinant or synthetic antigens have been developed for use in ELISA format (Chung *et al.*, 1999; Hancock *et al.*, 2003; Hancock *et al.*, 2004). Most recombinant antigens are component of the LLGP fraction employed in EITB (Greene *et al.*, 2000; Sako *et al.*, 2000; Hancock *et al.*, 2003). Evaluation of an 8 kDa synthetic antigen in ELISA format using serum from confirmed cysticercosis patients showed a sensitivity and specificity of 90.4% (n=52) and 90.3%, respectively (Bueno *et al.*, 2005). Similar results were found by Scheel *et al.* (2005). The polypeptide was seen as a diagnostic protein component of GP14 to GP42-39 bands of LLGP (Hancock *et al.*, 2003). The ELISA using recombinant GP50 antigen was 94.7% (n=57) sensitive and 93.8% specific. There was no significant difference in performance of the assay from using synthetic 8 kDa and recombinant GP50 antigens (Bueno *et al.*, 2005). The ELISAs using glycoproteins purified according to Ito *et al.* (1998) from *T. solium* cyst fluid and their recombinant antigens (Ag1V1/Ag2 chimeric protein) were compared in the serologic detection of human cysticercosis. The recombinant antigen detected 93.3% (n= 60) of human NCC cases, and the sensitivity did not statistically differ from the ELISA using affinity purified glycoproteins which was 100% (Sato *et al.*, 2006).

2.4.2.3.2. Antigen detection ELISA

The presence of antibodies does not constitute direct evidence of a living parasite within the host (Garcia *et al.*, 1997; Fleury *et al.*, 2007). It may indicate transient antibodies from

exposure to infection (Garcia *et al.*, 2001) and/or persisting antibodies of previously established infection after elimination due to immune mechanism and/or drug therapy (Harrison *et al.*, 1989; Garcia *et al.*, 1997). In order to overcome such limitations, several attempts were made to develop antigen detection ELISAs using monoclonal antibodies (Harrison *et al.*, 1989; Brandt *et al.*, 1992; Van Kerckhoven *et al.*, 1998). The monoclonal antibodies recognize repetitive epitopes of circulating glycoprotein antigens (40 to 200 kDa) present on the surface and in the secretion of metacestodes (Harrison *et al.*, 1989; Draelants *et al.*, 1995a). A mouse monoclonal antibody IgM coded HP10, developed against glycoproteins from surface enriched extract of *T. saginata* cysticerci (Harrison *et al.*, 1989), is used for the detection of these glycoproteins in the serum of *T. solium* infected people. The serum HP10 antigen detection ELISA was evaluated using serum from patients with severe NCC due to subarachnoidal and intracranial *T. solium* cysticerci. The assay displayed 84.8% (n=46) sensitivity and 94% specificity in serum from patients with active infection (Fleury *et al.*, 2007). In another study a similar sensitivity (85%) and specificity (92%) was found (Garcia *et al.*, 2000). The assay is used for serodiagnosis and follow-up of NCC (Garcia *et al.*, 2000; Garcia, 2007).

Another MoAb-based antigen detection ELISA system was developed by Brandt *et al.* (1992) and modified by Van Kerckhoven *et al.* (1998) and Dorny *et al.* (2000). Two MoAbs (158C11 and 60H8) of the IgG1 isotype were produced against the secretion and excretion (ES) of *T. saginata* cysticerci (Van Kerckhoven *et al.*, 1998). The (158C11 and 60H8) monoclonal antibody-based ELISA is being used for clinical management and epidemiological surveys of human cysticercosis (Erhart *et al.*, 2002; Prado-Jean *et al.*, 2007). The sharing of antigens between the metacestode and adult tapeworm might influence seropositivity in endemic areas (Draelants *et al.*, 1995a; Correa *et al.*, 1999). In the hamster model of taeniosis, adult antigens have been demonstrated to cross the intestinal epithelium and enter the circulation (Correa *et al.*, 2002).

The antigen detection assays that utilize polyclonal antibodies or monoclonal antibodies for the detection of immunodominant antigens (e.g. antigen B) lack reproducibility (Draelants *et al.*, 1995a). One reason for this observation could be the rapid sequestration of antigens in host tissues adjacent to the parasite, ingested by phagocytes or transported to lymphoid tissues, where they may thus escape detection (Correa *et al.*, 2002). Other monoclonal antibodies were also evaluated for use in antigen serodiagnostic assays and obtained variable results (Chen *et al.*, 1991; Lin *et al.*, 1999).

2.4.2.4. Dot ELISA

The performance of antibody detection dot-ELISAs depends on antigens and/or reference tests used. The complete homogenate of *T. solium* cyst antigen dotted on nitrocellulose membrane detected antibodies in 56.5% (n=23) sera from patients with CT/MRI confirmed NCC. The assay was 92% specific (Biswas *et al.*, 2004). In a clinically and laboratory confirmed NCC patient population dot-ELISA had 91.1% (n=45) sensitivity and 100% specificity. The assay detected 58.3% (n=12) of LLGP EITB positive individuals during immunological screening of endemic population (Agudelo *et al.*, 2005).

2.4.3. Diagnosis of cysticercosis in pigs

2.4.3.1. Carcass inspection

Meat inspection is the only diagnostic method carried out on large scale in slaughterhouses for the post-mortem detection of pig cysticercosis. The method is more sensitive to detect dead, degenerated, or calcified cysticerci; but is most likely to miss quite a number of viable cysticerci, as they have the same pinkish-red color as the meat (Wanzala *et al.*, 2003). The procedure is based on the partial incision and careful observation in the “predilection” sites (sites with higher density of cysts than elsewhere in the carcass): heart, masseters, tongue, and *triceps brachii* (Boa *et al.*, 2002). The technique has demonstrated low sensitivity. Dorny *et al.* (2004) estimated a sensitivity of 22.1% and specificity of 100%. In Zambia, meat inspection detected 38.7% (n=31) of total carcass dissection positive pigs (Phiri *et al.*, 2006). Boa *et al.* (2002) showed that routine meat inspection involving visual inspection of incised and intact surfaces of heart, tongue, external and internal masseter muscles, and *triceps brachii* muscles can only reveal 10.6% of the total carcass cysts. In other words, the inspection can only detect 10.6% of infected animals. The study also showed that the routine inspection sites contained 18.5% of the total carcass cysts.

2.4.3.2. Tongue palpation

Tongue examination for detection of *T. solium* cysts in live pigs by palpation and visual inspection is a low cost ante-mortem diagnostic method of porcine cysticercosis. The technique has a high specificity (100%), but generally low sensitivity (Dorny *et al.*, 2004; Phiri *et al.*, 2006). The sensitivity of the method depends on the level of infection. Approximately, 76 % and 78% of positive tongue palpation pigs were found seropositive in ELISA and EITB, respectively (Sato *et al.*, 2003). The diagnostic antigens were isoelectric-focusing purified glycoproteins according to Ito *et al.* (1998). In an endemic area, the tongue palpation detected up to 70.8% (n=24) of meat inspection positive pigs (Gonzalez *et al.*, 1990). In comparison to total carcass dissection as low as 16% sensitivity was also recorded (Phiri *et al.*, 2006). Despite the low sensitivity the method is used in epidemiological studies of porcine cysticercosis (Sarti *et al.*, 1992; Mutua *et al.*, 2007; Sikasunge *et al.*, 2008). In a pig population (n=1691) tongue palpation showed prevalence of 10.8% while this was 23.3% by Ag-ELISA (Sikasunge *et al.*, 2008).

2.4.3.3. Electroimmunotransfer Blot

The EITBs are the most sensitive and specific assays for the detection of antibodies specific to *T. solium* cysticercosis in pigs. The EITB using LLGPs (Tsang *et al.*, 1989) was evaluated on serum samples (n=45) from naturally infected pigs with *T. solium* and other heterologous infections including echinococcosis. The assay was determined to be 100% sensitive and specific. It detected antibodies in experimentally infected pigs between 5 and 8 weeks post infection (Tsang *et al.*, 1991). This assay has been extensively used in the epidemiological surveys of pig cysticercosis (Sakai *et al.*, 1998; Garcia *et al.*, 2003; Krecek *et al.*, 2008). The EITB using isoelectric-focusing purified GPs (Ito *et al.*, 1998) was also evaluated on serum samples (n=8) from naturally infected pigs and found similar results (Ito *et al.*, 1999). Another EITB assay using saline extracted *T. solium* metacestode crude somatic antigen mixture (8, 11, 16 and 23 kDa) had a sensitivity and specificity of 90% (n=20) and 100%

(n=25), respectively, in pig serum of natural infection (confirmed at necropsy). No heterologous sera (n=26) reacted to these bands. 46 kDa antigen band cross reacted with serum from *E. granulosus* infected pigs (Pathak *et al.*, 1994).

2.4.3.4. Enzyme-Linked Immunosorbent Assay

2.4.3.4.1. Antibody detection ELISA

The assay detects IgG. Antigens used for coating of most ELISAs for the detection of antibodies against *T. solium* cysticercosis in pig serum are from cyst fluid or crude homogenates of the *T. solium* cysticerci, or from the related parasite *T. crassiceps* (Nunes *et al.*, 2000). The excretory/secretory (ES) products of the parasite produced *in vitro* were also used (Ko & Ng, 1998; D'Souza & Hafeez, 1999). The ELISA using ES diagnostic antigens demonstrated 92% sensitivity and 100% specificity in naturally infected pigs (D'Souza & Hafeez, 1999). The sensitivities of the assays were higher when cyst fluid is used as diagnostic antigens (Nunes *et al.*, 2000; Pinto *et al.*, 2000). These crude antigens have shown cross reactions with sera from pigs infected with *T. hydatigena*, *E. granulosus*, *Ascaris suum*, *Fasiolopsis buski*, *Hymenolepis diminuta*, and *Diplydium caninum* (Kumar & Gaur, 1987; Cheng & Ko, 1991; Ko & Ng, 1998; Pinto *et al.*, 2000). Saline extracted crude somatic antigen of *T. solium* metacestode gave a sensitivity of 70% (n=20) and specificity 100% (n=25) when tested on Ab-ELISA format. However, four of 15 pigs infected with *E. granulosus* were positive (Pathak *et al.*, 1994).

Fractionation and/or purification of the antigens have improved the specificity of the assay (Ito *et al.*, 1998; Assana *et al.*, 2007). A fraction of 14 kDa antigen purified using an ion exchange column on high performance liquid chromatography from crude cyst fluid of *T. solium* was found specific (Assana *et al.*, 2007). The isoelectric-focusing purified glycoprotein antigens from cyst fluid (Ito *et al.*, 1998) have shown a specificity and sensitivity of 100% (n= 8) in detecting antibodies against *T. solium* metacestodes in pig serum (Ito *et al.*, 1999). The sensitivities of ELISAs using these glycoprotein antigens and their recombinant chimeric protein antigens showed no statistical difference. They detected 87% (n=30) of *T. solium* cysticercosis positive pigs (Sato *et al.*, 2006).

2.4.3.4.2. Antigen detection ELISA

The detection of viable cysts is achieved through capturing circulating antigens by MoAbs. The two MoAb-based ELISA systems (Harrison *et al.*, 1989; Brandt *et al.*, 1992; Van Kerckhoven *et al.*, 1998) explained in the paragraph on antigen detection ELISA for diagnosis of human cysticercosis (see 2.4.2.3.2) are also used for detection of circulating glycoprotein antigens of viable metacestodes in pig serum. The assays could detect antigens in serum of pigs harbouring live cysticerci from four weeks after infection onwards, in contrast no antigen is detected in those containing only dead cysticerci (Nguekam *et al.*, 2003). Both HP10 and (158C11 and 60H8) ELISAs are being used for the study of the epidemiology of *T. solium* infections in pig populations (Sciutto *et al.*, 1998; Sikasunge *et al.*, 2008). The genus-specificity of the assay limited differentiation between metacestode infections of *T. solium* and *T. hydatigena* in pigs (Dorny *et al.*, 2003).

The objectives of this study are:

1. To develop a MoAb-158C11 and 60H8-based dipstick ELISA for the detection of circulating antigens of *T. saginata*/*T. solium* metacestodes using the Nunc-immunoTM stick system (Maxisorp).
2. To evaluate the performance of the dipstick ELISA in comparison to the standard microplate Ag-ELISA.

Chapter III: MATERIALS AND METHODS

3.1. Serum samples

Four negative and three positive bovine serum samples were used in the tests done for the optimization of a dipstick ELISA protocol. The optical density (OD) measured after analysis with the standard microplate Ag-ELISA (Dorny *et al.*, 2002) for the three positive sera obtained from artificially infected cattle were about 1.9 (P₃) and 2.9 (P₁ and P₂) and that of all negative sera were below 0.05. More sera from slaughter cattle (positive=11 and negative=19) and pigs (positive=7 and negative=8) were tested using the adapted protocol to evaluate its performance in comparison with the standard microplate Ag-ELISA. The number of samples was limited due to the availability of reference sera.

3.2. Trichloroacetic acid (TCA) treatment of serum

The TCA pre-treatment of serum samples was done as previously described by Dorny *et al.* (2000). An equal volume of serum and freshly prepared 5% TCA (Sigma, Chemical Co.) w/v in RO-DI water were vortex mixed, and incubated for 20 min at room temperature. After revortexing, the mixture was centrifuged for 9 min at 12,000g. Finally, the supernatant was mixed with an equal volume of neutralization buffer (0.156 M sodium carbonate/bicarbonate, pH 10.0), which gave a final dilution of 1:4.

3.3. Microplate Ag-ELISA

The microplate Ag-ELISA was done as previously described by Dorny *et al.* (2002). Polystyrene 96-microwell ELISA plates (Nunc® Maxisorp) were coated with 100 µl MoAb B₁₅₈C₁₁A₁₀ (5 µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6) and incubated for 30 min at 37°C while shaking. The plates were washed once with phosphate buffered saline (PBS) containing 0.05% w/v Tween 20 (Merck, Germany), pH 7.3 (referred as PBS-T20). Blocking was done with 1% new born calf serum (NBCS) in PBS-T20 (150 µl/well) and incubated for 15 min at 37°C while shaking. After emptying the wells, 100 µl of the pre-treated serum was added and incubated at 37°C for 15 min while shaking. All samples were run in duplicate. After washing three times with PBS-T20, biotinylated MoAb B₆₀H₈A₄ (100µl at 1.25 µg/ml in 1% NBCS + PBS-T20) was added and incubated at 37°C for 15 min while shaking. After washing three times with PBS-T20, 100 µl of horseradish peroxidase conjugated streptavidin (Streptavidin-HRP) diluted 1/10000 in 1% NBCS + PBS-T20 was added and incubated for 15 min at 37°C while shaking. Then, the wells were washed three times with PBS-T20; and finally, 100 µl of substrate solution (orthophenylenediamine dihydrochloride (OPD) in RO-DI water) + 30% v/v H₂O₂ was added. After incubation for 15 min at 30°C in the dark without shaking, 50 µl of 4N H₂SO₄ was added to halt the reaction. The plates were read with the help of an automated spectrometer (ThermoLabsystems/Multiskan EX ELISA reader) at wavelengths of 492 nm and 655 nm. A sample was considered positive if the value of its OD reading was significantly above the average OD readings of the negative reference sera (n=8) at a probability level of p= 0.001 (Sokal & Rohlf, 1981).

3.4. Dipstick Ag-ELISA

3.4.1. Initial reference protocol

A dipstick device consisting of a polystyrene paddle stick (Nunc-Immuno™ Stick, Maxisorp) which fits into a polypropylene tube was used in a dipstick Ag-ELISA. This initial protocol was designed by modifying the standard microplate Ag-ELISA (Van Kerckhoven *et al.*, 1998; Dorny *et al.*, 2000; Dorny *et al.*, 2002) and used as a reference protocol for tests done during protocol optimization. Briefly, sticks were coated with 300 µl MoAb B₁₅₈C₁₁A₁₀ (5µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6), and incubated for 1 h at 37°C while shaking. MoAb B₁₅₈C₁₁A₁₀ was added into tubes and then the corresponding sticks were tightly fitted before incubation (works for all incubations). Unbound antibody was washed: sticks were dipped five times into washing solution (PBS-T20) and tubes were rinsed three times with PBS-T20. Fresh PBS-T20 was used to wash each stick; the containers (small glass beakers) were thoroughly washed under tap water to avoid cross contaminations (works for all washings). Blocking was done with 1% NBCS in PBS-T20 (1 ml/tube), and incubated for 15 min at 37°C while shaking. Only the tubes were washed. TCA pre-treated serum (150 µl per tube) was then added into the tubes, and incubated for 1 h at 37°C while shaking. After washing, 300 µl biotinylated MoAb B₆₀H₈A₄ (1.25 µg/ml in 1% NBCS + PBS-T20) was added, and incubated at 37°C for 1 h while shaking. After washing, 300 µl horseradish peroxidase conjugated streptavidin (1:10 000 in 1% NBCS + PBS-T20) was added, and incubated at 37°C for 1 h while shaking. After final washing, 300 µl precipitating substrate solution (15 mg o-dianisidine dihydrochloride (ODN) in 2.5 ml DMSO + 22.5 ml RO-DI water + 25 ml PBS) +20 µl 30% v/v H₂O₂ was added, and incubated at room temperature for 30 min without shaking. The sticks were washed by inserting into RO-DI water to stop the reaction. Orange color on the tip sticks, as examined visually, indicated the presence of circulating antigen.

3.4.2. Protocol optimization tests

Series of tests were performed by modifying the initial protocol described under 4.4.1. The modified (index test) protocol and the initial reference protocol were always done in parallel, and their results were compared to determine which factor to consider in the new protocol. One factor was changed at a time. Ultimately, a new protocol was developed. Only procedures of the modified (index test) protocols are described in this section.

Test N°1: Washing under a stream of PBS-T20

Five known (three positive and two negative) serum samples were prepared in duplicate to compare dipping washing and under a stream washing. The latter was done by washing the sticks under a gentle stream of PBS-T20 from a wash bottle for a few seconds. The tubes were rinsed three times with PBS-T20 from the same bottle. The details of the procedure of the stream washing are presented in Annex I.

Test N°2: Incubation time

Five known samples (two negative and three positive) were prepared in three replicates and incubated in three incubation time settings: 30 min (group 1), 45 min (group 2) and 60 min. The details of the procedure of 30 min (group 1) and 45 min (group 2) incubation times are presented in Annex II.

Test N°3: Incubation temperature

Five known samples (two negative and three positive) were prepared in duplicate and incubated at two incubation temperatures: 25 °C and 37°C. The details of the procedure of 25 °C are presented in Annex III.

Test N°4: Incubation without shaking

Five known samples (three positive and two negative) were prepared in duplicate. One group was incubated with shaking and the other incubated without shaking. The details of the procedure of the incubation without shaking are presented in Annex IV.

Test N°5: Water washing

Five known samples (three positive and two negative) were prepared in duplicate to compare PBS-T20 washing and tap water washing. In the latter, tap water was used to wash one group of sticks. The sticks were dipped into tap water five times. The tubes were also washed three times with tap water. The details of the procedure are presented in Annex V.

Test N°6: Diaminobenzidine (DAB) substrate

Five known samples (three positive and two negative) were prepared in duplicate, and tested with two substrates: ODN and DAB. One gram of DAB powder was diluted in 20 ml PBS from which aliquots of 100 µl were prepared. The 100 µl aliquot was further diluted in 10 ml PBS for use. The details of the procedure are presented in Annex VI.

Test N°7: TCA non-treated serum

Five known samples (three positive and two negative) were tested in duplicate using TCA treated serum and TCA non-treated serum. In the latter, sera were diluted ¼ in PBS and used directly without TCA pre-treatment. The details of the procedure are presented in Annex VII.

Test N°8: TCA treated non-centrifuged serum

Five known samples (three positive and two negative) were prepared in duplicate to compare TCA treated centrifuged serum and TCA treated non-centrifuged serum. In the latter, sera were pre-treated with an equal volume of TCA. Without centrifugation, an equal volume of neutralization buffer was added to the TCA precipitated serum and thoroughly mixed. The details of the procedure are presented in Annex VIII.

Test N°9: Increased incubation times for coating and sample

Five known (three positive and two negative) samples were prepared in duplicate to study if a longer incubation time for the coating and sample steps could improve the test performance compared to 1 h incubation time without shaking. The sticks were coated overnight at 4°C. The incubation time for the sample step was 1.5 h. The details of the procedure are presented in Annex IX.

3.5. Adapted protocol for dipstick Ag-ELISA

Sticks were coated with 300 µl MoAb B₁₅₈C₁₁A₁₀ (5µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6), and incubated for 1 h at 25°C while shaking. Unbound antibody was washed by dipping the sticks five times in PBS-T20 and rinsing the tubes three times with PBS-T20. Fresh PBS-T20 was used to wash each stick, and the containers were thoroughly washed under tap water to avoid cross contaminations. Blocking was done with 1% NBCS in PBS-

T20 (1 ml/tube), and incubated for 15 min at 25°C while shaking. Only the tubes were rinsed three times. TCA pre-treated serum (150 µl per tube) was then added into tubes, and incubated for 1 h at 25°C while shaking. After washing, 300 µl biotinylated MoAb B₆₀H₈A₄ (1.25 µg/ml in 1% NBCS + PBS-T20) was added; and incubated at 25°C for 1 h while shaking. After washing, 300 µl horseradish peroxidase conjugated streptavidin (1:10 000 in 1% NBCS + PBS-T20) was added; and incubated at 25°C for 1 h while shaking. After final washing, 300 µl precipitating substrate solution (15 mg o-dianisidine dihydrochloride (ODN) in 2.5 ml DMSO + 22.5 ml RO-DI water + 25 ml PBS) + 20 µl 30% v/v H₂O₂ was added; and incubated at room temperature for 30 min without shaking. The sticks were washed by dipping into RO-DI water to stop the reaction. Red color on the tip of the sticks, as examined visually, indicated the presence of circulating antigen.

3.5.1. Determination of the detection level

A serial dilution of a serum sample was done to evaluate the detection level of the adapted protocol described under 4.5. All dilutions of the serum were also tested in parallel using the microplate Ag-ELISA (cut off= 0.146).

3.5.2. Evaluation of the dipstick Ag-ELISA

The adapted protocol was used to test more samples for the detection of circulating *T. saginata* and *T. solium* ES antigens. A total of 30 cattle sera and 15 pig sera were tested. These samples were simultaneously tested with the microplate Ag-ELISA. The cut offs were 0.151(cattle) and 0.083 (pig).

Chapter IV: RESULTS

4.1. Protocol optimization tests

Test N°1: Washing under a stream of PBS-T20

The test identified all positive (3/3) samples from negative (2/2) samples. The color of positive sticks did not differ between dip washing and stream washing methods (figure II).

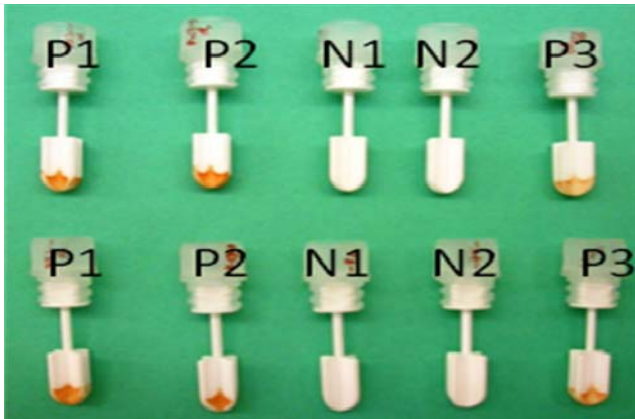


Figure II. A photo image showing the results of a dip washing (top) and a stream washing (bottom) methods.

Test N°2: Incubation time

Three incubation times (30 min, 45 min and 60 min) were compared. All incubation times identified positive (3/3) samples from negative (2/2) samples. However, the color resolution of positive sticks was increased with increasing incubation time. The two highly positive samples (P₁ and P₂) showed similar colorations at 45 min and 60 min incubation times while one relatively low positive sample (P₃) produced slightly a less intense color at 45 min incubation time (figure III).

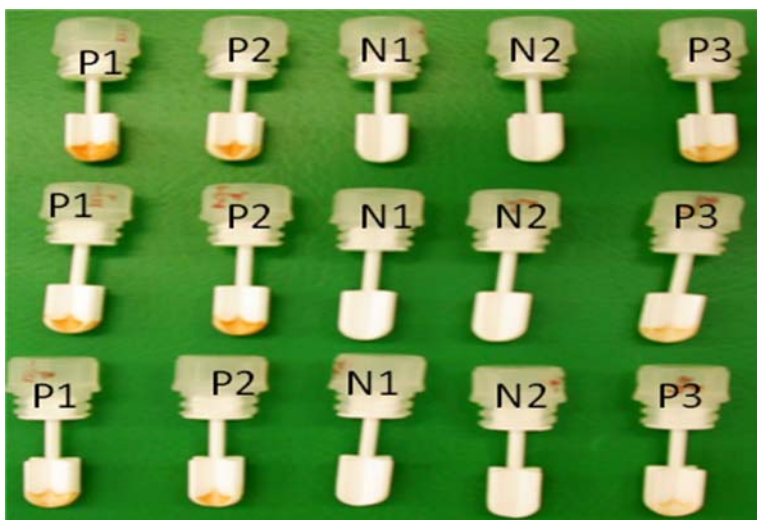


Figure III. A photo image showing the results of 60 min (*top*), 45 min (*middle*) and 30 min (*bottom*) incubation times.

Test N°3: Incubation temperature

37°C and 25 ° C incubation temperatures were compared. Both incubation temperatures identified positive samples (3/3) from negative (2/2) samples. Similar results were observed in color intensity of positive sticks between the incubation temperatures (figure IV).

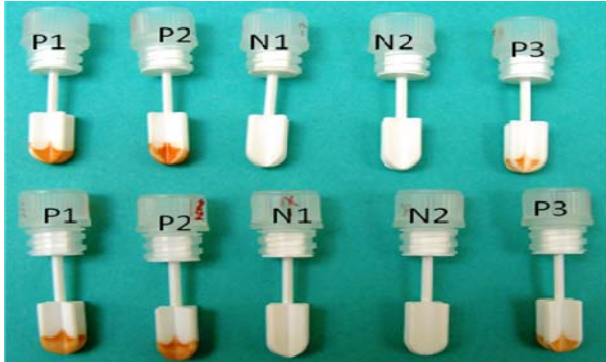


Figure IV. A photo image showing the results of 37°C (*top*) and 25 ° C (*bottom*) incubation temperature.

Test N°4: Incubation without shaking

The use of shaking during incubation was assessed. Both shaking incubation and non-shaking incubation identified positive samples (3/3) from negative (2/2) samples. The shaking incubation allowed the formation of narrow bands on the top border of the colored area of positive sticks. The significance of the band was higher to detect the low positive sample (P₃) (figure V).

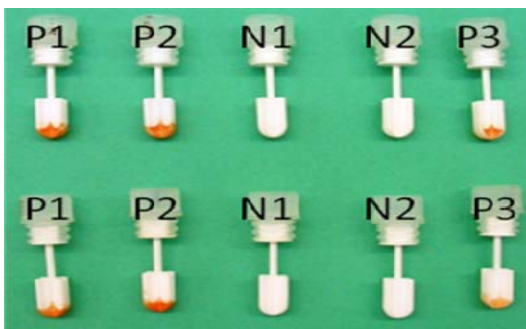


Figure V. A photo image showing results of incubation with shaking (*top*) and incubation without shaking (*bottom*).



Figure VI. A close up photo image of a positive stick showing the narrow band formed due to shaking during incubation

Test N°5: Tap water washing

Tap water was evaluated for washing as an alternative to PBS-T20. Both water and PBS-T20 identified positive samples (3/3) from negative (2/2) samples. Tap water washing showed less colored positive sticks. Moreover, erosive washings of water left white lines on the colored surface of the positive sticks (figure VII).

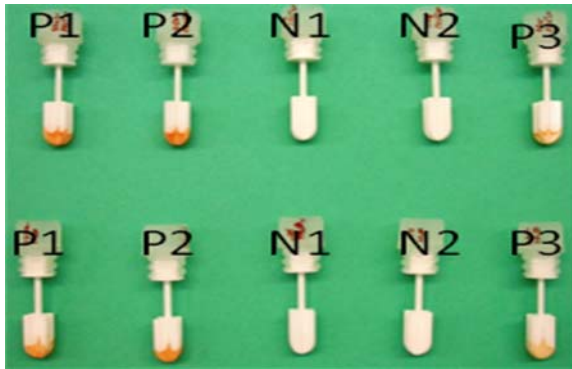


Figure VII. A photo image showing the results of PBS-T20 wash (*top*) and tap water wash (*bottom*).

Test N°6: Diaminobenzidine (DAB) substrate

Two substrates were compared. Both ODN and DAB identified positive samples (3/3) from negative (2/2) samples. The red color of ODN was easier to detect on the white paddle of the sticks. DAB gave a light purple color with poor contrast, and the color was hardly detected on the low positive sample sticks (figure VIII).

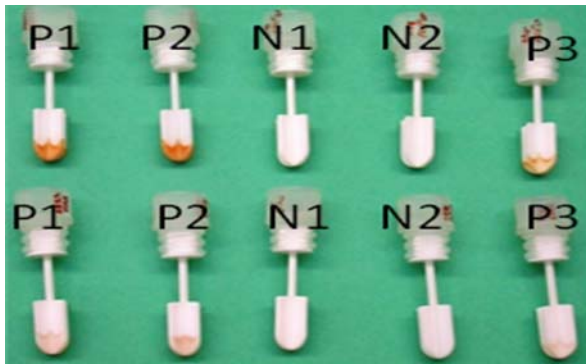


Figure VIII. A photo image showing the results of substrates: ODN (*top*) and DAB (*bottom*).

Test N°7: TCA non-treated serum

Serum TCA treatment and non-treatment were compared. Both of them identified positive samples (3/3) from negative (2/2) samples. TCA treated serum showed a more intense color on the positive sticks. One low positive sample from TCA non-treated serum became very light (figure IX).



Figure IX. A photo image showing the results of TCA treated serum (*top*) and TCA non-treated serum (*bottom*)

Test N°8: TCA treated non-centrifuged serum

TCA pre-treatment of serum either centrifuged or non-centrifuged, identified positive samples (3/3) from negative (2/2) samples. Centrifugation after TCA treatment produced a more clear color on the sticks of positive samples. The non-centrifuged serum appeared dirty on the sticks of negative and positive samples (figure X).



Figure X. A photo image showing the results of TCA pre-treated centrifuged serum (*top*) and TCA pre-treated non-centrifuged serum (*bottom*)

Test N°9: Overnight coating

Overnight coating (4°C) and 1.5 h sample incubation at 25°C produced a less intense color compared with 1 h coating (25°C) and 1 h sample incubation at 25 °C (figure XI).

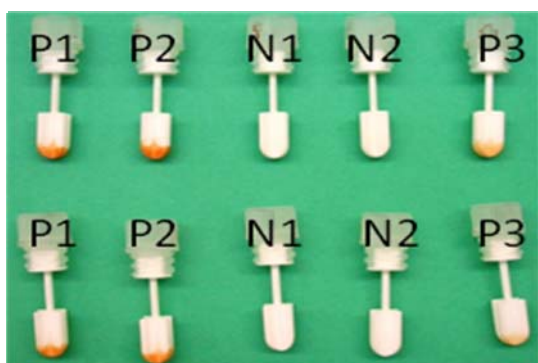


Figure XI. A photo image showing results of overnight (4°C) coating incubation (*top*) and 1 h coating incubation (*bottom*).

4.2. Determination of the detection level

The detection level of the adapted dipstick ELISA was interpreted in OD value. The new dipstick Ag-ELISA protocol detected an OD value of 0.348; while the traditional microplate Ag-ELISA detected further dilutions with an OD value of 0.170. The picture of the dipsticks for serial dilutions is presented (figure XII).

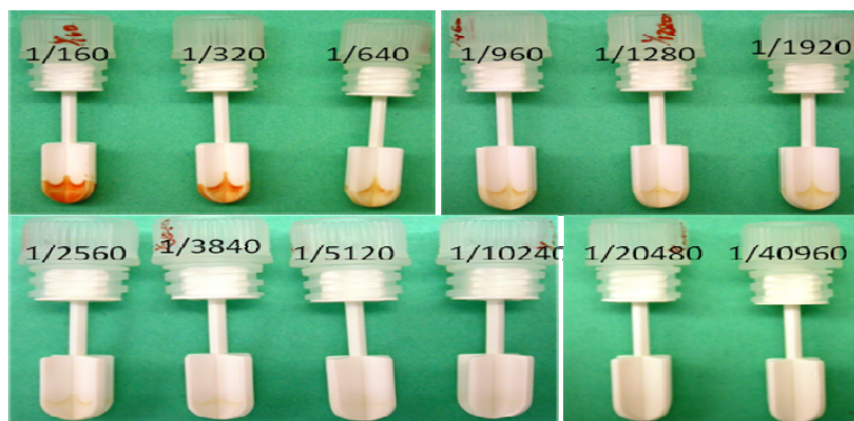


Figure XII. A photo image showing the results of the dipstick Ag-ELISA detection level on serial dilutions of a *T. saginata* cysticercosis positive serum sample (P₂) obtained from artificially infected cattle.

Table I. Results of the microplate Ag-ELISA and dipstick Ag-ELISA tests on a serial dilution of a *T. saginata* cysticercosis positive serum sample (P₂) obtained from artificially infected cattle.

Dilution	OD	Plate Ag-ELISA	Dipstick Ag-ELISA	Dilution	OD	Plate Ag-ELISA	Dipstick Ag-ELISA
1/160	2.784	+	+	1/2560	0.557	+	+
1/320	2.681	+	+	1/3840	0.348	+	+
1/640	1.844	+	+	1/5120	0.303	+	-
1/960	1.273	+	+	1/10240	0.170	+	-
1/1280	1.024	+	+	1/20480	0.100	-	-
1/1920	0.656	+	+	1/40960	0.061	-	-

4.3. Evaluation of the dipstick Ag-ELISA

The dipstick Ag-ELISA detected 11/11 positive serum samples obtained from naturally infected cattle, and all negative samples (19/19) were negative. The same samples were detected by the plate Ag-ELISA. The assay had 100% relative sensitivity and specificity as compared to the microplate Ag-ELISA (table II). The dipstick Ag-ELISA detected 7/7 positive serum samples from pigs, and all negative samples (8/8) were negative. The same samples were detected by microplate Ag-ELISA. The assay had 100% relative specificity and sensitivity as compared to the microplate Ag-ELISA (table III).

Table II. Results of the microplate Ag-ELISA and dipstick ELISA for the detection of ES antigens of *T. saginata* in serum samples from naturally infected cattle using the adapted protocol.

Sample Code	OD	Plate Ag-ELISA	Dipstick Ag-ELISA	Sample Code	OD	Plate Ag-ELISA	Dipstick Ag-ELISA
B01	0.031	-	-	B16	0.038	-	-
B02	0.065	-	-	B17	1.314	+	+
B03	0.031	-	-	B18	2.952	+	+
B04	0.031	-	-	B19	2.947	+	+
B05	0.032	-	-	B20	0.065	-	-
B06	0.055	-	-	B21	0.863	+	+
B07	0.034	-	-	B22	2.151	+	+
B08	0.077	-	-	B23	2.903	+	+
B09	0.038	-	-	B24	0.862	+	+
B10	0.033	-	-	B25	0.644	+	+
B11	0.035	-	-	B26	0.150	-	-
B12	0.043	-	-	B27	0.486	+	+
B13	0.038	-	-	B28	0.035	-	-
B14	0.048	-	-	B29	0.831	+	+
B15	0.036	-	-	B30	3.036	+	+

Table III. Results of microplate Ag-ELISA and dipstick Ag-ELISA for the detection of ES antigens of *T. solium* in serum samples from naturally infected pig using the adapted protocol.

Sample code	OD	Plate Ag-ELISA	Dipstick Ag-ELISA	Sample code	OD	Plate Ag-ELISA	Dipstick Ag-ELISA
P01	2.526	+	+	P09	0.062	-	-
P02	2.864	+	+	P10	0.046	-	-
P03	2.867	+	+	P11	0.064	-	-
P04	2.888	+	+	P12	0.044	-	-
P05	2.770	+	+	P13	0.048	-	-
P06	1.042	+	+	P14	0.044	-	-
P07	2.725	+	+	P15	0.050	-	-
P08	0.073	-	-				

Chapter V: DISCUSSION AND CONCLUSION

It has been reported that most field laboratories in developing countries are poorly equipped (Gray *et al.*, 1997; Tegbaru *et al.*, 2004). In such laboratory settings it is difficult to perform a test that requires sophisticated equipment. In the present study we have adapted the existing ES antigen detection ELISA for the diagnosis of *T. saginata* and *T. solium* cysticercosis to a dipstick system using the Nunc-immunoTM sticks (Maxisorp) in order to reduce the requirement of relatively expensive equipment. Most dipstick ELISA systems in the previous studies utilize nitrocellulose or polyvinylidene difluoride membrane made sticks (Kim & Doyle, 1992; Allan *et al.*, 1993). Few studies on the use of Nunc-immunoTM sticks for the detection of pathogens in food samples are published (Aldus *et al.*, 2003; Blazkova *et al.*, 2006). To our knowledge no article describing the use of such dipsticks on serum samples is published. In order to further simplify the test requirements, we have tried to adapt the procedure step by step.

In this study the result of 25°C incubation was similar to the conventional 37°C incubation. The same performance was also shown on the serial dilution tests of a positive sample (P₂) both at 25°C and 37°C. The performance of the assay at 25°C indicates that the assay can be used in an environment with temperatures between 25°C and 37°C. Allan *et al.* (1993) also reported the use of dipstick ELISA for the detection of coproantigens at room temperature. However, in most ELISAs incubation of the reactants were done at 37°C (Van Kerckhoven *et al.*, 1998; Dorny *et al.*, 2000; Aldus *et al.*, 2003; Blazkova *et al.*, 2006). In some microplate Ag-ELISA for the diagnosis of cysticercosis the plates were coated at 37°C (conventional incubation) followed by overnight incubation at 4°C to improve the binding of capture antibodies (Van Kerckhoven *et al.*, 1998; Dorny *et al.*, 2000). Therefore, 25°C for 1 h coating incubation followed by overnight coating incubation at 4°C might also improve the sensitivity of the dipstick Ag-ELISA. The low coloration of positive sticks coated overnight at 4°C without prior 25°C coating incubation indicates that low temperature could affect sensitivity of the dipstick assay. In a study conducted for the diagnosis of leprosy, incubation of serum samples overnight at 4°C alone improved the sensitivity of the assay. Moreover, in the same study the overnight incubation at 4°C after conventional incubation had improved the sensitivity even more (Parkash *et al.*, 2007). In our case the increased incubation time of serum samples by only 30 min did not improve the sensitivity of the present dipstick assay. This is likely due to the inadequate binding of capture antibodies at 4°C.

Shaking during incubation gave better results over non-shaking, especially in light positive samples due to the formation of a more intensely colored narrow band on the top border of the colored area of positive sticks. This suggests that the use of a shaker improves the detection of lightly infected animals compared to when a non-shaking incubation is used. The importance of these bands is minimal in strong positive samples as the whole immersed tips produce intense color. Dorny *et al.* (2002) also described that shaking improves the sensitivity of traditional microplate Ag-ELISA.

The color intensity of the positive sticks was increased with increasing incubation time. The 1 h incubation time gave a better result for low positive sample. In the original microplate ELISA for the detection of circulating antigen of *T. saginata* cysticerci 1 h was optimal incubation time (Van Kerckhoven *et al.*, 1998). Using TCA pretreated serum it was reduced

to 15 min except for capture antibody coating, which was 30 min (Dorny *et al.*, 2002). Therefore, it might be possible to improve sensitivity of the dipstick assay by increasing the incubation time especially for coating of the sticks with capture antibody and/or for sample step.

Similar results were obtained between washing under a gentle stream of PBS-T20 from a wash bottle and a dip washing in PBS-T20 indicating that washing under a running PBS-T20 can be an alternative method for washing the dipstick devices (sticks and tubes). The methods were applied before in studies using the same Nunc-immunoTM stick system (Maxisorp) for the detection of pathogens in food samples (Aldus *et al.*, 2003; Blazkova *et al.*, 2006). However, none of them compared the two washing methods. Therefore, the choice is merely the technical simplicity of the methods to perform.

TCA pre-treated serum samples gave a more intense coloration of the positive sticks in comparison with the non-TCA treated ones. Again, the importance was clear on light positive samples. The use of TCA improves the sensitivity and specificity of the assay by dissociation of the immune-complexes liberating the TCA soluble glycoprotein epitopes and precipitating interfering serum proteins (Draelants *et al.*, 1995a; Draelants *et al.*, 1995b; Dorny *et al.*, 2000). The TCA pre-treated serum samples should be centrifuged before use to remove precipitated materials (Draelants *et al.*, 1995a; Dorny *et al.*, 2000). The precipitated component of serum was bound to even negative sticks. This might complicate the differentiation between light positive and negative samples.

The dipstick ELISA developed in this study detected circulating *T. saginata* antigens in a maximum dilution having 0.348 OD reading on microplate Ag-ELISA (Cut off=0.151). Two further two-fold dilutions were detected by the standard microplate Ag-ELISA, suggesting a lower sensitivity of the dipstick Ag-ELISA. In previous studies dipstick assays have also demonstrated a lower sensitivity when compared to the microplate ELISA system (Allan *et al.*, 1993; Wu *et al.*, 1997). For example, dipstick dot-ELISA, using the same antisera as microplate ELISA, failed to detect low OD microplate ELISA positive samples (Allan *et al.*, 1993). This is probably due to the differences in the means of setting a negative cut-off for each assay, visually in the dipstick ELISA and statistically based on OD values in the microplate ELISA. Low OD positive samples probably fell below the visual cut-off for the dipstick ELISA. Because of positive correlation between OD readings and live cyst counts (Onyango-Abuje *et al.*, 1996; Van Kerckhoven *et al.*, 1998) dipstick ELISA more likely fail to detect light infections than the standard Ag-ELISA. As light grade infections are quite common under natural conditions (Wanzala *et al.*, 2003), this is an important weakness and requires further studies.

The dipstick ELISA identified 11 (n=11) microplate Ag-ELISA positive serum samples obtained from *T. saginata* infected cattle (with an unknown number of living cysts). The assay also identified 7 (n=7) microplate Ag-ELISA positive serum samples obtained from *T. solium* infected pigs (with an unknown number of living cysts), suggesting that the test could also be used for the diagnosis of animals with *T. solium* cysticercosis. The same number of positive results between dipstick ELISA and microplate Ag-ELISA is probably due to the fact that the OD values of all samples on microplate Ag-ELISA were higher than the determined detection limit of the dipstick Ag-ELISA. Samples having OD values between the detection limits of the two ELISAs were not included in the study. In this study we tried to determine sensitivity of the dipstick Ag-ELISA using the standard Ag-ELISA as a reference test. The actual sensitivity and specificity of the assay needs to be established using parasitologically

confirmed positive and negative samples, including sera from heterologous infections to evaluate the presence of cross-reactions.

From this study it can be concluded that the dipstick Ag-ELISA is helpful for the identification of animals infected with *T. saginata* (cattle) and *T. solium* (pig) cysticerci. The visual reading of the sticks for the color change avoids the use of an expensive ELISA reader needed for reading of the standard ELISA plates. The visual reading and the possibility of performing the test at 25°C allow substantial reduction in the laboratory equipment required (incubator, ELISA reader, and washer). Such assays are useful in the resource deprived laboratories in the developing countries. The assay can be performed in settings where the microplate ELISA is impractical. However, the assay is less sensitive than the standard microplate Ag-ELISA, which still needs improving.

Chapter VI: RECOMMENDATIONS

The described sensitivity of the dipstick ELISA is relative to the standard microplate Ag-ELISA. Therefore, it is necessary to know the true sensitivity and specificity of the assay, and we recommend further studies for the determination of overall test performance using serum samples from parasitologically confirmed positive and negative animals as well as serum samples from animals with heterologous infections. The sensitivity of the dipstick ELISA obtained in the present study by serial dilution was low compared to the conventional microplate Ag-ELISA and requires further improvement. The use of capture antibody precoated sticks decreases the time taken to accomplish a test. Therefore, we recommend further study on the maintenance of the diagnostic sticks without affecting the performance of the assay.

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Annexes

Annex I. Test procedure for washing under a stream of PBS-T20 from a wash bottle

Steps	Reagents	Dilutions	Volume/tube	Incubation
Capture Antibody	B ₁₅₈ C ₁₁ A ₁₀	5 µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6	300 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks/tubes washed under a stream of PBS-T20</i>			
Block	NBCS	1% NBCS in PBS-T20	1 ml	37°C, 15 min, shaking
<i>Washing</i>	<i>Only tubes rinsed 3 times under a stream of PBS-T20</i>			
Serum	TCA pre-treated serum	1:4 final dilution	150 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks/tubes washed under a stream of PBS-T20</i>			
Detecting Antibody	B ₆₀ H ₈ A ₄	1.25 µg/ml in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks/tubes washed under a stream of PBS-T20</i>			
Conjugate	Streptavidin-HRP	1:10 000 in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks/tubes washed under a steam of PBS-T20</i>			
Substrate	ODN	(15 mg in 2.5 ml DMSO + 22.5 ml RO-DI water + 25 ml PBS)+ 20 µl 30% v/v H ₂ O ₂	300 µl	Room temperature, 30 min, No shaking
<i>Washing</i>	<i>Wash sticks 2-3 times by dipping into RO-DI water</i>			

Annex II. Test procedure for 30 min incubation time and 45 min incubation time

Steps	Reagents	Dilutions	Volume/tube	Group 1 30 min	Group 2 45 min
Capture Antibody	B ₁₅₈ C ₁₁ A ₁₀	5µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6	300 µl	37°C, 30 min, shaking	37°C, 45 min, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>				
Block	NBCS	1% NBCS in PBS-T20	1 ml	37°C, 15 min, shaking	37°C, 15 min, shaking
<i>Washing</i>	<i>Tubes rinsed 3 times with PBS-T20</i>				
Serum	TCA pre-treated serum	1:4 final dilution	150 µl	37°C, 30 min, shaking	37°C, 45 min, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>				
Detecting Antibody	B ₆₀ H ₈ A ₄	1.25 µg/ml in 1% NBCS + PBS-T20	300 µl	37°C, 30 min, shaking	37°C, 45 min, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>				
Conjugate	Streptavidin-HRP	1 µl in 10 ml 1% NBCS + PBS-T20	300 µl	37°C, 30 min, shaking	37°C, 45 min, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>				
Substrate	ODN	(15 mg in 2.5 ml DMSO + 22.5 ml RO-DI water + 25 ml PBS)+ 20 µl 30% v/v H ₂ O ₂	300 µl	Room temperature, 30 min, No shaking	Room temperature, 30 min, No shaking
<i>Washing</i>	<i>Wash sticks 2-3 times by dipping into RO-DI water</i>				

Annex III. Test procedure for 25°C incubation temperature

Steps	Reagents	Dilutions	Volume/tube	Incubation
Capture Antibody	B ₁₅₈ C ₁₁ A ₁₀	5 µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6	300 µl	25°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Block	NBCS	1% NBCS in PBS-T20	1 ml	25°C, 15 min, shaking
Washing	<i>Tubes rinsed 3 times with PBS-T20</i>			
Serum	TCA pre-treated serum	1:4 final dilution	150 µl	25°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Detecting Antibody	B ₆₀ H ₈ A ₄	1.25 µg/ml in 1% NBCS + PBS-T20	300 µl	25°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Conjugate	Streptavidin-HRP	1: 10 000 in 1% NBCS + PBS-T20	300 µl	25°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Substrate	ODN	(15 mg in 2.5 ml DMSO + 22.5 ml RO-DI water + 25 ml PBS)+ 20 µl 30% v/v H ₂ O ₂	300 µl	Room temperature, 30 min, No shaking
Washing	<i>Wash sticks 2-3 times by dipping into RO-DI water</i>			

Annex IV. Test procedure for incubation without shaking of the sticks

Steps	Reagents	Dilutions	Volume/tube	Incubation
Capture Antibody	B ₁₅₈ C ₁₁₁ A ₁₀	5µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6	300 µl	37°C, 1 h, No shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Block	NBCS	1% NBCS in PBS-T20	1 ml	37°C, 15 min, No shaking
<i>Washing</i>	<i>Tubes rinsed 3 times with PBS-T20</i>			
Serum	TCA pre-treated serum	1:4 final dilution	150 µl	37°C, 1 h, No shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Detecting Antibody	B ₆₀ H ₈ A ₄	1.25 µg/ml in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, No shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Conjugate	Streptavidin-HRP	1: 10 000 in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, No shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Substrate	ODN	(15 mg in 2.5 ml DMSO + 22.5 ml RO-DI water + 25 ml PBS)+ 20 µl 30% v/v H ₂ O ₂	300 µl	Room temperature, 30 min, No shaking
<i>Washing</i>	<i>Wash sticks 2-3 times by dipping into RO-DI water</i>			

Annex V. Test procedure for tap water washing

Steps	Reagents	Dilutions	Volume/tube	Incubation
Capture Antibody	B ₁₅₈ C ₁₁ A ₁₀	5µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6	300 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into tap water and tubes rinsed 3 times with tap water</i>			
Block	NBCS	1% NBCS in PBS-T20	1 ml	37°C, 15 min, shaking
<i>Washing</i>	<i>Tubes rinsed 3 times with tap water</i>			
Serum	TCA pre-treated serum	1:4 final dilution	150 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into tap water and tubes rinsed 3 times with tap water</i>			
Detecting Antibody	B ₆₀ H ₈ A ₄	1.25 µg/ml in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into tap water and tubes rinsed 3 times with tap water</i>			
Conjugate	Streptavidin-HRP	1: 10 000 in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into tap water and tubes rinsed 3 times with tap water</i>			
Substrate	ODN	(15 mg in 2.5 ml DMSO + 22.5 ml RO-DI water + 25 ml PBS)+ 20 µl 30% v/v H ₂ O ₂	300 µl	Room temperature, 30 min, No shaking
<i>Washing</i>	<i>Wash sticks 2-3 times by dipping into RO-DI water</i>			

Annex VI. Test procedure for DAB substrate

Steps	Reagents	Dilutions	Volume/tube	Incubation
Capture Antibody	B ₁₅₈ C ₁₁ A ₁₀	5µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6	300 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Block	NBCS	1% NBCS in PBS-T20	1 ml	37°C, 15 min, shaking
<i>Washing</i>	<i>Tubes rinsed 3 times with PBS-T20</i>			
Serum	TCA pre-treated serum	1:4 final dilution	150 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Detecting Antibody	B ₆₀ H ₈ A ₄	1.25 µg/ml in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Conjugate	Streptavidin-HRP	1:10000 in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Substrate	DAB	100 µl (1 g/20 ml PBS) DAB in 10 ml PBS + 3.5 µl 30% v/v H ₂ O ₂	300 µl	Room temperature, 30 min, No shaking
<i>Washing</i>	<i>Wash sticks 2-3 times by dipping into RO-DI water</i>			

Annex VII. Test procedure for TCA non-treated serum

Steps	Reagents	Dilutions	Volume/tube	Incubation
Capture Antibody	B ₁₅₈ C ₁₁ A ₁₀	5µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6	300 µl	37°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Block	NBCS	1% NBCS in PBS-T20	1 ml	37°C, 15 min, shaking
Washing	<i>Tubes rinsed 3 times with PBS-T20</i>			
Serum	TCA non pre-treated serum	1:4 final dilution in PBS	150 µl	37°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Detecting Antibody	B ₆₀ H ₈ A ₄	1.25 µg/ml in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Conjugate	Streptavidin-HRP	1:10000 in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Substrate	ODN	(15 mg in 2.5 ml DMSO + 22.5 ml RO-DI water + 25 ml PBS)+ 20 µl 30% v/v H ₂ O ₂	300 µl	Room temperature, 30 min, No shaking
Washing	<i>Wash sticks 2-3 times by dipping into RO-DI water</i>			

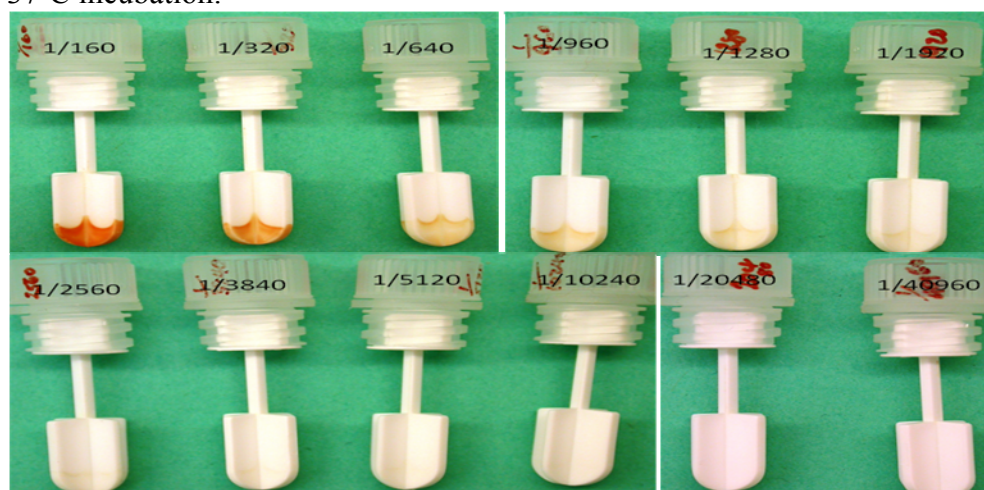
Annex VIII. Test procedure for TCA treated non-centrifuged serum

Steps	Reagents	Dilutions	Volume/tube	Incubation
Capture Antibody	B ₁₅₈ C ₁₁ A ₁₀	5µg/ml in 0.05M carbonate/bicarbonate buffer, pH 9.6	300 µl	37°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Block	NBCS	1% NBCS in PBS-T20	1 ml	37°C, 15 min, shaking
Washing	<i>Tubes rinsed 3 times with PBS-T20</i>			
Serum	TCA pre-treated non centrifuged serum	Diluted 1:4 in TCA + Neutralization buffer	150 µl	37°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Detecting Antibody	B ₆₀ H ₈ A ₄	1.25 µg/ml in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Conjugate	Streptavidin-HRP	1: 10 000 in 1% NBCS + PBS-T20	300 µl	37°C, 1 h, shaking
Washing	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>			
Substrate	ODN	(15 mg in 2.5 ml DMSO + 22.5 ml RO-DI + 25 ml PBS)+ 20 µl 30% v/v H ₂ O ₂	300 µl	Room temperature, 30 min, No shaking
Washing	<i>Wash sticks 2-3 times by dipping into RO-DI water</i>			

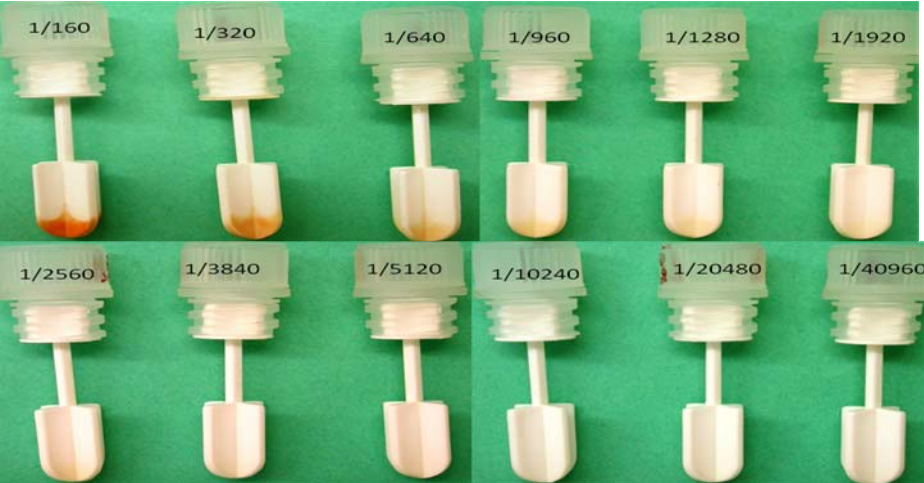
Annex IX. Test procedure for overnight coating and 1 h coating incubation

Steps	Reagents	Dilutions	Volume/ tube	Group 1 Overnight coating	Group 2 1 h coating incubation
Capture Antibody	B ₁₅₈ C ₁₁ A ₁₀	5µg/ml in 0.05M carbonate/bicarbon ate buffer, pH 9.6	300 µl	4°C, overnight, no shaking	25°C, 1 h, no shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>				
Block	NBCS	1% NBCS in PBS- T20	1 ml	25°C, 15 min, no shaking	25°C, 15 min, no shaking
<i>Washing</i>	<i>Tubes rinsed 3 times with PBS-T20</i>				
Serum	TCA pre- treated serum	1:4 final dilution	150 µl	25°C, 1.5 h, no shaking	25°C, 1 h, no shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>				
Detecting Antibody	B ₆₀ H ₈ A ₄	1.25 µg/ml in 1% NBCS + PBS-T20	300 µl	25°C, 1 h, no shaking	25°C, 1 h, no shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>				
Conjugate	Streptavidin- HRP	1:10000 in 1% NBCS + PBS-T20	300 µl	25°C, 1 h, no shaking	25°C, 1 h, no shaking
<i>Washing</i>	<i>Sticks were dipped 5 times into PBS-T20 and tubes rinsed 3 times with PBS-T20</i>				
Substrate	ODN	(15 mg in 2.5 ml DMSO + 22.5 ml RO-DI water + 25 ml PBS)+ 20 µl 30% v/v H ₂ O ₂	300 µl	Room temperature , 30 min, No shaking	Room temperature, 30 min, No shaking
<i>Washing</i>	<i>Wash sticks 2-3 times by dipping into RO-DI water</i>				

Annex X. A photo image showing the results of a serial dilution of a positive sample (P₂) at 37°C incubation.



Annex XI. A photo image showing the results of a serial dilution of a positive sample (P₂) at 25°C incubation



Short curriculum vitae

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