Serological markers for improved diagnosis of porcine cysticercosis

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"I have not failed, I have merely found $10,000$ ways that do not work."

Thomas A. Edison

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1

Diagnosis of *Taenia solium* infection: a literature review

1.1 General introduction

Cysticercosis in pigs is caused by the larval stage of the tapeworm *Taenia solium*. Man is the only definitive host, harboring the adult tapeworm in the small intestine (taeniosis). During defecation, gravid proglottids containing eggs are shed into the environment. These eggs are the source of infection with the larval stage, *Cysticercus cellulosae*. The natural intermediate host is the pig, harboring larval cysts anywhere in its body, mostly without clinical symptoms. Man can also act as intermediate host. Humans become infected with cysts by accidental ingestion of *T. solium* infective eggs by fecal-oral contamination (Figure 1.1).

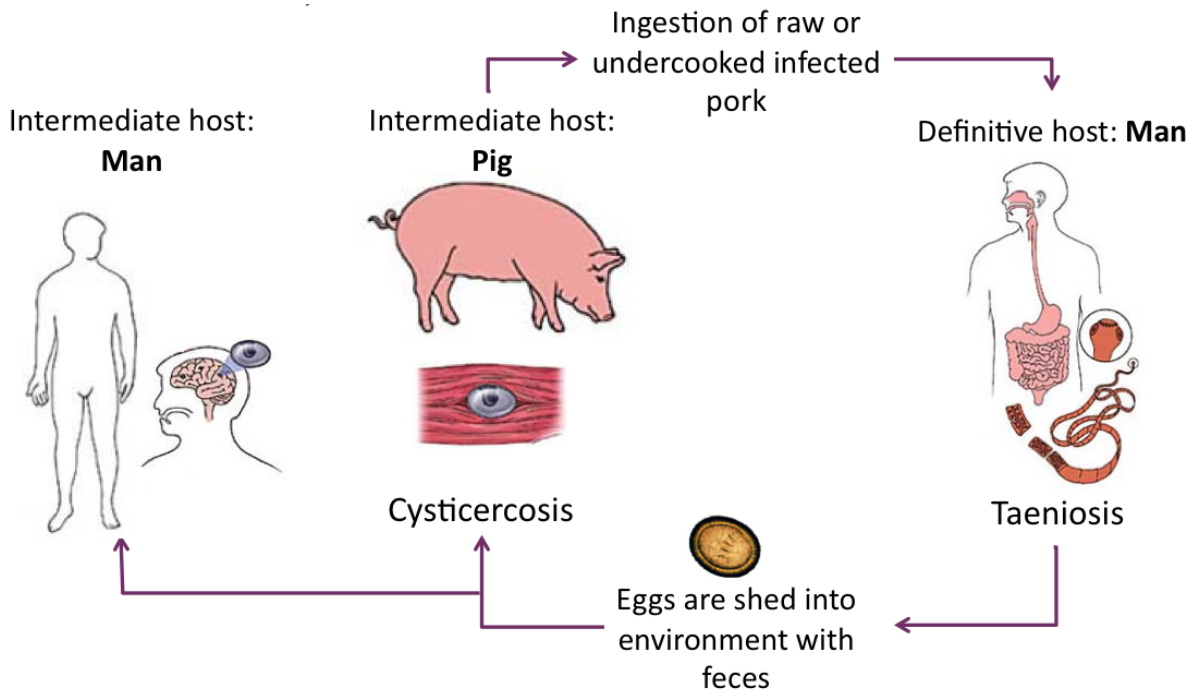

Figure 1.1: *Taenia solium* life cycle. Humans are the definitive host (harboring the adult *Taenia* tapeworm in the intestine); the infective eggs are shed into the environment with the feces. The natural intermediate host is the pig (harboring the larval stage or cysticercus). Humans can be also infected with the larval stage (accidental intermediate host). (Adapted from: `http://www.aafp.org/afp/20070701/91.html`)

In man, cysts can develop in different parts of the body (subcutaneous tissue, the eye, the muscles); however, cysts preferably tend to lodge in the central nervous system, causing neurocysticercosis (NCC). This can result in severe symptoms ranging from headache, various neurological symptoms, epilepsy, hydrocephalus and even death (Del Brutto, 2005).

Taenia solium cysticercosis is a major public health problem in developing countries,

where free-roaming pigs have access to human feces and hygienic conditions are poor. Figure 1.2 shows the global distribution of *T. solium* taeniosis/cysticercosis. According to data collected in Peru, there would be an estimated 23,500 to 39,000 inhabitants with symptomatic NCC among whom 19,000 to 31,000 would be associated with seizures (Bern et al., 1999). Extrapolating these results to other developing countries in Latin America, there are 75 million people living in areas of endemicity and 400,000 cases of symptomatic NCC in Latin America alone. NCC is considered as the main cause of epilepsy worldwide (International League Against Epilepsy, 1994). Due to increased travelling and immigration, there are more and more imported cases of NCC in developed countries (Schantz et al., 1998; Chatel et al., 1999; Townes et al., 2004). For instance, cysticercosis has emerged as a cause of severe neurological disease in the United States (Sorvillo et al., 2007). A total of 221 cysticercosis deaths were identified for 1990–2002. Fatal cysticercosis affected mainly immigrants from Mexico and other Latin American countries; however, US-born persons were also affected.

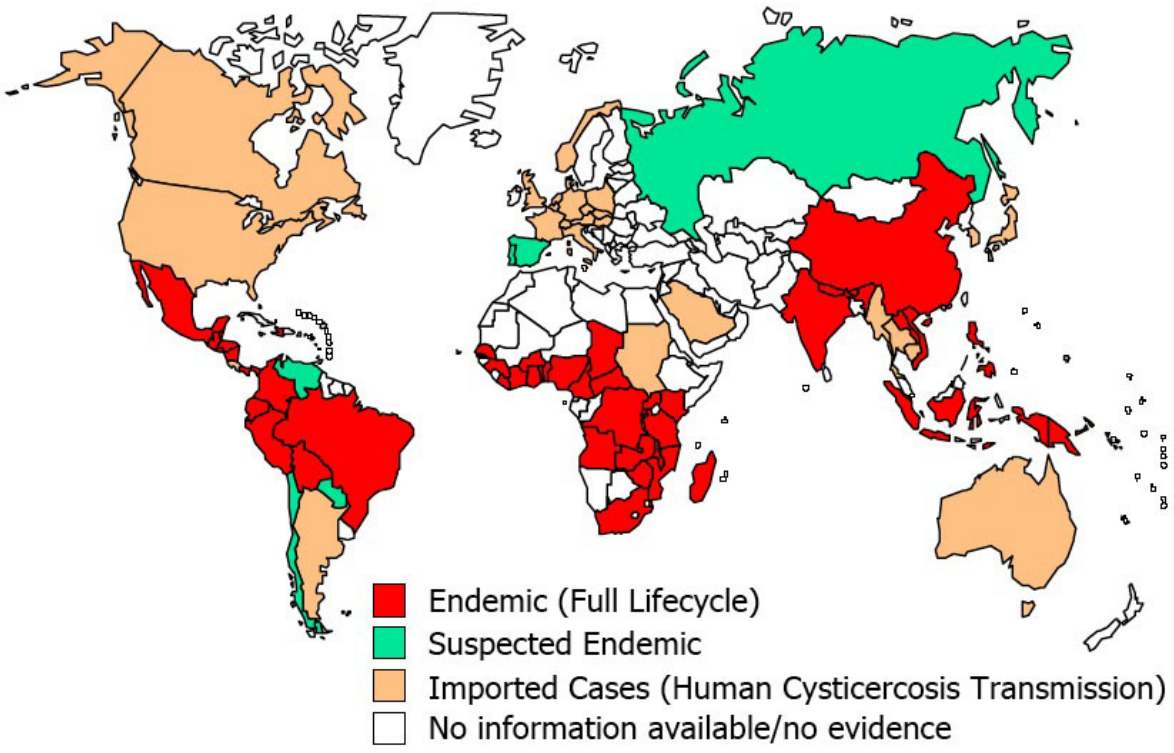

Figure 1.2: Global distribution of *Taenia solium* taeniosis/cysticercosis (Source: `http://www.who.int`).

Porcine cysticercosis poses also an economic constraint, because of condemned carcasses and reduced household income for poor farmers. In Eastern Cape Province of South Africa, the estimated overall monetary burden due to *T. solium* cysticercosis var-

ied from US $18.6 million to US $34.2 million depending on the method used to estimate productivity losses (Carabin et al., 2006).

There are major discrepancies in the treatment of neurocysticercosis in part due to the vast variation in clinical manifestations (Evans et al., 1997). Symptomatic therapy with antiepileptic drugs is indicated to control epilepsy, often in conjunction with anti-inflammatory drugs. Whether the use of antiparasitic drugs (albendazole or praziquantel) is advisory, is still subject to debate (Garcia et al., 2002). Some authors claim that their use is unnecessary because cysticidal therapy can result in severe brain inflammation and cysts will degenerate and heal by natural evolution of the disease (Carpio et al., 1998). However, meta-analysis of randomized trials of cysticidal drug therapy for neurocysticercosis showed that cysticidal drug therapy results in better resolution of cysts, lower risk for recurrence of seizures, and a reduction in the rate of generalized seizures (Del Brutto et al., 2006).

In 1993, *T. solium* cysticercosis was listed as a potentially eradicable disease by the International Task Force for Disease Eradication (Schantz et al., 1993). In developed countries, the main method of control was to eradicate porcine cysticercosis through improved pig husbandry and meat inspection. This approach has typically failed in developing countries. Corralling pigs in a pen where they cannot scavenge for food, requires that they be given additional feed, which is not affordable for many subsistence farmers. Nowadays, *T. solium* taeniosis/cysticercosis is no longer considered as potentially eradicable because of the insufficient operational experience (Molyneux et al., 2004).

Nevertheless, several other tools are available for the control of *T. solium* cysticercosis: mass treatment of tapeworm carriers (Allan et al., 1997; Garcia et al., 2006), treatment of pigs with oxfendazole (Gonzalez et al., 1996, 2001) or in the future, vaccination of pigs (Flisser et al., 2004; Gonzalez et al., 2005; Parkhouse et al., 2008; Sciutto et al., 2008). However, regardless of the chosen strategy, one of the most important aspects of control and elimination of cysticercosis is prevention of infection by improving public awareness and hygienic conditions. In this view, health and pig management education campaigns (Sarti et al., 1997; Sarti and Rajshekhar, 2003; Ngowi et al., 2007, 2008) should be implemented during control programs.

In order to control the disease, the first step is to assess the prevalence of taeniosis/cysticercosis. For porcine cysticercosis, tongue inspection and post mortem examinations can be used for diagnosis (Gonzalez et al., 1990). Clinical diagnosis of human cysticercosis is primarily done by (neuro)imaging techniques (Garcia and Del Brutto, 2003). The prevalence of human and pig infection can also be estimated using serology based on either antibody or antigen detection (Dorny et al., 2003).

This chapter provides an overview of the current techniques for diagnosis of *T. solium*

cysticercosis and taeniosis with emphasis on immunodiagnosis. From this, it will become clear that there is a need for improved diagnostic techniques, particularly for porcine cysticercosis.

1.2 Diagnosis of cysticercosis

1.2.1 Porcine cysticercosis: Tongue and carcass inspection

Tongue inspection and palpation consists of palpating nodules and/or visual identification of cysticercus cysts. The pig is placed on its side and restrained, and a rod is used to keep its mouth open. The tongue is gently pulled aside using a cloth and the base of the tongue is examined and palpated. Tongue inspection is often performed by farmers and pig traders at the informal markets (The Cysticercosis Working Group in Peru, 1993). The findings of the tongue inspection determine the price of the pig to be sold. The tongue test is very specific but lacks sensitivity: Gonzalez et al. (1990) reported a sensitivity of 70%; Pouedet et al. (2002) reported a sensitivity of 50-55% and in another study the sensitivity was as low as 21% (Dorny et al., 2004b). In any case, the tongue examination, being a test essentially without cost and having high specificity, can be useful as rapid assessment tool in epidemiological surveys.

Complete carcass dissection with enumeration of the cysts is regarded as the gold standard for diagnosing porcine cysticercosis (Dorny et al., 2004b) and can provide a tool for validation of immunodiagnostic tests. However, routine carcass inspection at the slaughterhouse lacks sensitivity and in many developing countries, regulations concerning meat inspection are inadequate or non-existent (Joshi et al., 2003). Furthermore, only a small percentage of pigs are slaughtered at an official slaughterhouse and as such subjected to carcass inspection (The Cysticercosis Working Group in Peru, 1993).

1.2.2 Human cysticercosis: Clinical diagnosis and imaging techniques

Neurocysticercosis may produce absolutely no clinical symptoms but it may also present a variety of clinical manifestations. This pleomorphism is due to differences in number, size, localization and stage of development of cysticerci, as well as to the host's immune response to the parasite (Takayanagui and Odashima, 2006). The clinical symptoms are mostly non-specific and defining a typical syndrome of neurocysticercosis is unrealistic. Seizures are reported to be the most common symptom, occurring in $70-90\%$ of patients (White, 1997). Other clinical manifestations include: focal neurological signs, intracranial hypertension, psychiatric disturbances, migraine-type headache, hydro-

cephalus, cranial nerve involvement and tumor-like syndromes, acute meningitis, stroke syndromes... (Sotelo and Del Brutto, 2000).

Modern neuroimaging techniques play the most important role in the diagnosis of NCC. Previous radiological procedures such as plain roentgenograms, isotope brain scans, pneumoencephalograms, cerebral angiography and myelography are now largely replaced by computed tomography (CT) and magnetic resonance imaging (MRI) (Del Brutto et al., 1998). Both CT and MRI provide objective evidence regarding the topography of the lesions and the degree of inflammation surrounding the cysticerci (Garcia et al., 2005). CT and MRI findings in parenchymal NCC depend on the stage of development of the cysticerci. Cystic lesions with an eccentric hyperdense nodule representing the scolex are characteristic of NCC. Other findings include single or multiple enhancing lesions and hydrocephalus — the most common CT finding in patients with subarachnoid cysticercosis (Garcia and Del Brutto, 2003). With the exception of cystic lesions, most imaging findings are non-specific and represent a diagnostic challenge (Sotelo and Del Brutto, 2000).

Recently, diagnostic criteria for neurocysticercosis were provided based on objective clinical, imaging, immunological and epidemiological data (Del Brutto et al., 2001). The criteria include four categories — absolute, major, minor and epidemiologic — stratified on the basis of their individual diagnostic strength. Interpretation of these criteria results in two degrees of diagnostic certainty: definite and probable (Table 1.1).

The gold standard for diagnosing NCC is pathological confirmation through biopsy or autopsy; obviously, these techniques have limitations (Dorny et al., 2003). Visualization of a cystic lesion with scolex on CT or MRI or direct visualization of the subretinal parasite during ophthalmic examination (absolute diagnostic criteria for NCC) can also be regarded as gold standard tests.

Another manifestation of cysticercosis in humans, common in Asian countries, is the presence of subcutaneous nodules (Willingham et al., 2003; Dorny et al., 2004c). Fine needle aspiration cytology provides a safe and rapid tool for diagnosis of subcutaneous cysticercosis (Adhikari et al., 2007).

1.2.3 Immunodiagnosis: Antibody detection

Infection with *T. solium* results in a specific antibody response. These antibodies can be detected in either serum, cerebrospinal fluid (CSF) in the case of NCC (Arruda et al., 2006) or even tear specimen in the case of ophtalmic cysticercosis (Sahu et al., 2008). Several techniques have been described to detect antibodies to *T. solium* infections in man and pigs, such as radioimmunoassay (Miller et al., 1984), hemagglutination (Larralde et al., 1986; Ferreira et al., 1997), the complement fixation test (Garcia et al., 1995), dipstick assay (Hayunga et al., 1991), latex agglutination (Rocha et al., 2002), ELISA

Table 1.1: Diagnostic criteria for neurocysticercosis (Source: Del Brutto et al., 2001).

Diagnostic criteria		
Absolute criteria	1.	Histologic demonstration of the parasite from biopsy of a brain or spinal cord lesion
	2.	Cystic lesions showing the scolex on CT or MRI
	3.	Direct visualization of subretinal parasites by fundoscopic examination
Major	1.	Lesions highly suggestive of NCC on neuroimaging studies
	2.	Positive serum EITB[a] for the detection of anticysticercal antibodies
	3.	Resolution of intracranial cystic lesions after therapy with albendazole or praziquantel
	4.	Spontaneous resolution of small single enhancing lesions
Minor	1.	Lesions compatible with NCC on neuroimaging studies
	2.	Clinical manifestations suggestive of NCC
	3.	Positive CSF[b] ELISA for detection of anticysticercal antibodies or cysticercal antigens
	4.	Cysticercosis outside the CNS
Epidemiologic	1.	Evidence of a household contact with *T. solium* infection
	2.	Individuals coming from or living in an area where cysticercosis is endemic
	3.	History of frequent travel to disease-endemic areas
Diagnostic certainty		
Definitive	1.	Presence of one absolute criterion
	2.	Presence of two major plus one minor and one epidemiological criterion
Probable	1.	Presence of one major plus two minor criteria
	2.	Presence of one major plus one minor and one epidemiological criterion
	3.	Presence of three minor plus one epidemiologic criterion

[a] Enzyme-linked immunoelectrotransfer blot

[b] Cerebrospinal fluid

and immunoblot techniques (Escalante et al., 1999).

Initially, antigens used in antibody detection assays were either cyst fluid, excretion-secretion (ES) products or crude homogenates from cysticerci from either *T. solium* or the related parasites *T. crassiceps* or *T. saginata* (Ko and Ng, 1998; D'Souza and Hafeez, 1999; Pinto et al., 2000; Arruda et al., 2005; Oliveira et al., 2007). However, unpurified antigens have moderate sensitivities and relatively poor specificities (Schantz and Sarti-Gutierrez, 1989). Improved protein purification techniques and research on antigenic properties of cyst fluid and surface proteins has led to the development of better serological tools (Gottstein et al., 1986; Parkhouse and Harrison, 1987; Hayunga et al., 1991; Ito et al., 1998; Assana et al., 2006).

The most specific test so far is the enzyme-linked immunoelectrotransfer blot (EITB) with an initially reported specificity of 100% and sensitivity of 98% (Tsang et al., 1989). This immunoblot uses an enriched fraction of glycoproteins obtained by purifying a raw cysticercus extract through chromatography with lentil-lectin (LLGP). Reaction with any one of seven specific bands was regarded as diagnostic for cysticercosis (Figure 1.3). However, Furrows et al. (2006) reported that the presence of a single positive 50 kDa band may not indicate infection and thus has to be regarded as not specific. Moreover, the sensitivity of this assay drops dramatically in cases with single cysts in the brain (Wilson et al., 1991; Singh et al., 1999). Recently, Prabhakaran et al. (2007) reported that using conformation-sensitive immunoassays — with urea-induced tertiary conformations of the lentil lectin glycoproteins — they were able to detect antibodies in 46% of 60 patients with solitary cysticercus granuloma who were serologically negative on standard immunoblot. The EITB test has been extensively used for the diagnosis of human and porcine cysticercosis (Tsang et al., 1991). The assay, however, has some drawbacks. It depends on infected pigs for supplying the source material. Preparation of the antigen and performance of the Western blot require considerable technical expertise. Furthermore, the antigen mixture is not suitable for use in an ELISA format due to the presence of aspecific fractions. Finally, a Western blot assay is not suitable for field studies, nor is it a suitable or affordable assay for diagnosis in countries where cysticercosis is endemic (Hancock et al., 2003). To address these issues, the seven diagnostic LLGP antigens have been identified, characterized and produced as either recombinant or synthetic proteins (Greene et al., 1999, 2000; Hancock et al., 2003, 2004, 2006). These proteins have been tested in ELISA and Western blot assays for the diagnosis of cysticercosis (Handali et al., 2004; Scheel et al., 2005; Bueno et al., 2005; da Silva et al., 2006). When tested in ELISA, the sensitivities and specificities of the individual proteins were well below that of the corresponding native LLGP proteins in the EITB, whereas results for Western blot format closely mirrored those of the LLGP EITB.

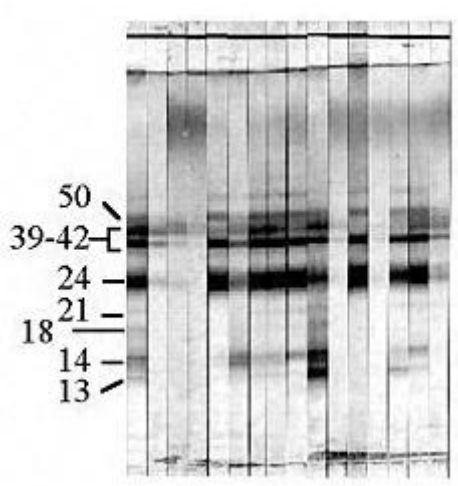

Figure 1.3: Typical antibody reactions in the enzyme-linked immunoelectrotransfer blot (EITB) for cysticercosis. The positions of the seven diagnostic glycoproteins are marked and designated according to their relative mobilities in SDS-PAGE (in kDa) (Adapted from: `http://www.dpd.cdc.gov/DPDx/HTML/ImageLibrary/Cysticercosis_il.htm`).

Other researchers purified glycoproteins from cyst fluid using isoelectric focusing electrophoresis (IEFE) (Ito et al., 1998). These antigens, applied both in immunoblot and ELISA, were highly specific and sensitive for differential serodiagnosis of NCC. Later, sera from rabbits immunized with these immunodiagnostic antigens were used to screen a *T. solium* metacestode cDNA library (Sako et al., 2000). Four clones (Ag1, Ag1V1, Ag2, Ag2V1) were characterized and the recombinant proteins were first tested in immunoblot. Ag1V1 and Ag2 were chosen as ELISA antigens, and the Ag1V1/Ag2 chimeric protein was expressed. With this ELISA using either IEFE purified glycoproteins or the chimeric protein, it was possible to detect antibody responses in pigs harboring 16 or more cysts from 30 days p.i.; also, no cross reactions were observed with pigs infected with *T. hydatigena* (Sato et al., 2003).

Recently, many other researchers reported the use of recombinant or synthetic proteins in immunodiagnosis of cysticercosis. A Pubmed Protein database search[1] on *T. solium* resulted in 311 proteins, of which several are used in immunodiagnosis (Table 1.2). The related parasites *T. crassiceps* and *T. saginata* are also the source of recombinant and synthetic proteins used for diagnosis of *T. solium* cysticercosis (Gevorkian et al., 1996; Hernandez et al., 2000; Fleury et al., 2003; Robles et al., 2005; Ferrer et al., 2007b).

Regardless of the technique used, detection of *T. solium* specific antibodies in serum only indicates exposure to the parasite and not necessarily established infection, resulting

[1] `http://www.ncbi.nlm.nih.gov/pubmed/`

in a transient antibody response (Garcia et al., 2001b). In this regard, human taeniosis/cysticercosis is said to resemble an "iceberg" with 3 tiers: the tip of this iceberg consists of people who harbor established symptomatic CNS disease—that is, NCC. Below this, there is a larger population with established cysticercosis infection outside the CNS or in the CNS but without discernable symptoms and below this tier, there are many people who were exposed to failed infections, but became seropositive (Bern et al., 1999; Garcia et al., 2001b). Furthermore, antibodies may persist long after the parasite has been eliminated by immune mechanisms and/or antiparasitic therapy (Harrison et al., 1989; Garcia et al., 1997). Detection of anti-parasite antibodies in a population in an endemic village does not necessarily reflect the true prevalence, leading to misdiagnosis of a proportion of neurological cases (Bern et al., 1999). It can also lead to superfluous use of antiparasitic therapy in a patient where the parasites are not viable (Garcia et al., 2000).

Recently, a new immunodiagnostic tool was described for the diagnosis of NCC (Prasad et al., 2008). Instead of measuring antibody responses resulting from the humoral immune response against the parasite, a cyst fluid antigen-based lymphocyte proliferation test was developed to measure the cellular immune response. The researchers reported a sensitivity and specificity of 93.8% and 96.2%, respectively. However, these results still need to be validated in other endemic areas.

Table 1.2: List of *Taenia solium* proteins used in antibody detection.

Protein name	GenBank accession no.	Reference
B1 variant	ABI23958	Bae, direct submission
M13h variant	ABI23957	Bae, direct submission
RS1 variant	ABI23956	Bae, direct submission
immunogenic protein	AAK07745	Bin, direct submission
cysticercosis specific antigen	AAF06716	Chung et al., 1999
small heat shock protein	CAD36617	Ferrer et al., 2005
secreted antigen Ts8B3	CAD48847	Ferrer et al., 2007a
secreted antigen Ts8B2	CAD48846	Ferrer et al., 2007a
secreted antigen Ts8B1	CAD48845	Ferrer et al., 2007a
antigenic protein	AAK18818	Gao, direct submission
H17g protein	CAE46111	Garate, direct submission
filamin	CAK96164	Garate, direct submission
low MW excretion secretion antigen m4	CAD44557	Garate, direct submission
low MW excretion secretion antigen b1	CAD44556	Garate, direct submission
low MW excretion secretion antigen m13h	CAD44555	Garate, direct submission
hypothetical protein	AAT98623	Gonzalez, direct submission
RS1	AAD51765	Greene et al., 1999, 2000
14 kDa glycoprotein TS14 precursor	AAD51764	Greene et al., 1999, 2000
18 kDa glycoprotein TS18 precursor	AAD51763	Greene et al., 1999, 2000
18 kDa glycoprotein TS18 variant 1 precursor	AAD51767	Greene et al., 2000

Table continues on next page

Table 1.2: List of *Taenia solium* proteins used in antibody detection (cont.).

Protein name	GenBank accession no.	Reference
14 kDa diagnostic antigen	AAF25005	Greene et al., 2000
18 kDa glycoprotein TS18 variant 3 precursor	AAD51769	Greene et al., 2000
18 kDa glycoprotein TS18 variant 2 precursor	AAD51768	Greene et al., 2000
8 kDa diagnostic antigen Ts14	AAM00205	Greene et al., 2000; Hancock et al., 2001, 2003
8 kDa diagnostic antigen Ts14	AAM00204	Greene et al., 2000; Hancock et al., 2001, 2003
Ts14 variant 3	AAM00208	Greene et al., 2000; Hancock et al., 2003
Ts14 variant 2	AAM00207	Greene et al., 2000; Hancock et al., 2003
Ts14 variant 1	AAM00206	Greene et al., 2000; Hancock et al., 2003
8 kDa diagnostic antigen Ts18 variant 1	AAM00199	Greene et al., 2000; Hancock et al., 2003
8 kDa diagnostic antigen TS18 variant 8	AAM00214	Hancock et al., 2003
8 kDa diagnostic antigen TsRS2	AAM00213	Hancock et al., 2003
8 kDa diagnostic antigen TsRS2 variant 1	AAM00212	Hancock et al., 2003
8 kDa diagnostic antigen TsRS1 variant 2	AAM00211	Hancock et al., 2003
8 kDa diagnostic antigen TsRS1	AAM00210	Hancock et al., 2003
8 kDa diagnostic antigen TsRS1 variant 2	AAM00209	Hancock et al., 2003
8 kDa diagnostic antigen Ts18 variant 7	AAM00203	Hancock et al., 2003
8 kDa diagnostic antigen Ts18 variant 6	AAM00202	Hancock et al., 2003
8 kDa diagnostic antigen Ts18 variant 5	AAM00201	Hancock et al., 2003
8 kDa diagnostic antigen Ts18 variant 4	AAM00200	Hancock et al., 2003
diagnostic antigen GP50	AAP49284	Hancock et al., 2004
diagnostic antigen GP50c precursor	AAP49288	Hancock et al., 2004
diagnostic antigen GP50b precursor	AAP49287	Hancock et al., 2004
diagnostic antigen GP50a precursor	AAP49286	Hancock et al., 2004
T24	AAP47268	Hancock et al., 2006
diagnostic antigen GP50	AAP49285	Hancock, direct submission
immunogenic protein	CAB42637	Hubert et al., 1999
immunogenic protein	CAB42638	Hubert et al., 1999
18 kDa glycoprotein mature form	AAX32919	Lee et al., 2005
14 kDa glycoprotein mature form	AAX32918	Lee et al., 2005
immunogenic protein Ts76	AAF60976	Liu, direct submission
immunogenic protein Ts21	AAF60975	Liu, direct submission
immunogenic protein Ts11	AAF60974	Liu, direct submission
hypothetical protein	CAD53347	Montero et al., 2003
hypothetical protein	CAD38264	Montero et al., 2003
18 kDa glycoprotein variant 1	AAK21959	Obregon-Henao et al., 2001
14 kDa glycoprotein	AAF67357	Obregon-Henao et al., 2001
HP6 protein	CAO82075	Parkhouse et al., 2008
low MW antigen 2 variant 1 (Ag2V1)	BAB18646	Sako et al., 2000
low MW antigen 2 (Ag2)	BAB18645	Sako et al., 2000
low MW antigen 1 variant 1 (Ag1V1)	BAB18644	Sako et al., 2000
low MW antigen 1 (Ag1)	BAB18643	Sako et al., 2000
low MW antigen 1 variant 1 (Ag1V1)	BAE96735	Sato et al., 2006b
low MW antigen 1 variant 1 (Ag1V1)	BAE96734	Sato et al., 2006b
low MW antigen 1 variant 1 (Ag1V1)	BAE96733	Sato et al., 2006b
low MW antigen 1 variant 1 (Ag1V1)	BAE96732	Sato et al., 2006b
low MW antigen 1 variant 1 (Ag1V1)	BAE96731	Sato et al., 2006b
low MW antigen 1 variant 1 (Ag1V1)	BAE96730	Sato et al., 2006b
low MW antigen 1 variant 1 (Ag1V1)	BAE96729	Sato et al., 2006b
low MW antigen 2	BAE96728	Sato et al., 2006b
low MW antigen 2	BAE96727	Sato et al., 2006b

Table continues on next page

Table 1.2: List of *Taenia solium* proteins used in antibody detection (cont.).

Protein name	GenBank accession no.	Reference
low MW antigen 2	BAE96726	Sato et al., 2006b
low MW antigen 2	BAE96725	Sato et al., 2006b
low MW antigen 2	BAE96724	Sato et al., 2006b
low MW antigen 2	BAE96723	Sato et al., 2006b
low MW antigen 2	BAE96722	Sato et al., 2006b
Cysticercus cellulosae-specific antigenic polypeptide	AAS55469	Yang, direct submission
membrane protein T24	ABI20734	Zhang, direct submission
excretion secretion antigen b1	ABI20733	Zhang, direct submission
18 kDa glycoprotein TS18 precursor	ABI20732	Zhang, direct submission
14 kDa glycoprotein TS14 precursor	ABI20731	Zhang, direct submission
excretion secretion antigen M13	ABI20730	Zhang, direct submission
excretion secretion antigen M4	ABI20729	Zhang, direct submission
cysticercosis 10 kDa antigen	ABI20728	Zhang, direct submission
calcium-binding protein	AAM34786	Zurabian et al., 2005

1.2.4 Immunodiagnosis: Antigen detection

Considering the drawbacks of antibody detection in clinical settings, antigen detection can provide a valuable alternative since it reflects the presence of viable parasites. In this respect, it can also provide a tool for serological monitoring of antiparasitic therapy (Garcia et al., 2000; Nguekam et al., 2003b). Several assays have been developed to detect parasite antigens in serum, CSF or urine using either polyclonal or monoclonal antibodies (Correa et al., 1989; Choromanski et al., 1990; Wang et al., 1992; Pardini et al., 2001; Espindola et al., 2002; Parija et al., 2004; Parija and Rajesh, 2006). Because of the localization of the cysts in the brain, antigen detection in CSF may be more appropriate for diagnosis than in serum; however, sampling of CSF is more cumbersome than blood sampling (Dorny et al., 2003).

Two monoclonal antibody-based tests (B158/B60 Ag-ELISA and HP10 Ag-ELISA) have been validated and are used routinely for the detection of parasite antigens (Harrison et al., 1989; Brandt et al., 1992; Garcia et al., 1998; Van Kerckhoven et al., 1998; Erhart et al., 2002; Nguekam et al., 2003a,c; Dorny et al., 2004b; Somers et al., 2006; Fleury et al., 2007; Sikasunge et al., 2008a). The sensitivity of Ag-ELISA in humans is reported to be high: $> 80\%$ for serum and even $> 90\%$ for CSF (Garcia et al., 1998, 2000; Fleury et al., 2007). Also, Erhart et al. (2002) noted a very high agreement between Ag-ELISA, CT scanning and biopsy results. However, when applied to rurally reared pigs with low cyst burdens, these well-established and reliable serodiagnostic procedures seem to be less sensitive (Sciutto et al., 1998) although other authors were able to detect pigs harboring one single cyst (Nguekam et al., 2003a; Dorny et al., 2004b). In addition, the genus

specificity of these tests does not allow the differentiation between infections of *T. solium* and *T. hydatigena* in pigs (Dorny et al., 2003).

In a recent community-based study in pigs, the B158/B60 and HP10 Ag-ELISAs were used in a Bayesian approach (together with tongue inspection and EITB) to calculate prevalence and sensitivity and specificity of each test (Krecek et al., 2008). This resulted in sensitivity and specificity (assuming absence of *T. hydatigena*) of 76% and 84%, respectively for the B158/B60 Ag-ELISA and 55% and 83%, respectively for the HP10 Ag-ELISA.

1.2.5 Molecular techniques

For the clinical diagnosis of NCC, histopathological examination of biopsy specimens can be a useful confirmation method. However, it is not always easy to make a definitive diagnosis of the cysticercus as a result of the preparation of tissue sections and the degree of degeneration and/or calcification of the tissue (Yamasaki et al., 2006b). Mitochondrial DNA analysis of biopsied lesions can confirm the initial diagnosis. Until recently, there was only one report on DNA diagnosis of cysticercosis found in 3 black bears in California (Theis et al., 1996). Since then, two cases of NCC and one case of intramuscular cysticercosis were confirmed by mitochondrial DNA analysis (Yamasaki et al., 2004b, 2006a; Sudewi et al., 2007). Other researchers confirmed the diagnosis of NCC by PCR detection of *T. solium* DNA in CSF (Almeida et al., 2006).

Molecular identification of biopsy specimens is not only important for definitive diagnosis in the case of doubtful outcome of histopathological examination, but also for differential diagnosis with other potentially zoonotic taeniid species. Indeed, cysticercosis due to *T. crassiceps* has been reported in patients with HIV-AIDS (Chermette et al., 1995; Maillard et al., 1998).

Molecular techniques such as multiplex PCR can also be important tools for molecular epidemiology in order to determine the genotype of *T. solium* (Afro-American or Asian) (Sato et al., 2006a).

1.3 Diagnosis of taeniosis

Taeniosis in man can be caused by *T. solium*, *T. saginata* or *T. saginata asiatica*. In control and/or elimination programs differentiation between these *Taenia* spp. may not be a critical point because any tapeworm infection in humans needs to be eliminated (Pawlowski, 2006). However, for individual diagnosis and in epidemiological studies, species differentiation is preferable.

1.3.1 Questioning

The easiest method by far to diagnose a tapeworm carrier, is asking if a person observed releasing tapeworm proglottids. *Taenia saginata*/*T. s. asiatica* carriers are often aware of the presence of a worm, considering the motility of the proglottids that actively exit the host's intestine. However, this is not the case for *T. solium* carriers, where the expulsion of proglottids occurs with the feces. False positive answers occur, since patients cannot always distinguish between tapeworm proglottids and other parasites (Dorny et al., 2005). However, after providing training to health care practitioners and informing the general population, self-detection of tapeworm carriers can be a useful strategy (Flisser et al., 2005).

1.3.2 Coproscopy

Using conventional fecal examinations both proglottids and eggs can be found in stool samples. As expulsion does not occur daily, repeated fecal examinations are advised (Dorny et al., 2005). Still, sensitivity remains low: 30–50% for finding tapeworm proglottids or eggs (Pawlowski, 2006). Anthelmintic treatment and the use of a purge with electrolyte-polyethyleneglycol salt (EPS) before and after treatment improves the recovery of the scolex and the quality of the expelled proglottids (Jeri et al., 2004). However, the scolex is recovered in only ±30% of patients.

Taeniid somata can be distinguished in several ways: based on morphological differences (Table 1.3), based on enzyme electrophoresis (Le Riche and Sewell, 1977) and based on molecular techniques (p.17). Taeniid eggs and proglottids of *T. saginata* and *T. s. asiatica* can only be distinguished using molecular tools. For morphological differentiation, mature proglottids need to be fixed and stained in order to examine the ovarian lobes and the vaginal sphincter. Gravid proglottids can be distinguished based on the number of unilateral uterine branches (Mayta et al., 2000). The presence or absence of rostellar hooks on the scolex does not automatically indicate that it is *T. solium* or *T. saginata*/*T. s. asiatica* since morphological abnormalities can occur (Rodriguez-Hidalgo et al., 2002).

Enzyme electrophoresis based on glucose phosphate isomerase zymogrammes is a faster and less labor intensive technique than staining somata. However, it requires fresh or frozen material since preservation of proglottids in alcohol or formalin destroys enzyme activity (Le Riche and Sewell, 1978).

1.3.3 Antibody detection

Adult tapeworm carriers can be diagnosed by detecting antibodies directed against ES antigens collected from immature *T. solium* tapeworms cultured in immunosuppressed

Table 1.3: Morphological differences between *Taenia solium*, *Taenia saginata* and *Taenia saginata asiatica* (Source: Dorny et al., 2005).

	T. solium	*T. saginata*	*T. s.asiatica*
Scolex			
- rostellum	present	absent	present
- hooks	22–32	absent	absent
Mature proglottids			
- number of testes	375–575	800–1200	324–1216
- ovary	3 lobes	2 lobes	2 lobes
- vaginal sphincter	absent	present	present
Gravid proglottids			
- number of unilateral uterine branches	7–16	14–32	11–32
- branching pattern	dendritic	dichotomous	dichotomous
- expulsion from host	passively[a] (in groups)	actively[b] (single)	actively[b] (single)

[a] Usually with feces

[b] Outside defecation

hamsters (Wilkins et al., 1999). The test, an immunoblot assay, was determined to be 95% sensitive and no cross reactions with other parasitic infections, including *T. saginata*, were observed. The ES antigens were characterized and two recombinant antigens of 33 kDa and 38 kDa were produced in order to replace the native proteins (Levine et al., 2004). Recently, these antigens were expressed by baculovirus in insect cells and tested in an EITB format. Field testing in Peru resulted in sensitivities of 97% for rES33 and 98% for rES38 and specificities of 100% and 91% for rES33 and rES38, respectively (Levine et al., 2007). The major advantages of this test are that it allows species-specific diagnosis and avoids the potential biohazard of handling feces contaminated with *T. solium* eggs (Allan et al., 2003). A drawback may be that antibodies still persist even though a person may no longer harbor the tapeworm.

Other researchers used oncosphere antigens to diagnose tapeworm carriers (Verastegui et al., 2003). Antibodies could be detected in 95% of tapeworm carriers but also in 20% of active NCC cases.

1.3.4 Copro-antigen detection

Antigen capture ELISA tests have been developed for the detection of parasite specific products in the feces of the host. The antibodies used in these tests have been raised by the hyperimmunization of rabbits. For the generation of polyclonal antibodies, rabbits were immunized with different *T. solium* or *T. saginata* antigens such as homogenized proglottids, ES antigens or surface fraction of adult tapeworms (Allan et al., 1990; Pawlowski et al., 2005). These tests have all shown consistent results: they are genus-specific — *T. solium* and *T. saginata* are both positive in the tests but no cross reactions occur with other intestinal parasites, antigen can be detected several weeks prior to patency, antigen levels are independent of egg output, coproantigen is stable for days at room temperature and for several months in formalin-fixed samples, coproantigen levels drop rapidly following successful treatment (Allan and Craig, 2006).

These tests have been used in field studies: they gave a good specificity (over 99%) and consistently detected 2.4 times more taeniosis cases than microscopy (Allan et al., 1993, 1996; Garcia et al., 2003a; Margono et al., 2006).

However, there are still some practical issues to be resolved (Pawlowski et al., 2005). The performance of the test in field studies on *T. saginata* remains largely unknown. The test is still not widely available and currently no commercially available test is on the market. Given the fact that the technology itself is not complicated, it should be feasible to establish a system of reference laboratories that can provide the test.

1.3.5 Molecular techniques

Various molecular techniques have been developed to identify to species level the expelled tapeworm proglottids or eggs, including the use of DNA probes (Rishi and McManus, 1987; Harrison et al., 1990; Gonzalez et al., 2000), PCR and PCR coupled to restriction fragment length polymorphism (RFLP) (Mayta et al., 2000; Rodriguez-Hidalgo et al., 2002; Gonzalez et al., 2002; Nunes et al., 2005), single strand conformation polymorphism (SSCP) (Gasser et al., 1999), random amplified polymorphic DNA (RAPD)-PCR (Maravilla et al., 2003), multiplex PCR (Yamasaki et al., 2004a), nested PCR (Mayta et al., 2008) and sequence characterized amplified region (SCAR) markers (Dias et al., 2007). Each of these techniques has advantages and disadvantages, e.g. the use of DNA probes, PCR-RFLP and SSCP are relatively time-consuming; however, PCR using species-specific primers rapidly provides sensitive and reliable results (Yamasaki et al., 2006b).

The use of molecular techniques has been mostly focused on the differentiation of *T. solium* from *T. saginata*, genetic characterization and intraspecific genetic polymorphism (Bowles and McManus, 1994; Gasser et al., 1999; Maravilla et al., 2003). Recently, tests for the differential diagnosis of *T. saginata*, *T. s. asiatica* and two genoypes of *T. solium* have been developed based on mitochondrial DNA (Yamasaki et al., 2002, 2004a; Somers et al., 2007).

Methods have been developed to extract DNA of *Taenia* spp. from fecal samples which can be used in a copro-PCR for the diagnosis of taeniosis (Nunes et al., 2003; Yamasaki et al., 2004a). There are still some limitations to be overcome: current DNA extraction methods are too expensive for use as a routine test (Dorny et al., 2005); non-amplification of target DNA can occur due to competition between template DNA and inhibitory substances present in the feces, sample heterogeneity on stool collection and preservation of stool samples (Yamasaki et al., 2006b). However, in a recent evaluation of copro-PCR, Wandra et al. (2006) reported that when stool samples were stored in ethanol properly after collection, taeniid DNA was detected in all the examined tapeworm carriers.

1.4 Conclusion: application of immunodiagnostic tools for diagnosis of cysticercosis

Immunodiagnostic assays are a useful tool in epidemiological studies to estimate the prevalence and identify the risk factors associated with transmission of *T. solium* (Widdowson et al., 2000; Phiri et al., 2002; Moro et al., 2003; Sikasunge et al., 2007); they also offer the possibility of surveillance of the infection during and after control programs (Sarti et al., 2000). The application of antibody or antigen detection depends on the information that

is needed. Antibody detection assays only reflect exposure to the parasite whereas antigen detection assays indicate the presence of living parasites. Ideally, a combination of both tests is best for seroepidemiological studies and for supporting diagnosis by neuro-imaging techniques (Dorny et al., 2004a). So far, only a few studies on NCC and epilepsy used both antigen and antibody detection (Newell et al., 1997; Correa et al., 1999b; Zoli et al., 2003; Prado-Jean et al., 2007).

Validation of immunodiagnostic tests is required in order to obtain accurate data on sensitivity and specificity. In the absence of gold standard, a Bayesian approach may be useful to draw assumptions about test characteristics (sensitivity and specificiy) and prevalence (Pouedet et al., 2002; Dorny et al., 2004b; Krecek et al., 2008).

Recently, in a joint effort FAO, WHO and OIE [2] published a comprehensive report with the guidelines for the surveillance, prevention and control of taeniosis/cysticercosis (Murrell, 2005). Research priorities include the development of more sensitive and specific diagnostic tests for use in pigs and a better way to identify people infected with the disease (Eddi et al., 2003). Indeed, reliable tests for diagnosis of *T. solium* cysticercosis in pigs are still lacking. Antibody detection only reflects exposure and not necessarily an established infection. The use of the current antigen detection tests in pigs is hampered by the cross reactions with other taeniid species. Furthermore, up to now there is no test available that can distinguish between infections with viable cysts (active cysticercosis) and infections with degenerated cysts (inactive cysticercosis). A diagnostic test differentiating between active and inactive cysticercosis in pigs would be an important tool to study transmission dynamics and immunity in epidemiological studies and to monitor the effect of anthelmintic treatment or vaccination in control programs. In humans, this differentiation would be a practical aid for clinical diagnosis and case management. Also, a quantitative diagnostic assay would be a useful tool in epidemiological studies to assess the infection burden and the risk of transmission of the disease. Moreover, the biggest challenge would most likely be the development of a diagnostic assay with sufficient sensitivity to detect cysticercosis in rural pigs with generally low levels of cyst burden (Sciutto et al., 1998).

There is a clear need for better diagnostic assays for cysticercosis, especially for diagnosis in pigs. To develop such assays, serological markers that can be incorporated in a diagnostic test need to be identified. Chapter 2 gives an overview of two recent technological developments applied in the experimental work of this thesis that can assist in identifying such markers.

[2]FAO: Food and Agriculture Organization of the United Nations; WHO: World Health Organization; OIE: World Organization for Animal Health

2

Laser beams and dromedaries...
Review of SELDI-TOF MS and Nanobodies

2.1 General introduction

This chapter gives a short overview of the two techniques applied in the experimental work of this thesis. Surface enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a recent MS-based technology that can be applied to identify biomarkers e.g. for different disease states. Because of the promising results obtained with SELDI-TOF MS for diagnosis of human African trypanosomiasis (sleeping sickness) (Papadopoulos et al., 2004), this approach was chosen for the present work to search for biomarkers that can distinguish between active and inactive cysticercosis, i.e. infections with viable and degenerated cysts, respectively (Chapter 4). The first part of this chapter shortly reviews the general methodology, along with some applications and the current controversy on the use of SELDI-TOF MS.

The second part of this chapter focuses on the properties, production and applications of so-called "Nanobodies" (Nb), recombinant single-domain antibody fragments derived from the Heavy-chain antibodies naturally occurring in camelids. Since Kohler and Milstein (1975) introduced the hybridoma technology, the availability of monoclonal antibodies opened the way to the development of diagnostics and therapeutics. Nowadays, focus tends to shift to the cloning and engineering of smaller antibody fragments by genetic engineering. Because of their particular properties, Nbs are a valuable tool for the development of antibody-based diagnostic and therapeutic applications. Moreover, Nbs often recognize novel epitopes that are not recognized by conventional monoclonal antibodies. We therefore chose this approach to isolate and characterize Nbs that can be used for specific diagnosis of *T. solium* cysticercosis (Chapter 5).

2.2 Surface enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF MS)

2.2.1 Introduction

The search for specific biomarkers to diagnose disease has evolved rapidly since the advent of proteomics. Proteomics in general deals with the large-scale study of gene and cellular function directly at protein level. Proteins are vital parts of living organisms, as they are the main components of the physiological metabolic pathways of cells. Understanding the proteome, the structure and function of each protein and the complexities of protein-protein interactions will be critical for developing the most effective diagnostic techniques and disease treatments in the future. During the early years of proteomics and until relatively recently, profiling of protein expression in disease relied primarily

on the use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), which was later combined with mass spectrometry (Hanash, 2003). The potential of MS to yield comprehensive profiles of peptides and proteins in biological fluids without the need to first carry out protein separations has attracted interest. In principle, such an approach is highly suited for biomarker identification because of reduced sample requirements and high throughput. A recent development in this field, particularly for serum analysis, is the technology referred to as surface enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF MS), first introduced by Hutchens and Yip (1993).

2.2.2 General methodology

The SELDI-TOF MS technology consists of three major components: the ProteinChip Array, the reader, and the data analysis software. The ProteinChip Array consists of either 8 or 16 spots composed of a specific chromatographic surface (Figure 2.1). The chromatographic surfaces are uniquely designed to retain proteins from a complex sample mixture according to general or specific protein properties. Each spot is composed of a chemically (anionic, cationic, hydrophobic, hydrophilic or metal ion) or biochemically (antibody, receptor, DNA, enzyme, etc.) active surface (Merchant and Weinberger, 2000). Biochemical surfaces are designed to interact specifically with a single, target protein and are custom-made by using an open, preactivated platform on which a bait molecule is immobilized. Chemically active surfaces retain whole classes of proteins and are commercially available (Issaq et al., 2003). As such, chemically active surfaces are preferred for biomarker discovery. The sample is dispensed onto the ProteinChip surface and to achieve protein specificity a series of washes is applied with an appropriate buffer designed to elute unbound proteins and interfering substances while retaining the proteins of interest (Issaq et al., 2002). Energy-absorbing molecules, often referred to as "matrix", are added and the ProteinChip is inserted in the reader.

The ProteinChip Reader is a laser desorption ionization (LDI) time-of-flight (TOF) mass spectrometer equipped with a pulsed, UV, nitrogen laser (Figure 2.2). The thermal energy from the laser is transferred from the matrix to the sample proteins which irradiate and then are desorbed/ionized. The ionized gaseous molecules enter the TOF MS region of the instrument, which measures the mass-to-charge ratio (m/z) of each protein, based on the time required for the ions to fly down the vacuum tube towards an oppositely charged electrode (lighter ions travel faster through the TOF tube then heavier ions). Each ion that strikes the electrode is registered as a component of the data spectrum that emerges from the analysis. The output generated from the TOF MS analysis is a series of peaks showing the relative abundance versus the molecular weights (MW) of the detected proteins.

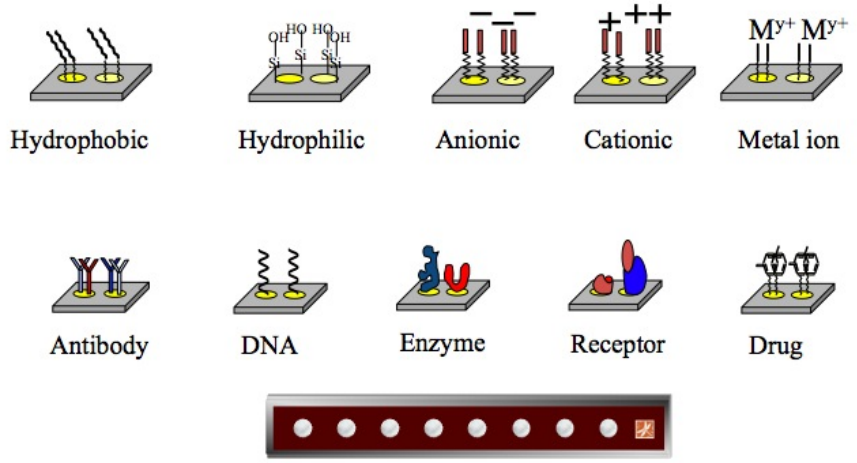

Figure 2.1: ProteinChip Array with its different chromatographic surfaces. The upper arrays represent chemically modified surfaces and the bottom arrays are biochemically modified surfaces (Source: Ciphergen Basic Course).

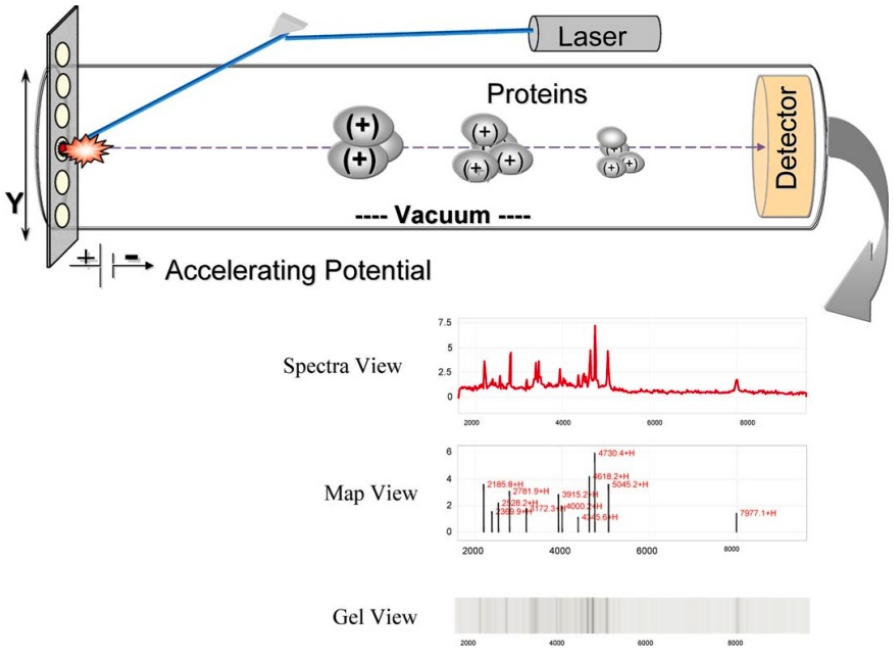

Figure 2.2: Schematic of SELDI-TOF mass spectrometer. The time-of-flight mass spectrometer measures the MW of the proteins retained on the ProteinChip Array. The software associated with the instrument can display the result either as a spectrum, map or gel view (Source: Issaq et al., 2002).

One of the primary uses of SELDI-TOF MS is to identify differences in the protein expression profiles of two or more distinct samples. The samples are often quite complex, particularly in the field of biomarker discovery where protein profiles of clinical samples are analyzed. The software can convert the MS peak traces to more simplified map views or one-dimensional gel electrophoresis displays to simplify the identification of unique peaks or those that display differences in protein abundance in one of the samples (Figure 2.2). The end result of a SELDI-TOF MS analysis is a list of the MW of proteins whose relative abundance differs between two or more samples. The next step depends on the specific application of the SELDI-TOF platform. If actual identification of the differentially expressed proteins is required, most often the proteins need to be purified using classical chromatography followed by identification using conventional high-resolution MS (Issaq et al., 2003). However, a new ion source has been developed that allows the proteins to be tryptically digested directly on the ProteinChip Array and the resultant fragments identified by tandem MS (Issaq et al., 2002). An application that has generated much interest in SELDI-TOF MS is its potential use as a diagnostic tool in the early detection of diseases (Conrads et al., 2003). By surveying different population cohorts (i.e. diseased vs healthy), unique protein abundance profiles can be produced. Specific proteomic signatures can be distinguished by statistical analysis using powerful data-mining tools (Petricoin et al., 2002c). Proteomic pattern analysis relies on the pattern of proteins observed and does not rely on the identification of a traceable biomarker.

2.2.3 Applications

The versatility of SELDI-TOF MS has allowed its use in projects ranging from the identification of disease biomarkers to different biological applications such as the study of biomolecular interactions (Hinshelwood et al., 1999), the characterization of posttranslational modifications and phosphorylation states of proteins involved in signal transduction pathways (Thulasiraman et al., 2004).

With regard to disease biomarker discovery, extensive research has been done on the identification of biomarkers for the (early) diagnosis of different forms of cancer. A non-exhaustive list can be found in Table 2.1. SELDI-TOF MS has also been used to discover biomarkers for other diseases such as Alzheimer's disease (Carrette et al., 2003), HIV-1-associated dementia (Luo et al., 2003), Gaucher disease (Boot et al., 2004) and hepatitis (Morra et al., 2007).

Examples of studies employing SELDI-TOF MS in the field of bacterial and parasitic diseases are relatively scarce. Thulasiraman et al. (2001) studied virulence factors in *Yersinia pestis*. The differential expression of proteins in *Y. pestis* grown at 26°C and 37°C was monitored. Two antigens were identified as catalase-peroxidase and Antigen 4.

Hynes et al. (2003) performed proteomic profiling of *Helicobacter* species. They found distinct profiles for the different strains. Also, *Helicobacter pylori* strains form culture collections that had undergone numerous subcultures were almost identical, whereas the profiles from fresh isolates were markedly different.

Papadopoulos et al. (2004) used proteomic signature analysis for the diagnosis of human African trypanosomiasis (HAT). The serum proteomic profiles of 85 patients with HAT and 146 controls were compared and data analysis was done using 3 distinct tools (tree-classifier, neural network and genetic algorithm). They reported an overall sensitivity and specificity of 100% and 98.6%; no individual biomarkers were identified.

Rioux et al. (2007) studied biomarkers during the first twelve weeks of *Fasciola hepatica* infection in sheep. The goal was to identify biomarkers for early parasite invasion and define biomarkers expressed within 5 weeks of infection since these could theoretically be involved in establishment or suppression of host immunity. They noted significant changes in biomarker patterns beginning within 3 weeks of infection; two biomarkers were identified as transferrin and apolipoprotein A-IV.

Another study identified collagen-binding proteins produced by 4 different *Lactobacillus* spp. (Howard et al., 2000). It has been proposed that the release of these and other extracellular matrix-binding proteins prevent the colonization by infectious pathogens and this might explain the beneficial effects of probiotic bacteria.

2.2.4 SELDI: subject of debate

SELDI-TOF MS technology is potentially a promising tool for the rapid identification of disease-specific biomarkers and proteomic patterns. SELDI technology has significant advantages over other proteomic technologies in that it has a very high throughput and the amount of input material required for analysis is miniscule compared with more traditional 2D-PAGE (Wulfkuhle et al., 2003). Proteomic-based studies have offered real hope of new marker discovery, but many initially promising studies have not been followed by wider-scale validation and application (Banks, 2008).

Recently, there has been considerable controversy concerning the SELDI profiling approach. A major concern has been whether the SELDI-based approaches are reproducible, other concerns deal with study design bias (Diamandis, 2004a; Hu et al., 2005; Coombes et al., 2005a). Indeed, analysis of published data for ovarian cancer revealed the existence of significant experimental bias (Sorace and Zhan, 2003; Baggerly et al., 2004a,b).

When comparing different studies on ovarian, prostate and breast cancer, it was noted that there were discrepancies between the results of the different research groups (Diamandis, 2003, 2004b; Xiao et al., 2005; Skytt et al., 2007). However, it was suggested that different peaks were identified because of different methods and different approaches

Table 2.1: Examples of studies applying SELDI-TOF MS for discovery of cancer biomarkers.

Cancer type	Study type	Identified markers	Reference
ovarian cancer	pattern[a]		Petricoin et al., 2002a
	marker ID[b]	transferrin, haptoglobin precursor fragment, immunoglobulin heavy chain	Rai et al., 2002
	marker ID	haptoglobulin-α	Ye et al., 2003
	pattern		Vlahou et al., 2003
	marker ID	transthyretin, beta-hemoglobin, apolipoprotein A-I, transferrin	Kozak et al., 2003, 2005
	marker ID	apolipoprotein A-I, truncated transthyretin, inter-α-trypsin inhibitor fragment	Zhang et al., 2004b
prostate cancer	pattern		Petricoin et al., 2002b
	pattern		Adam et al., 2002
	pattern		Qu et al., 2002
	pattern		Li et al., 2004
	pattern		Skytt et al., 2007
breast cancer	pattern		Li et al., 2002
	marker ID	complement component $C3a_{desArg}$, truncated $C3a_{desArg}$	Li et al., 2005
	pattern		Mendrinos et al., 2005
	marker ID	treatment responsive proteins: kininogen, apolipoprotein A-II	Heike et al., 2005
colon cancer	marker ID	prothymosin-α	Shiwa et al., 2003
head and neck cancer	marker ID	annexin V	Melle et al., 2003
lung cancer	pattern		Zhukov et al., 2003
pancreas cancer	pattern		Koopmann et al., 2004
bladder cancer	pattern		Liu et al., 2005
colorectal cancer	marker ID	apolipoprotein C-I, albumin fragment, apolipoprotein A-I	Engwegen et al., 2006

[a] Diagnosis based on differential proteomic patterns, no biomarker identification

[b] Individual biomarkers were identified

to statistical analysis (Grizzle and Meleth, 2004). From this, it is clear that differences in experimental set-up (pre- and post-analytical aspects) could be responsible for the lack of agreement between different studies (Karsan et al., 2005). This was reviewed by Bons et al. (2005) and a summary of these aspects is presented in Table 2.2.

Table 2.2: Pre- and post-analytical aspects important for protein profiling studies (Source: Bons et al., 2005).

Pre-analytical aspects	
Storage effects:	freeze-thaw cycles, storage temperature
Use of serum or plasma	
Sampling time:	clotting time, spinning time, time between spinning and storage
Sample preparation:	denaturation, fractionation, depletion of high abundance proteins
Calibration	
Matrix	
Post-analytical aspects	
Patient population	
Bioinformatics and biostatistics:	peak detection, laser settings, data analysis software

The National Cancer Institute/Early Detection Research Network sponsored a multicenter validation study of markers for the early detection of prostate cancer. In the first-stage study analytical reproducibility of the SELDI platform was evaluated and good cross-laboratory reproducibility could be achieved under strict operating procedures (Semmes et al., 2005). In the next stage the intention was to test if the same algorithm could discriminate between cancer and noncancer samples derived from independent and geographically distinct nonoverlapping populations (McLerran et al., 2008a). This could not be achieved because the diagnostic performance was compromised by bias. When analyses were repeated using a new cohort of specimens and eliminating this bias, discrimination between cancer and noncancer was again not achieved (McLerran et al., 2008b). The authors concluded that the results from the previous studies (Adam et al., 2002; Qu et al., 2002) were not generalizable. This validation study again emphasizes the critical role of pre-analytical factors that can introduce bias.

Liotta and Petricoin (2008) stated that for proper clinical validation, the diagnostic

protocol and the instrument must be fixed and standardized. Commercial MS technology is highly competitive and new instruments and technologies are emerging several times a year. Consequently, to prevent a diagnostic fingerprint profile, that is strictly dependent on a particular MS technology, to become obsolete, diagnostic biomarker readouts need to be generated that are independent of the measurement platform. In this case that means sequencing and identification of the proteins underpinning the diagnostic peaks. Once the proteins are identified, they can be measured by any suitable immunoassay. This approach is now the major one used for biomarker discovery, rather than solely relying on proteomic pattern analysis. However, another concern raised in this respect, is that most, if not all, identified proteins thus far represent acute-phase reactants present in high abundance in serum (see Table 2.1) and as such are of questionable clinical value (Diamandis and van der Merwe, 2005; Diamandis, 2006).

So, what is the way forward? Careful study design, standardization/calibration and a systematic characterization of pre-analytical and analytical effects will be required (Master, 2005), as well as improving instrumentation and applying rigourous quality control and quality assessment of SELDI data (Coombes, 2005; Hong et al., 2005). Protein profiling can only become a reliable diagnostic tool if it fulfils the criteria for reproducibility and standardization that are generally accepted for diagnostic tests in clinical chemistry. There is a need for better characterization and careful description of the methods, including technical details, to allow comparison between studies (Bons et al., 2005). Standard protocols for proteomic studies using SELDI-TOF MS would be useful, however, recommendations are not yet available. To summarize with the words of Coombes et al. (2005a): serum proteomics profiling — a young technology that begins to mature...

2.3 Nanobodies

2.3.1 Introduction

The discovery of hybridoma technology announced a new era in antibody research (Kohler and Milstein, 1975). By fusing mouse plasma cells with myeloma cells, monoclonal antibodies to different antigens can be produced and isolated. Besides the importance as research tool, the availability of murine monoclonal antibodies opened the way to the development of diagnostic and therapeutic applications. However, the application of monoclonal antibodies on a large scale suffers from some technical drawbacks such as expensive production methods, inability to optimise the antibody by genetic engineering and the potential immunogenicity of mouse antibodies when administered to humans (Carter, 2001). Strategies have been developed to avoid this immune response, including fusion of mouse variable regions to human constant regions as "chimeric" antibodies, "de-immunization" by removal of T-cell epitopes and "humanization" by grafting mouse complementarity determining region (CDR) onto human antibody scaffolds (Hudson and Souriau, 2003). Some commercially available therapeutic antibodies include monoclonal antibodies to CD25 (IL-2 receptor subunit) for management of allograft rejection (daclizumab, Zenapax; basiliximab, Simulect), TNF-α for treatment of Crohn's disease and rheumatoid arthritis (infliximab, Remicade) and to respiratory syncytial virus (palivizumab, Synagis) (Elliott et al., 1994; van Dullemen et al., 1995; The IMpact-RSV Study Group, 1998; Vincenti et al., 1998). Alternative strategies now allow selection of fully human monoclonal antibodies from transgenic mice carrying human immunoglobulin loci (He et al., 2002; Babcock et al., 2006).

Recombinant DNA technology has allowed the cloning and genetic engineering of antibody genes, which can then be expressed as recombinant antibodies in eukaryotic cells. However, the first trials to produce antibodies in bacteria yielded disappointingly low levels of functional molecules (Boss et al., 1984). To obtain good yields of active antibody protein, it was necessary to change the antibody format from the full length molecule, consisting of two multidomain heavy and light chains, to smaller versions like the antigen binding fragment (Fab) or variable domain fragment (Fv) and in a later stage single-chain Fv fragment (scFv, Fv fragment with a peptide linker connecting the domains) (Figure 2.3) (Ward, 1993). Unfortunately, these scFv fragments have a reduced affinity compared to the parent antibody, are prone to aggregation and proteolysis of the linker sequence (Muyldermans, 2001).

In the past, antigen-binding fragments comprising the single conventional heavy chain variable domain (VH) have also been generated (Ward et al., 1989). However, apart from the reduced affinity for the antigen, removing the VL domain exposes the large

hydrophobic surface of the VH rendering the VH molecule "sticky" and difficult to produce in soluble form. Consequently, it has not been possible to generate single-domain VH antibodies as a valid alternative to monoclonal antibodies.

2.3.2 Heavy-chain antibodies

Hamers-Casterman et al. (1993) noticed that a significant proportion of the natural antibody repertoire of camels, dromedaries and llamas consists of a unique type of antibodies lacking light chains (Figure 2.3). These so-called Heavy-chain antibodies (HCAbs) are fully functional in antigen binding, as opposed to mouse or human HCAbs, produced as a result of a pathological disorder, known as heavy-chain disease (Seligmann et al., 1979). Homodimeric HCAbs have also been documented in nurse shark (Greenberg et al., 1995) and spotted ratfish (Rast et al., 1998). The heavy chains of HCAbs have a lower MW than their conventional antibody counterparts due to the absence of the first constant domain (CH1). This explains the absence of the light chain in HCAb, as the CH1 domain is the anchoring point for the constant domain of the light chain (Padlan, 1994). Consequently, the HCAbs bind their antigen by one single domain, the variable domain of the heavy chain, referred to as VHH (to distinguish it from classic VH) instead of the paired VH and VL domains (Muyldermans et al., 1994). As such, the VHH is the smallest functional antigen-binding fragment (15 kDa) derived from a functional immunoglobulin. By definition, Nanobodies (Nb) are recombinant VHHs with a known affinity for a particular antigen. Adhering to this definition, in the next paragraphs we will use the term "VHH" instead of "Nanobody".

Approximately 50% of the serum IgG repertoire of dromedaries consists of Heavy-chain antibodies (Hamers-Casterman et al., 1993); in llamas this is only 25–45% (van der Linden et al., 2000). Different IgG fractions can be isolated by differential adsorption on protein A and protein G immuno-adsorbents. According to the decreasing MW of the heavy chain of the antibodies within these fractions, the IgGs were named IgG1, IgG2 and IgG3. The IgG1 fraction contains the conventional antibodies whereas the IgG2 and IgG3 fractions are HCAbs (De Genst et al., 2006a). Differences in the hinge sequences allow further differentiation of these IgG subclasses.

2.3.3 Properties

2.3.3.1 Sequence and structure of VHH

Sequence analysis demonstrated that the specific VHH gene is encoded in the germline by a distinct set of V genes (Nguyen et al., 1998). Analysis of genomic and cDNA VHH sequences shows that the CH1 domain is present in the genome but is spliced out during

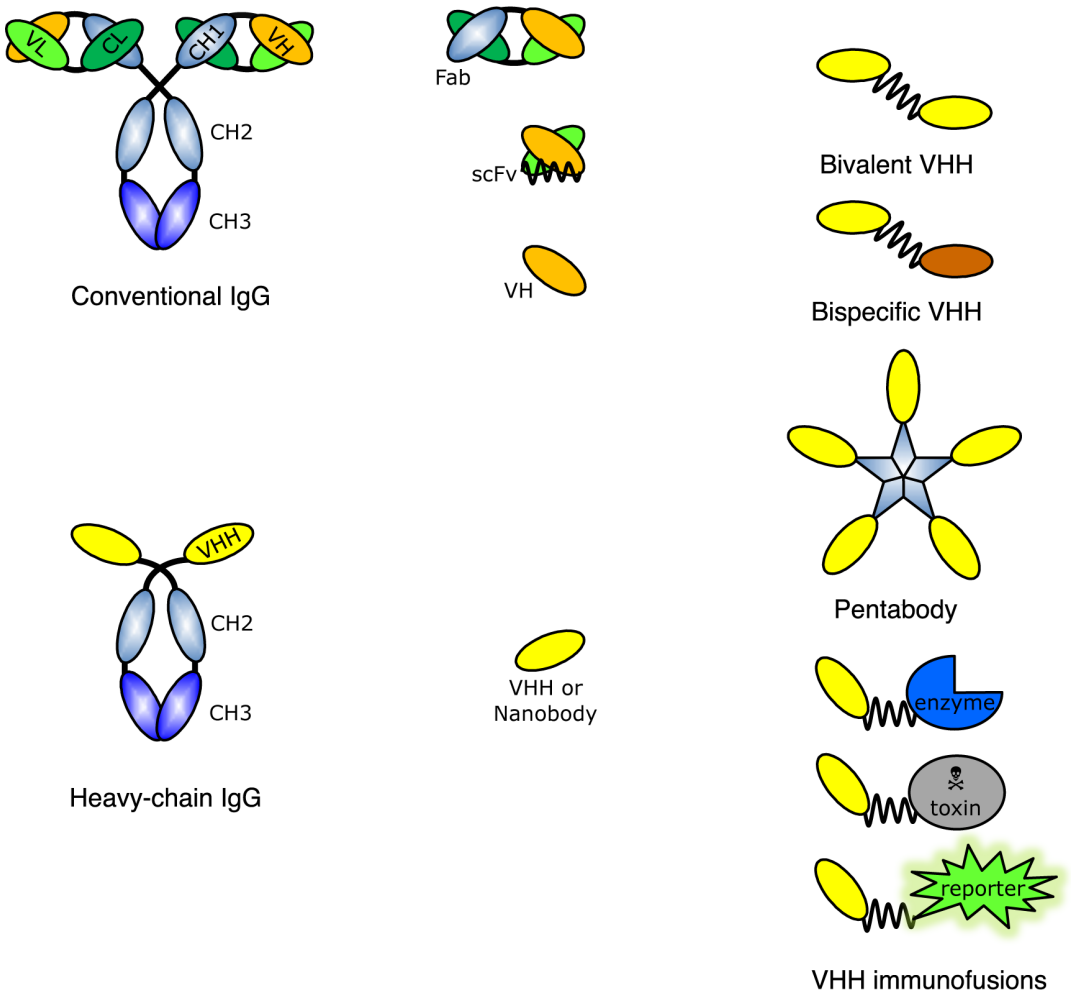

Figure 2.3: Schematic representation of conventional and Heavy-chain antibodies and derived antigen binding fragments. VH, VHH = variable domain of the heavy chain, VL = variable domain of the light chain, CH = constant domain of the heavy chain, CL = constant domain of the light chain.

mRNA processing probably due to point mutations in the donor splice site at the 3' end of the CH1 (Nguyen et al., 1999). Due to the fact that the light chain is missing in HCAbs, combinatorial diversity (random joining of VH and VL) is not possible. Nevertheless, VHH germline segments are highly diverse and rearranged VHHs are extensively diversified by somatic mutation processes, leading to a broad antigen-binding repertoire (Nguyen et al., 2000).

With regard to the amino acid sequence, VH and VHH domains share a high degree of identity. The amino acid residues determining the typical immunoglobulin fold are very well conserved (Vu et al., 1997). However, VHH carry a number of remarkable amino acid substitutions in the framework-two region (Figure 2.4). These residues are extremely well conserved in VH domains and are involved in forming the hydrophobic interface with VL domains. The Val37Phe (or Tyr), Gly44Glu, Leu45Arg and Trp47Gly substitutions (in going from VH to VHH) render this side of the domain more hydrophilic to compensate for the absence of the VL domain (Muyldermans et al., 1994). Another noteworthy substitution is the Leu11 to Ser in framework-1 region. This amino acid normally interacts with the CH1 domain, which is missing in HCAbs. This suggests that the amino acids located in this part of the VHH will be solvent exposed. The Leu11Ser mutation increases the hydrophilicity and helps in keeping the VHH domain soluble (Muyldermans, 2001).

Besides the VH/VHH hallmark substitutions in the framework regions, the hypervariable regions of VHH also differ from VH (Vu et al., 1997). First, the hypervariable regions CDR1 and CDR3 of VHH are on average longer than those of VH. Secondly, the long CDR3 is most often connected by a disulfide bond to the CDR1 (Figure 2.4). Structural analysis provides evidence that the antigen-binding loops of VHH exhibit a much larger structural repertoire than observed for VH. The most striking difference in this respect is that in VHH the long CDR3 loop tends to protrude form the VHH surface (Muyldermans, 2001).

2.3.3.2 Unique features of VHHs as compared to conventional antibody fragments

The single domain nature of VHH gives rise to several unique features and advantages for biotechnological applications. Besides the easy cloning and selection of VHHs from an in vivo matured immune library using display technologies (See Production of antigen-specific VHH, pg.34), there are other advantages such as the high expression yield and ease of purification, the solubility and stability, the generation of antigen-specific, high-affinity binders, the recognition of unique conformational epitopes and the homology to human VH III sequences.

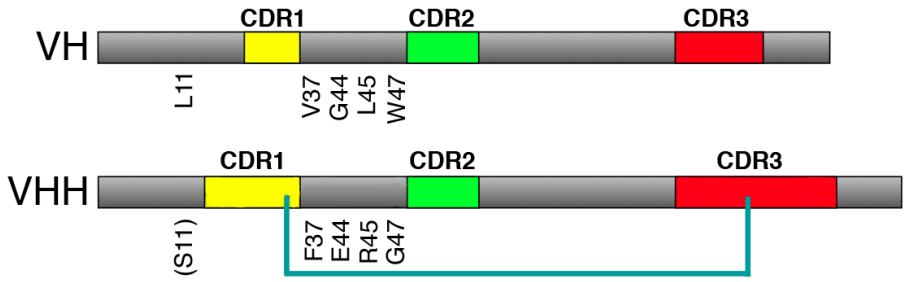

Figure 2.4: Schematic representation of the gene sequence differences between VH and VHH. The position of the CDR in between the framework regions is indicated. The CDR1 and CDR3 of a VHH are larger than in VH genes, and they are often connected by a disulfide bond (blue line). The hallmark amino acid substitutions in framework 1 and 2 are given. The numbering is according to the Kabat numbering (Kabat, 1988).

High expression yields and ease of purification. VHHs are expressed efficiently as soluble and non-aggregating recombinant proteins due to their small size and hydrophilic substitutions in framework-2. When expressed in *Escherichia coli* grown in shaking culture flasks, the yield is on average 10 times higher for VHH than most scFv constructs (Arbabi-Ghahroudi et al., 1997). With other expression systems (e.g. *Saccharomyces* yeast culture) it is possible to obtain even higher production yields of more than 100 mg/l (Frenken et al., 2000). Recombinant VHH can easily be purified by directing the expressed VHH toward the periplasm and subsequently extracting the periplasmic proteins by simple osmotic shock (Skerra and Plückthun, 1988). When the recombinant VHH contains a C-terminal histidine tag, further purification is done with immobilized metal affinity chromatography (IMAC). Other procedures such as purification by Protein A chromatography for llama VHH or production of VHH as inclusion bodies are also possible (Muyldermans, 2001).

Highly soluble and stable single domain immunoglobulin fold. In contrast to VH domains, VHHs are naturally soluble because of the hydrophilic amino acid substitutions in framework 2 region. Furthermore, due to their single domain nature, the absence of engineered structures such as linker sequences (which can cause aggregation as observed for scFv) enhances solubility which results in better functional expression. VHH antibody fragments are also extremely temperature stable: they retain most of their binding capacity after incubation at 37°C (Arbabi-Ghahroudi et al., 1997). Some VHHs have even been shown to remain functional at 90°C (van der Linden et al., 1999). This high apparent stability is mainly attributed to their efficient refolding after chemical or thermal denaturation and to a lesser extent to an increased resistance to denaturation (Dumoulin

et al., 2002).

Antigen-specific, high-affinity binders. VHHs, cloned from an immunized dromedary, have been matured in vivo against the antigen. Therefore, no engineering is required to produce high affinity binders and VHHs that bind antigen with affinity in the nanomolar range are normally acquired (Muyldermans and Lauwereys, 1999). The affinities of VHHs are generally comparable to those of conventional antibody fragments. Occasionally, VHHs with affinity constants (K_D) as low as 100 pM are isolated (Saerens et al., 2004; De Genst et al., 2006b).

Recognition of unique conformational epitopes. The antigen-binding site of conventional antibodies forms a planar or concave surface, depending on the antigen, being a protein or hapten, respectively (Padlan, 1996). Besides these standard architectures, the antigen-binding site of VHH also includes protruding loops, so that small hidden epitopes can be targeted. Indeed, VHHs recognize unique epitopes that are currently out of reach for conventional antibodies, e.g. conserved cryptic epitopes of infectious agents (Stijlemans et al., 2004). Also, VHHs preferentially interact with the substrate pocket of enzymes and can thus act as potential receptor agonist/antagonist. Several VHHs that act as competitive enzyme inhibitors have been isolated against a variety of enzymes including lysozyme, carbonic anhydrase, α-amylase, β-lactamase and T-cell ecto-ADP-ribosyltransferase (Lauwereys et al., 1998; Conrath et al., 2001b; Koch-Nolte et al., 2007). The ability to recognize these recessed antigenic sites has been attributed to their smaller size and the ability of the protruding CDR3 loop to penetrate such sites (Desmyter et al., 2001). However, the latter is possibly not the only mechanism to block the antigenic enzyme: the non-canonical conformation of the CDR1 loop might also interact with cavities on the antigen surface (Muyldermans, 2001).

With respect to the antigen binding, the single-domain nature could be a disadvantage for binding to small antigens such as haptens and peptides because these normally bind in a groove or cavity at the VH-VL interface (Sundberg and Mariuzza, 2002). However, VHHs that are capable of binding low MW haptens have also been isolated (Alvarez-Rueda et al., 2007; Doyle et al., 2008).

Close homology to human VH fragments. As mentioned in the introduction, a major drawback of current diagnostic and therapeutic antibodies is that they are of murine origin, thus eliciting an immune response in the patient. The construction of chimeric, humanized or veneered antibodies has resolved some of this toxicity. In this respect, the unique features of VHH offer added value over conventional antibody fragments. The sequence comparison of the human VH and dromedary VHH demonstrates their high

degree of identity. The "humanization" of camel VHH is therefore more straightforward as compared to their murine counterparts because they are smaller and more homologous to human antibodies (Vu et al., 1997). Most of the substitutions other than the key camel-specific substitutions in framework 2 can be replaced by the human sequence without altering the performance of the VHH. The VHH will therefore be a good candidate to humanize by veneering (Muyldermans, 2001). A universal humanized VHH framework can then be employed with grafting of different antigen-binding loops (Saerens et al., 2005b).

2.3.4 Production of antigen-specific VHH

In principle, antigen-specific VHH can be obtained by proteolysis of HCAb (polyclonal VHH and VHH_2), by direct cloning of the VHH genes from B-cells of immunized animals, and from naïve or synthetic libraries (Muyldermans, 2001). This review will focus on cloning of recombinant VHH from an immunized animal, screening for antigen-specific binders by phage display and panning, and production in *E. coli* (Figure 2.5), as demonstrated by Arbabi-Ghahroudi et al. (1997). This is regarded as a general route to obtain small antigen-specific binders. Other selection platforms and library types are reviewed by Hoogenboom (2005).

2.3.4.1 VHH repertoire cloning

A dromedary (or another member of the Tylopoda) is immunized with the antigen of interest according to standard immunization protocols. The blood of the animal is collected and the peripheral blood lymphocytes are isolated. Saerens et al. (2004) demonstrated that lymph node lymphocytes can also serve as B-cell source to construct antibody libraries. However, puncture and aspiration of a lymph node is a more cumbersome technique than simple blood collection.

Subsequently, mRNA is isolated from the lymphocytes and used as a template for cDNA synthesis. Since the antigen-binding VHH is encoded by a single exon with homologous border sequences, the complete in vivo matured VHH repertoire of an immunized animal can be amplified by one single set of primers contrary to cloning of scFv where independent PCR are needed to amplify VH and VL and different sets of primers are required to amplify the different gene VH and VL families (Muyldermans, 2001). The method of cloning VHH is slightly complicated by the requirement to avoid the cloning of camelid VH originating from conventional antibodies. Elimination of the VH genes from the VHH gene pool can be accomplished by either using PCR primers that anneal selectively on the hinge of the HCAb (van der Linden et al., 2000) or by first employing

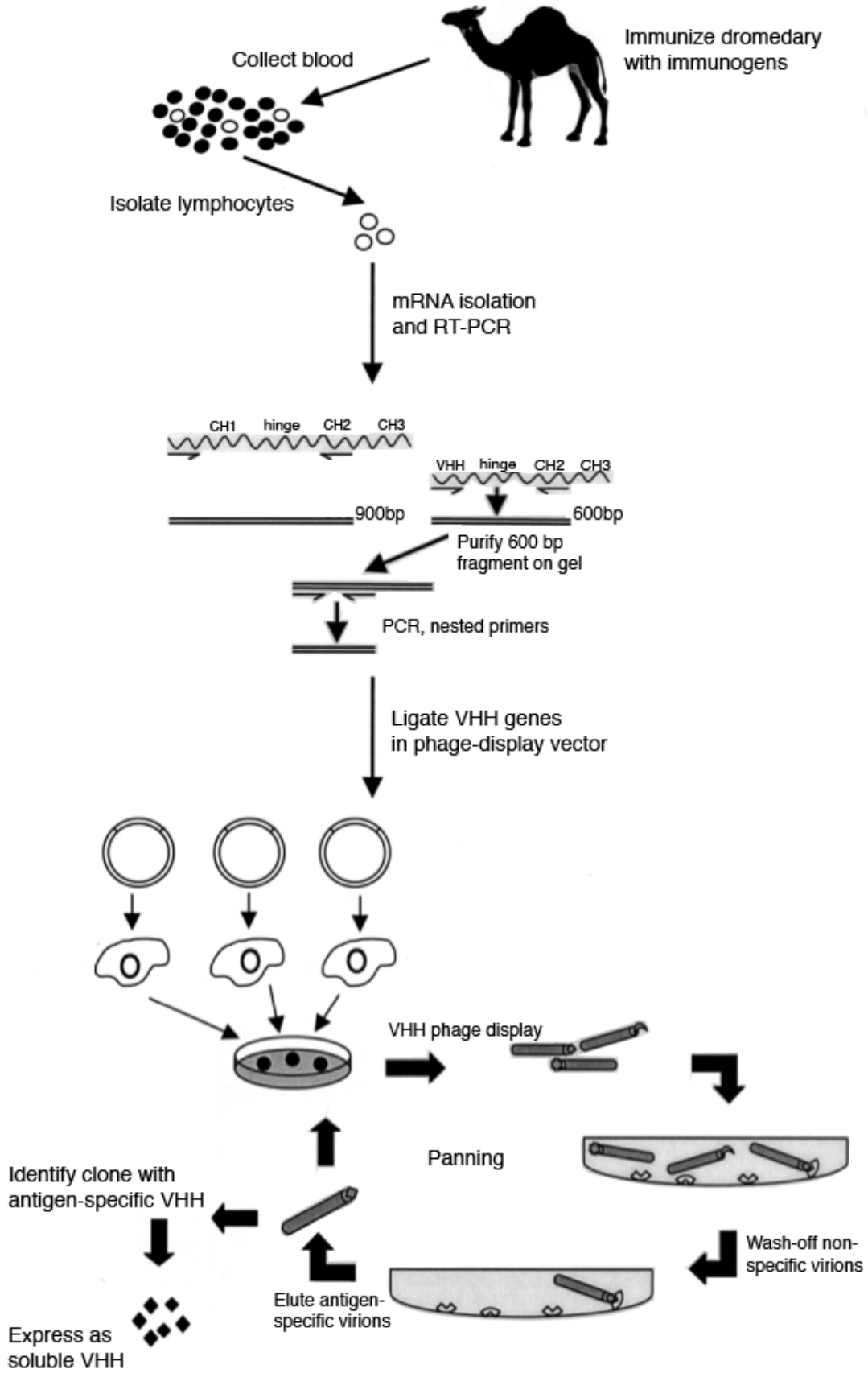

Figure 2.5: Schematic overview of cloning and selection of antigen-specific VHHs from an immunized dromedary. See text for explanation (Adapted from: Muyldermans, 2001).

a universal primer that amplifies all VHH and VH segments, the former lacking and the latter containing the CH1 exon. These PCR fragments are then separated on agarose gel and a second PCR with nested primers is employed to amplify the VHH and introduce restriction enzyme sites. The amplified VHH genes are then cloned in the appropriate expression vector, e.g. the pHEN4 phagemid vector (Figure 2.6), and a VHH library containing the repertoire of the intact in vivo matured antigen binding sites is obtained.

2.3.4.2 Selection of antigen-specific VHH

The VHH libraries can be screened for presence of antigen-specific binders by a combination of phage display and biopanning. Display systems in general provide a physical linkage between genotype (the VHH gene) and phenotype (antigen binding) to allow simultaneous selection of the genes that encode a protein with the desired binding function. In phage display (McCafferty et al., 1990) the VHH is displayed on the surface of philamentous phage as a fusion with the minor coat protein 3 of the virus, and the VHH gene is housed within the virion (Figure 2.7). Co-infection with helper phages, that contain the wild type genome, is necessary to produce complete phage particles displaying the VHH at their tip. Using a process of biopanning, one can rescue phages that display VHH that specifically binds to a target of interest (Hoogenboom and Chames, 2000). Briefly, a simple method for biopanning involves incubating the phage particles on immobilized antigen to allow phages displaying an antigen-specific VHH to bind. Non-binding phage particles are washed away and those that are bound, are eluted by pH-shock. Infection of bacteria with the binding phages results in phage amplification. Successive rounds of biopanning enrich the pool of phages, with clones that specifically bind the target antigen.

Next, individual clones are screened e.g. in ELISA for antigen recognition. Soluble VHH can be produced after either subcloning into a soluble expression vector (e.g. pHEN6, Figure 2.6) or through the use of bacterial non-suppressor strains and appropriate stop codons between the VHH fusion and the phage pIII gene (Muyldermans, 2001). Production in *E. coli* can be done by secretion in the periplasmic space and release of the soluble VHH by simple osmotic shock (Skerra and Plückthun, 1988).

2.3.5 Applications

The reduced size, improved solubility and higher stability of the camelid Heavy-chain antibody fragments are of special interest for biotechnological and medical applications. Several examples are given in the following paragraphs.

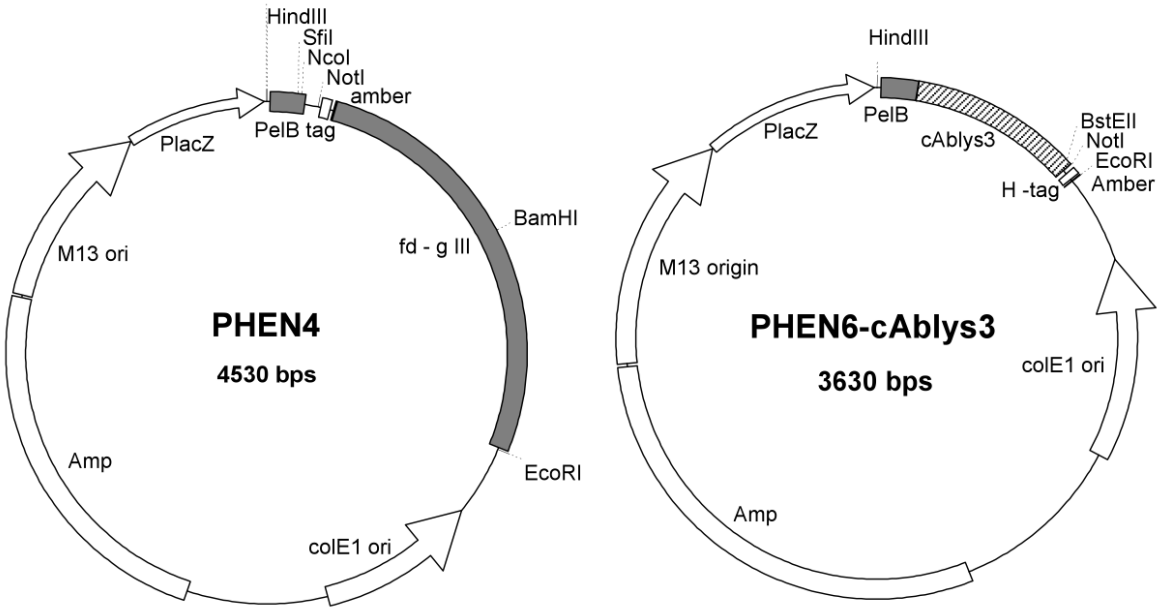

Figure 2.6: Schematic representation of the phagemid vector pHEN4 and expression vector pHEN6, the latter containing the Nanobody cAblys3 gene.

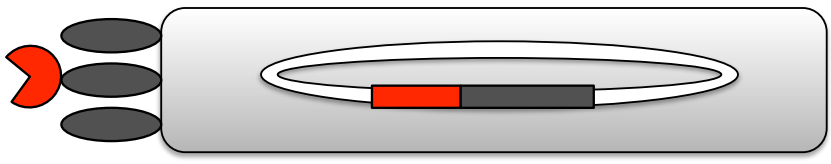

Figure 2.7: Schematic representation of M13 philamentous phage with a VHH (in red) fused to its minor coat protein 3. The VHH gene is included in the phagemid genome.

2.3.5.1 Biotechnological applications

Nanobodies as in vivo imaging agents The superior tissue penetration potential of Nbs due to their small size, combined with high affinity target binding and rapid clearance from the blood circulation favors their use as imaging agents. Cortez-Retamozo et al. (2002) indeed demonstrated that Nbs efficiently target tumors and metastatic lesions and that the excess of antibody is rapidly cleared from the blood circulation. Later on, Nbs were successfully employed for in vivo radioimmunodetection of tumors (Gainkam et al., 2008; Huang et al., 2008).

Detection probes in biosensors and usage in immuno-adsorbents The robust property of Nbs against various denaturing conditions makes them extremely powerful as immuno-adsorbents or as detection probes in biosensors. Probe regeneration sometimes requires harsh regeneration conditions. The efficient refolding of Nbs from strongly denaturing conditions further supports their use in biosensor applications involving multiple detection cycles (Saerens et al., 2008c).

Huang et al. (2005) developed a human prostate-specific antigen (PSA) biosensor based on Nbs that could measure clinically relevant concentration of PSA. In other research, Nbs were employed in competition ELISA assays to detect chloramphenicol and to detect caffeine in beverages (Ladenson et al., 2006; Wesongah et al., 2007). Sherwood et al. (2007) developed an antigen-capture assay for Marburg virus and demonstrated that Nbs could be employed for effective and fast diagnostic development.

Modular building blocks in manifold constructs Bivalent (or multivalent) and bispecific antibodies have many practical applications in immuno-diagnosis and therapy (see 2.3.5.2 for examples). Due to the single domain nature of Nbs, they can be easily tailored in different constructs (Figure 2.3). Bivalency or multivalency can allow antibodies to bind to antigens with great avidity. Zhang et al. (2004a) constructed so-called "pentabodies" by linking Nbs to the B-subunit of *E. coli* verotoxin, which self-assembles to form a homopentamer. This pentamerization resulted in introduction of avidity and dramatic increase of the functional affinity.

Bispecificity permits the cross-linking of two antigens. A bispecific construct can be produced by tethering two antibody fragments by the structural upper hinge of a natural antibody. The individual moieties fully retained their binding capacity and binding characteristics (Conrath et al., 2001a).

An extension of the above approach consists of replacing one of the antibodies by a toxin or enzyme resulting in functional immunofusions, where the antibody is used as delivery vehicle for the toxin or enzyme (Muyldermans, 2001).

Intrabodies The intracellular expression of antibodies ("intrabodies") is a powerful strategy to inhibit in vivo function of selected molecules. Rothbauer et al. (2006) fused Nbs to fluorescent proteins ("chromobodies") and expressed them in living cells. These chromobodies could recognize and trace antigens in different subcellular compartments. Gueorguieva et al. (2006) constructed intrabodies directed against Bax, a proapoptotic protein implicated in cell death involved in several neurodegenerative diseases. These intrabodies were stably expressed in mammalian cells, were nontoxic to their host cells and rendered them highly resistant to oxidative-stress-induced-apoptosis.

2.3.5.2 Therapeutic applications

Several examples of therapeutic Nbs are presented in Table 2.3. The Nbs exert their therapeutic capacity through different mechanisms: either by simply neutralizing the exogenous pathogen or toxin (Harmsen et al., 2005; Hmila et al., 2008), or through receptor or enzyme inhibition (Roovers et al., 2007). Nanobodies were also isolated that prevent protein aggregation (and sometimes even reduce already existing aggregates) which is characteristic of certain protein aggregation disorders (Dumoulin et al., 2003; Verheesen et al., 2006).

Another type of antibody-based therapy where Nbs can be a valuable tool is antibody-dependent enzyme prodrug therapy (ADEPT) for the treatment of cancer (Cortez-Retamozo et al., 2004). In ADEPT, an antibody specific to a tumor marker protein delivers a drug-activating enzyme to the cancer. Subsequent intravenous administration of an inactive prodrug results in drug activation and cytotoxicity only within the locale of the tumor. There are more examples of Nbs directing drug activity to a specific site. For instance, Nbs were isolated that target conserved, cryptic epitopes of the variant surface glycoprotein (VSG) of trypanosomes. The Nbs were conjugated to a trypanolytic factor to generate an immunotoxin with potential for trypanosomiasis therapy (Baral et al., 2006). For their use in targeting drugs across the blood-brain barrier (BBB), Nbs were selected that transmigrate the human BBB in an in vitro model and accumulate in the brain after intravenous injection in mice (Muruganandam et al., 2002). These could be used for treatment of neurological disorders.

Although the short serum half-life of Nbs favors their use as imaging agents, this limits their efficacy in many parenteral applications. Therefore, Nbs have been targeted to serum proteins (e.g. albumin or immunoglobulin) using bispecific Nbs recognizing these serum proteins in addition to the therapeutic target to prolonge serum half-life (Coppieters et al., 2006; Roovers et al., 2007; Harmsen et al., 2008). Another approach to increase serum half-life is ligation of the antibody to polyethylene glycol (PEG) (Harmsen et al., 2007).

Table 2.3: Examples of therapeutic applications of Nanobodies (Nb).

Disease and/or pathogen	Target	Fusion partner	Reference
Cancer	carcinoembryonic antigen	β-lactamase	Cortez-Retamozo et al., 2004
Cancer	epidermal growth factor receptor	anti-albumin Nb	Roovers et al., 2007
Human African trypanosomiasis	*Trypanosoma* spp.	truncated apo L-I	Baral et al., 2006
Foot and mouth disease	FMD virus	PEG	Harmsen et al., 2007
Foot and mouth disease	FMD virus	anti-porcine Ig Nb	Harmsen et al., 2008
Rotavirus diarrhea	rotavirus	none	van der Vaart et al., 2006
Rotavirus diarrhea	rotavirus	*Lactobacillus* cell-surface anchor	Pant et al., 2006
E. coli diarrhea	F4 fimbriae	none	Harmsen et al., 2005
S. mutans caries	I/II adhesin	none	Kruger et al., 2006
Amyloid diseases	lysozyme	none	Dumoulin et al., 2003
Oculopharyngeal muscular dystrophy	nuclear poly(A)-binding protein 1	none	Verheesen et al., 2006
Neurodegenerative diseases	Bax	none	Gueorguieva et al., 2006
Rheumatoid arthritis	TNFα	anti-albumin Nb	Coppieters et al., 2006
Scorpion toxin	Aahl' toxin	none	Hmila et al., 2008
N. meningitidis sepsis	lipopolysaccharide	none	El Khattabi et al., 2006
Brain diseases	cerebromicrovascular endothelial cells	none	Muruganandam et al., 2002

2.3.6 Conclusion

Since the discovery of Heavy-chain antibodies in 1993, the field of single-domain antibody fragments has been rapidly growing. Nanobodies have many advantages for biotechnological applications. They can be economically produced in micro-organisms and have a high stability. Furthermore, they are highly suited for expression as multivalent, including bispecific, constructs. Besides their therapeutic applications, they offer a valid alternative to monoclonal antibodies for use in diagnostic assays. As such, Nbs could also be used to develop a specific diagnostic test for cysticercosis.

Objectives

The **main objective** of the thesis is to study serological responses in pigs and to identify and develop tools for improved serodiagnosis of porcine cysticercosis. Indeed, there is a need for diagnostic tests that can *(1)* quantify the infection level, *(2)* differentiate between active and inactive cysticercosis and *(3)* specifically detect living *T. solium* cysts in pigs.

Therefore, the **specific sub-objectives** are:

1. To link serological data (antibody and antigen responses) to parasitological findings in experimentally infected pigs in order to study the host-parasite relationship
2. To study the relationship between number of cysts and titer of circulating antigen in order to develop a quantitative diagnostic test
3. To identify novel biomarkers in serum of infected pigs that can distinguish between active cysticercosis and inactive cysticercosis
4. To produce Nanobodies specific for *T. solium* that can be used for specific diagnosis of porcine cysticercosis

Chapter 3 (*"Serological responses in porcine cysticercosis: a link with the parasitological outcome of infection"*) deals with the first two sub-objectives. Chapter 4 (*"Identification of biomarkers for different stages of porcine cysticercosis using SELDI-TOF MS"*) addresses the third sub-objective. The fourth sub-objective is addressed in Chapter 5 (*"Nanobodies, a promising tool for species-specific diagnosis of* Taenia solium *cysticercosis"*).

3

Serological responses in porcine cysticercosis: a link with the parasitological outcome of infection

Adapted from:

N. Deckers, K. Kanobana, M. Silva, A.E. Gonzalez, H.H. Garcia, R.H. Gilman, P. Dorny (2008) Serological responses in porcine cysticercosis: a link with the parasitological outcome of infection. Int. J. Parasitol. 38, 1191–1198.

3.1 Introduction

Immunodiagnostic assays can provide a useful tool for surveillance of cysticercosis infection during and after control programs. Transmission of the disease can be interrupted by increased hygienic measures which are, however, difficult to implement in developing countries. Therefore, alternative measures for reduction of transmission are being developed, such as health education, mass chemotherapy in humans and pigs, and vaccination of pigs (Sarti et al., 1997; Garcia et al., 2007). Infected pigs treated with a single dose of 30 mg/kg of oxfendazole, became refractory to re-infection 3 months later (Gonzalez et al., 1996, 1997, 1998). Another approach initiated by different research groups is vaccination of pigs aiming at interrupting the parasite's life cycle by preventing pigs from acquiring the larval stage (Nascimento et al., 1995; Sciutto et al., 1995; Flisser and Lightowlers, 2001; Flisser et al., 2004; Gonzalez et al., 2005). A synthetic peptide-based vaccine has also proved to have therapeutic activity (de Aluja et al., 2005).

Interventions based on immunization of pigs are not yet commonly applied. This is partly due to a lack of understanding of the host-parasite interactions and the immune mechanisms involved in resistance to the disease. Prerequisites to evaluate results of immunization are *(1)* to understand whether all pigs equally acquire the disease upon infection and *(2)* to determine how long metacestodes remain viable without intervention.

Thus, reproducible and standardized experimental infections with *T. solium* in pigs are needed, both for vaccination studies and to learn more about the course and progress of infection. Recently, the Cysticercosis Working Group in Peru succeeded in developing a reproducible experimental model in pigs in which pigs are orally infected with a whole proglottid embedded in a banana ball. The advantage of oral infection is that it mimics the natural mode of infection. The use of a complete proglottid rather than eggs in solution has the disadvantage that the number of eggs ingested by the animals might differ. However, the infectivity of the eggs will also likely be more comparable with what occurs under natural conditions.

In this chapter we report on the serological response of experimentally infected pigs and an attempt was made to link the serological and the parasitological responses to infection. In a second part of the study, we extended this link by investigating whether a quantitative relationship could be established between the titer of circulating antigen in the serum of animals and the number of viable cysts at necropsy.

3.2 Materials and methods

3.2.1 Animals and experimental design 1

Eighteen piglets were purchased from a commercial cysticercosis-free farm in Lima, Peru and maintained at specific pathogen free facilities of the School of Veterinary Medicine of the San Marcos University (UNMSM) in Lima. Animals were housed individually and had free access to water and feed. The cysticercosis-free status of the animals was confirmed by analysis of pre-infection serum with the EITB (Tsang et al., 1991). Six piglets were infected at the age of 1 month, six piglets at the age of 3 months, and six piglets at the age of 5 months.

Animals received a single infective dose of *T. solium* eggs. Briefly, following a 1-week period of becoming accustomed to eating banana and honey balls, all animals were infected with one gravid proglottid embedded in a banana and honey ball. Following ingestion of the banana ball, animals were observed for 2 h to ensure the infective dose was not regurgitated. Animals were bled weekly during the first month p.i. and every 2 weeks thereafter, until the end of the experiment. Animals were euthanized approximately 3 months p.i. A systematic dissection of the full carcass was then performed. The number of cysts was enumerated and viable and degenerated cysts were differentiated. Viable cysticerci had well-limited thin-walled cystic structures containing clear vesicular fluid and a visible whitish protoscolex. Degenerated cysticerci were non-cystic but rather had semi-solid caseous masses with no discernible parasite structures (Phiri et al., 2006). This experiment was reviewed and approved by the Committee on Animal Research of the School of Veterinary Medicine, UNMSM.

3.2.2 Animals and experimental design 2

In a second part of the study, we aimed to investigate whether a quantitative relationship could be established between the total number of viable cysts found at necropsy and the level of circulating antigen in serum. For this purpose, serum samples from different experiments were used. All experiments were part of the research performed by the Department of Preventive Medicine of the School of Veterinary Medicine, UNMSM.

The criteria for inclusion of the serum samples in the study were *(1)* the availability of sufficient material and *(2)* the precise determination of the parasite burden (as well as differentiation between degenerated and viable cysts) by detailed carcass dissection following slaughter. This study included 98 porcine serum samples originating from 98 experimentally infected animals. The age of the animals varied between 3–12 months at slaughter.

3.2.3 Detection of circulating antigen

For experimental design 1, the B158/B60 Ag-ELISA (Brandt et al., 1992; Dorny et al., 2000) was performed twice. First, the standard protocol was used as described previously (Dorny et al., 2000) with a few modifications: incubation steps were reduced from 1 h to 30 min (coating) or 15 min (other steps); all incubations were done on a shaking plate except for the last step (substrate); streptavidin-horseradish peroxidase (HRP) (Jackson) diluted 1/10,000 was used as the conjugate.

In a second step, serum samples from infected animals were not pre-treated with trichloro acetic acid (TCA) but used at dilutions of 1/10 and 1/100 in PBS supplemented with 0.5% Tween 20 (Merck) (PBS-T) to enable a quantitative determination of levels of circulating antigen. Preliminary results (data not shown) demonstrated that omitting the pre-treatment did not affect the test results. The cut-off for the first assay was determined using serum samples of Peruvian pigs known to be negative for porcine cysticercosis. The cut-off value was calculated based on the mean ODs of eight negative reference pig sera (Sokal and Rohlf, 1981). The cysticercosis-negative status of the pigs used for negative control samples was determined by *(1)* detailed carcass dissection and *(2)* no antibodies in the EITB for *T. solium* cysticercosis (Tsang et al., 1991).

In the second assay, a reference standard curve was constructed using serial dilutions of ES products of *T. saginata* (*Tsag*ES) that were initially used for the development of the monoclonal antibodies (Brandt et al., 1992). The dilutions were made in PBS-T. A reference curve was included on each ELISA plate. The concentration of circulating antigen was calculated following the transformation of the OD value using the equation of the reference curve (quantitative ELISA for circulating antigen). Results from the different groups were compared.

For experimental design 2, the quantitative ELISA for circulating antigen was used with serum samples diluted 1/200.

3.2.4 Total IgG detection by ELISA

The ELISA for detection of serum antibodies was performed using crude metacestode antigen of *T. crassiceps*. *Taenia crassiceps* cysticerci (Toi strain, kindly provided by Chernin, (1975), and maintained in laboratory mice by two to four passages per year) were harvested from the peritoneal cavity of mice (Swiss A strain) approximately 90 days after experimental infection. The antigen was prepared according to Nunes et al. (2000). This test is based on the cross-reactivity of antigens between *Taenia* species and also yields a positive reaction in pigs infected with *T. hydatigena*. However, this ELISA was only performed on serum samples from the animals from experimental design 1 that were

known to be free of *T. hydatigena* infection. The ELISA was performed according to Nunes et al. (2000) with some minor modifications: all steps except the incubation of the substrate were done at 37°C on a shaking plate, 30 min incubation for the coating of the antigen, 15 min for the other incubation steps; blocking was done using PBS, pH 7.2 containing 0.05% Tween 20 and 2% newborn calf serum; test serum was diluted 1/200 in blocking fluid; anti-pig IgG (whole molecule) peroxidase conjugate (Sigma A-9417) was used at a dilution of $1/20,000$ in blocking fluid; OD was read at 492/655 nm. All samples were run 2-fold. On each plate two cysticercosis positive pig serum samples and eight negative pig serum samples were run. The cut-off was determined as the mean OD of the eight negative controls plus $3SD$.

3.2.5 Data analysis

The calculation of the standard curve and the statistical analyses were performed using the STATA statistical software, release 7.0 (Stata Corporation 2001, College Station, TX). Correlations between parameters were measured with the Pearson's correlation coefficient for linear correlation on normally distributed data. The confidence level was set at $P < 0.05$.

3.3 Results

3.3.1 Infection

The total number of cysts as well as the number of viable and calcified cysts found at necropsy are shown in Table 3.1. Both within and between age groups, a large variation in the number of cysts was found in each animal. The total numbers of cysts (number of viable cysts) ranged between 0–6246 (0–6210), 0–1364 (0–556) and 136–2913 (0–105) in the animals infected at 1, 3 and 5 months of age, respectively. At least one cyst was found in four of the six pigs infected at 1 month, in five out of the six pigs infected at 3 months and in all pigs infected at 5 months of age. From the pigs that had at least one parasite, these included viable cysts in only one of the pigs infected at 5 months of age, compared with eight of the 10 pigs infected at earlier age levels ($P < 0.05$). From the total cysts recovered at necropsy, 98% were degenerated in the animals infected at 5 months of age, compared with 55% in the animals infected at 3 months of age and only 2.7% in the animals infected at 1 month of age.

Table 3.1: Number and classification of cysts following necropsy of piglets infected with *Taenia solium* at the age of 1, 3 or 5 months.

Age[a]	Animal no.	Total no. of cysts	No. of viable cysts	No. of degenerated cysts
1 month	8115	6,246	6,210	36
	8116	3,199	3,195	4
	8113	2,038	1,946	92
	8114	302	112	190
	8117	0	0	0
	8118	0	0	0
	average	1,964.2	1,910.5	53.7
	% of total no.		97.3%	2.7%
3 months	8112	1,364	167	1,197
	8108	581	556	25
	8110	367	228	139
	8109	314	231	83
	8111	7	0	7
	8107	0	0	0
	average	438.8	197.0	241.8
	% of total no.		44.9%	55.1%
5 months	8101	2,913	105	2,808
	8105	1,724	0	1,724
	8102	831	0	831
	8103	597	0	597
	8106	582	0	582
	8104	136	0	136
	average	1,130.5	17.5	1,113.0
	% of total no.		1.5%	98.5%

[a] Age of animal when infected with *T. solium*. All animals were euthanized 3 months p.i.

3.3.2 Levels of circulating antigen

The kinetics of the serum antigen levels measured by the standard assay revealed a steep increase of circulating antigen levels in four of the six animals infected at 1 month of age, resulting in a plateau level in the antigen ratio between 1 and 2 weeks p.i., which was maintained until necropsy of the animals (Figure 3.1). No circulating antigen was detected in the serum of pigs numbers 8117 and 8118, infected at 1 month of age.

Four of the six animals infected at 3 months of age had high levels of circulating antigen. The increase in their circulating antigen levels was slower compared with the animals infected at 1 month of age, with a plateau level in the antigen ratio reached 3 weeks p.i.

Antigen levels in animals infected at 5 months of age remained low except in two animals (pigs numbers 8106 and 8101) in which they started to increase 3 weeks p.i. In only one of these two animals was a high antigen ratio measured up to necropsy.

To enable the measurement of a quantitative difference in antigen levels between the different age groups, serum samples obtained at 3, 6 and 10 weeks p.i. were diluted 1/10 and 1/100 and used in the quantitative ELISA for circulating antigen. The lowest concentration of detectable antigen was 3.9 ng/ml. The individual calculated values are depicted in Table 3.2 showing that levels reached were markedly higher in the animals infected at 1 month of age compared with animals infected at 3 and 5 months of age.

3.3.3 Total IgG ELISA with *T. crassiceps* antigen

The kinetics of the total Ig levels is shown in Figure 3.1. Serum antibody levels appeared to follow a reverse kinetics compared with circulating antigen. Antibody titers were highest in animals infected at 5 months of age, starting to increase as early as 3 weeks p.i. Following a steep increase, the antibody response in these animals declined from 8 weeks p.i. onwards (Figure 3.1C). In the animals infected at 1 and 3 months of age (Figure 3.1A and B, respectively) antibody levels increased slowly and remained substantially lower compared with the older age group. In addition, a decline in antibody titers at the time of slaughter was only observed in pigs numbers 8110 and 8112 (infected at 3 months of age).

3.3.4 Relationship between antigen titers and number of cysts

A total of 98 serum samples obtained from pigs with variable numbers of viable cysts (= 45), degenerated cysts only (= 14) and no cysts (= 39) were tested in duplicate. A serial dilution of *Tsag*ES yielded a standard curve that was subsequently used to calculate the concentration of antigen in the serum samples. The relationship between the number

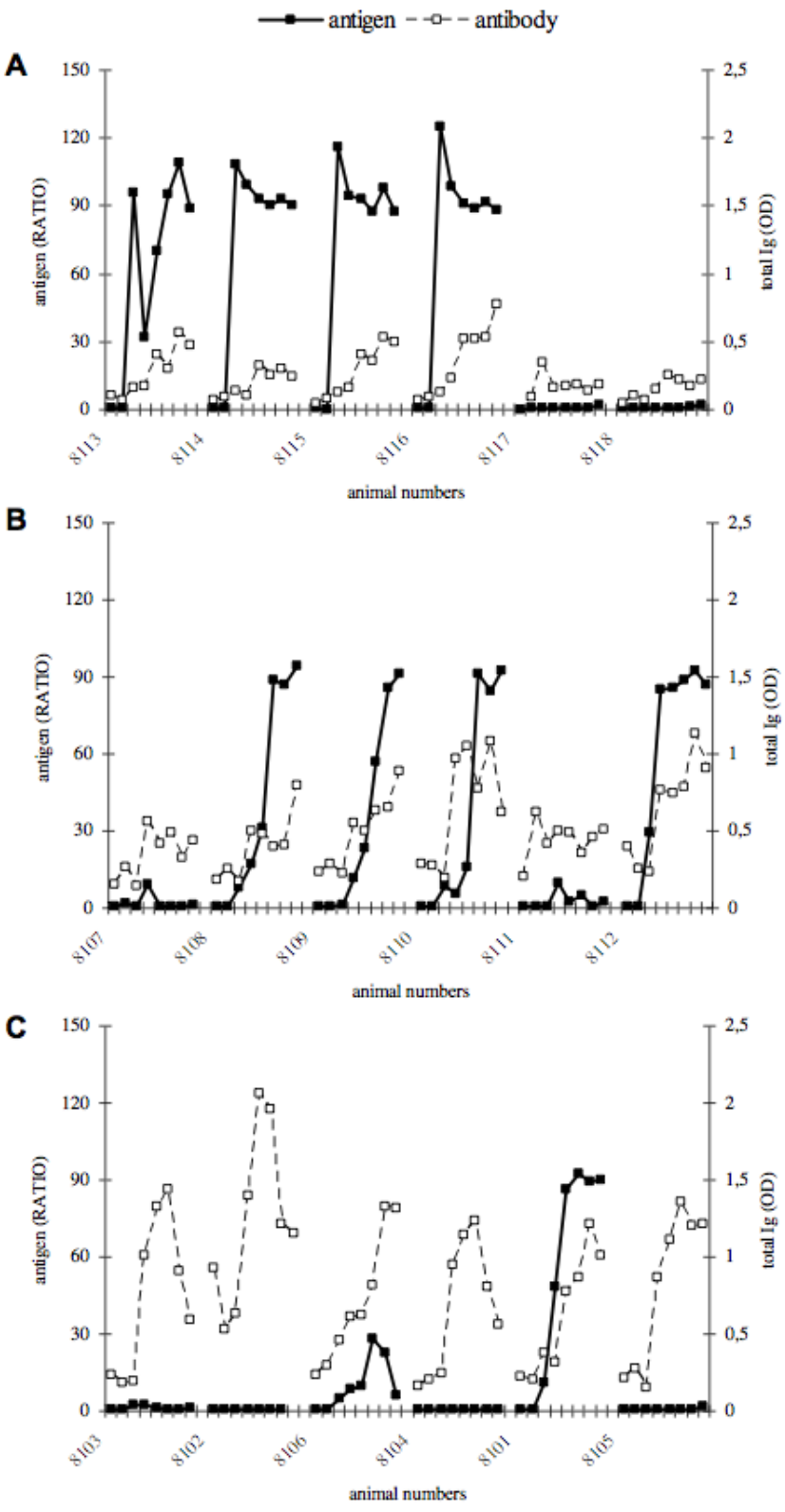

Figure 3.1: The serological antigen (■) and antibody (□) response of 1-month-old *(A)*, 3-months-old *(B)* and 5-months-old *(C)* piglets after oral infection with one *Taenia solium* proglottid. Sera were analyzed weekly during the first month p.i., and every 2 weeks thereafter: time points depicted are 0, 1, 2, 3, 4, 6, 8, 10 and 12 weeks p.i. Antigen levels are expressed as ratio = OD value of sample/cut-off value (calculated based on the mean OD value (492 nm) of eight non-infected control samples). Antibody values are expressed as OD values as measured with the *Taenia crassiceps* antibody ELISA.

Table 3.2: Concentration of circulating antigen (ng/mL) at 3, 6 and 10 weeks p.i. (WPI) in serum of piglets infected with *Taenia solium* at the age of 1, 3 or 5 months.

Age[a]	Animal no.	3 WPI	6 WPI	10 WPI
1 month	8115	119.32	154.39	147.7
	8116	13.49	127.52	132.69
	8113	≤ 3.90	15.28	87.5
	8114	8.71	82.3	142.5
	8117	≤ 3.90	4.1	≤ 3.90
	8118	≤ 3.90	≤ 3.90	≤ 3.90
3 months	8112	≤ 3.90	≤ 3.90	13.1
	8108	≤ 3.90	6.43	40.4
	8110	≤ 3.90	4.87	13.27
	8109	≤ 3.90	≤ 3.90	13.67
	8111	≤ 3.90	≤ 3.90	4.12
	8107	≤ 3.90	≤ 3.90	4.1
5 months	8101	≤ 3.90	21.37	79.73
	8105	≤ 3.90	≤ 3.90	4.1
	8102	≤ 3.90	≤ 3.90	≤ 3.90
	8103	≤ 3.90	≤ 3.90	4.1
	8106	≤ 3.90	≤ 3.90	≤ 3.90
	8104	≤ 3.90	≤ 3.90	≤ 3.90

[a] Age of animal when infected with *T. solium*. All animals were euthanized 3 months p.i.

of viable cysts and the concentration of circulating antigen is shown in Figure 3.2, resulting in a regression line with a Pearson's correlation coefficient of 0.89 ($R^2 = 0.81, n = 98, P < 0.001$).

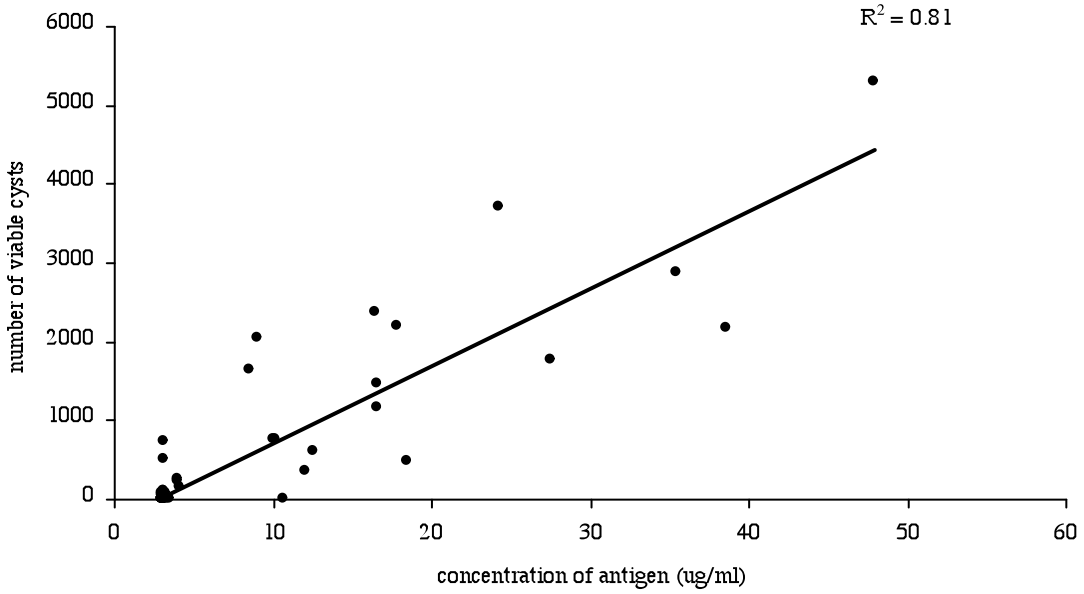

Figure 3.2: The correlation between the concentration of circulating antigen and the number of viable cysts in *Taenia solium* infected animals and non-infected control animals ($n = 98$). Antigen concentrations were calculated following transformation of the OD value using the equation of a reference curve obtained after serial dilution of *Taenia saginata* excretion-secretion products.

3.4 Discussion

Experimental infections with *T. solium* in pigs are a prerequisite to improve current understanding of the pathogenesis of the disease (Flisser et al., 2002). Recently, an oral infection model was developed in which piglets of defined age levels (1-, 3- and 5-months-old) are infected with a whole proglottid embedded in a banana ball. Although it does not allow determination of the exact level of infection, the use of a whole proglottid has the advantage that it mimics the natural mode of infection. Animals infected at 1 month of age harbored mainly viable cysts upon oral infection with one proglottid, whereas in piglets infected at 5 months of age most cysts were degenerated. The outcome of the infection in animals infected at 3 months of age was intermediate, with a more equili-

brated distribution of viable and degenerated cysts. The current study was carried out to investigate whether this model could also be used to study host-parasite relationships and the mechanisms underlying resistance to infection in more detail. An attempt was made to link the parasitological findings to serological data. In a second part of the study, the preliminary observation that levels of circulating antigen seemed to be related to the number of viable cysts was tested more extensively using more animals.

Although detailed carcass dissections at necropsy revealed a high variation in numbers of cysts, the trend was that the number of viable cysts decreased with the age at which the animals were infected. The presence of viable cysts in tissues of infected pigs was monitored by means of an ELISA for the detection of circulating antigen (Brandt et al., 1992). The kinetics of the antigen levels throughout the course of the infection differed markedly between the three age groups of the experimental infection model. In the younger animals a fast increase (starting 1 week p.i.) in titers of circulating antigen was observed in most animals, reaching a plateau as early as 2 weeks p.i. This demonstrates that antigen can be produced well before viable cysts are fully developed, which takes approximately 3 months (Pawlowski, 2002). An interesting observation was that animals with viable cysts at necropsy (nine animals) had invariably high antigen levels, whereas animals with no cysts (three animals) or only degenerating cysts (six animals) had low or nil antigen levels. The transient rise of circulating antigen in pig number 8107, which was negative at necropsy, probably suggests that the infection was aborted early in its course. The lack of circulating antigen in the other necropsy-negative animals indicates that cysts never established in these animals.

Antigen levels in pigs infected at younger ages not only increased faster but also reached higher levels than in older animals, and were associated with weaker antibody responses. Based on the presence of viable cysts and the higher antigen levels in the younger animals one could hypothesize that cysts circumvent a host immune response in the younger animals, develop faster and remain viable for a longer period of time. In addition, the rapid increase in antibodies in the older animals may be responsible for the abrogation of the development of cysts, which then immediately evolve to a caseous and degenerated status. The classical view of the evolution of cysticercosis lesions assumes that they all pass through the established viable stage, but recently the hypothesis that a substantial proportion of parasites are destroyed in early phases was also put forward to explain the distinct outcome of infection of human NCC (Garcia et al., 2003b).

Overall, our observation that higher total Ig titers are present in animals with a higher proportion of degenerated cysts contrasts with other observations showing that antibodies were mostly found in cases with live and involuting parasites, but only very few antibodies were detected in conjunction with degenerated cysts (de Aluja et al., 1996;

Flisser et al., 2002). Another striking difference in the antibody response was that in all animals infected at 5 months of age, and in two out of the six animals infected at 3 months of age, antibody levels were already decreasing at the time of necropsy (84 days p.i.). Upon experimental infection of pigs with 100,000 *T. solium* eggs de Aluja et al. (1996) also observed a decrease in antibody titers starting from 92 days p.i. However, even at a lower level, antibody titers remained positive up to 280 days p.i. A full comparison between experiments is not feasible as differences may have occurred at the level of the animals (breed), the parasites (difference in infectivity) and infection mode (100,000 eggs versus a full proglottid).

The conclusion that the presence of antibodies is influenced by the stage of the parasite is also true for our study, but we would associate higher antibody responses with cyst degeneration. In addition, a role for antibodies is suggested by the rapid increase to high antibody titers associated with an abrogated development of cysts in the older animals. Although evidence for a role of antibodies in inhibition of the development of *T. solium* parasite stages has not yet been reported for porcine cysticercosis, in mice infected with *T. crassiceps*, antibodies were shown to have a crippling effect (Garcia et al., 2001a). In *T. saginata* infection in cattle, the evidence for age resistance has been conflicting (reviewed by Rickard and Williams, 1982). Early reports, using animals that may have been free of prior exposure, demonstrated that although resistance (as related to the ability to become infected and the number of developing cysts) did not develop with age, older animals appeared to have an increased proportion of degenerated cysts (Penfold, 1937; Urquhart, 1961 cited by Rickard and Williams, 1982). This coincides with our findings. Whether the lower numbers of cysts in the older animals are artefacts or reflect the true situation is difficult to interpret as experiments with out-bred animals are inherently exposed to a high amount of variability (Hein and Griebel, 2003) and only a small number of animals were included in this study. Alternatively, the reduction in numbers of cysts in the older animals may have resulted from more effective innate immune responses in these animals. All animals were naive prior to infection, originating from a cysticercosis-free area, excluding exposure to *T. solium*. This implies an important role for the innate immune response in the initial struggle between the host and the parasite. This is further supported by the abrogation of the viability of cysts at a very early stage of infection, too early to be the result of acquired immunity.

In a second part of the study, we investigated whether the suggested relationship between levels of circulating antigen and numbers of viable cysts, as was observed in the experimental infection model, could be strengthened with the analysis of a higher number of serum samples obtained from a more heterogeneous set of pigs infected with *T. solium*. Despite the variation in ages of animals, in experimental versus natural infections a signif-

icant correlation between the number of viable cysts and the concentration of circulating antigen was found. A significant correlation does not necessarily implicate a causal relationship but we may conclude that the variation in antigen concentration in the serum of animals can be partly explained by the number of viable cysts recovered at necropsy. A more detailed analysis revealed that a threshold in number of cysts is required for finding this relationship (data not shown). In animals with degenerated cysts only, or less than a total number of 100 cysts, the relationship was less consistent. A threshold of 100 cysts was arbitrarily chosen and might need to be refined. The necessity of a threshold can easily be explained by the sensitivity of our assay. Although it proved able to detect one viable cyst (Dorny et al., 2004b), up to now, we do not know whether this is entirely reproducible. Maybe the age and development stage of the cysts also play a role in this regard. In addition to the number of viable cysts, the concentration of circulating antigen could be influenced by both host-derived and parasite factors such as the immune status of the host, the location and size of the cysts or genetic differences.

4

Identification of biomarkers for different stages of porcine cysticercosis using SELDI-TOF MS

Adapted from:

N. Deckers, P. Dorny, K. Kanobana, J. Vercruysse, A.E. Gonzalez, B. Ward, M. Ndao (2008) Use of ProteinChip technology for identifying biomarkers of parasitic diseases: the example of porcine cysticercosis (*Taenia solium*). Exp. Parasitol. 120, 320–329.

4.1 Introduction

The advent of proteomics has brought with it new technologies enabling the discovery of biomarkers that can be used to diagnose diseases. A recent development in this field is SELDI-TOF MS, a technology that enables the generation of protein profiles from complex biological samples such as serum (Merchant and Weinberger, 2000). The analytical platform used is the ProteinChip Biomarker System-II (BioRad). In this technique, proteins are bound to the chromatographic surface of the chip array through different physicochemical interactions. A nitrogen laser desorbs the protein/energy-absorbing molecule mixture from the array surface, enabling the detection of the proteins captured by the array. By comparing the protein profiles in samples under different biological conditions (e.g. healthy versus infected) specific biomarkers can be identified (Xiao et al., 2005).

Here, the ProteinChip platform was used to discover biomarkers for different stages of *T. solium* cysticercosis in pigs. Up to now, there are no diagnostic tests available that can differentiate between infections with viable cysts (active cysticercosis) and degenerated cysts (inactive cysticercosis). A diagnostic test differentiating between active and inactive cysticercosis in pigs would be an important tool to study transmission dynamics and immunity in epidemiological studies and to monitor the effect of anthelmintic treatment or vaccination in control programs. In humans, this differentiation would be a practical aid for clinical diagnosis and case management.

In the previous chapter, we reported different antibody and antigen kinetics in experimentally infected piglets linked to the age of infection and the parasitological findings. Consequently, we hypothesized that there are biomarkers present in the serum specific for these different phenotypes (active and inactive cysticercosis) that could be used as a diagnostic tool. To test this hypothesis, we analyzed serum samples from experimentally infected pigs using the ProteinChip platform to identify novel biomarkers that could contribute to the differentiation between active and inactive cysticercosis.

However, questions have been raised about SELDI-TOF technology for protein profiling, particularly in terms of reproducibility and experimental bias (Sorace and Zhan, 2003; Baggerly et al., 2004b; Diamandis, 2004b). To address these issues, a second sample set comprising 86 serum samples from naturally infected pigs was analyzed to validate the biomarkers discovered in the experimentally infected animals.

4.2 Materials and methods

4.2.1 Serum samples

4.2.1.1 Experimental infections

Serum samples from experimentally infected pigs described in the previous chapter, were also used in this study (Chapter 3, 3.2.1 "Animals and experimental design 1", p.46). Briefly, the samples were collected after experimental infection of three groups of six piglets each of 1, 3 and 5 months old, respectively. The animals were orally infected with a full proglottid of *T. solium*. Serum samples were collected weekly during the first month p.i. and every two weeks thereupon. Twelve weeks p.i. the animals were slaughtered. A total dissection of the carcasses was performed to determine parasite burden and a differentiation was made between viable and degenerated cysticerci. Serum samples collected prior to infection and at week 12 p.i. were used in this study.

4.2.1.2 Field infections

Serum samples from 86 naturally infected pigs were collected in Zambia ($n = 27$) and Peru ($n = 59$). The Zambian pigs had been purchased at the Chibolya slaughter slab in Lusaka and from villages in the Eastern Province. After slaughter, a complete dissection of half the carcasses together with the complete heart, tongue, head and neck muscles, psoas muscles, diaphragm, lungs, kidneys, liver, brains and eyes was performed to count cysticerci. Each slice was less than 0.5 cm thick so that all fully developed cysts would be revealed. For those muscle groups where cysts were only counted in half of the carcasses, the total number of cysts was calculated by multiplying the detected unilateral number by two. Cysts were classified according to their macroscopic appearance as either viable (translucent, fluid-filled with invaginated protoscolices) or degenerated (caseous or calcified). The estimated age of the pigs varied between 6 months and several years; pigs of both sexes were sampled. All the pigs from the Lusaka market were crosses of Large White and Landrace breeds. Pigs from the Eastern province were all of local breed.

The Peruvian serum samples were gathered from the serum bank at the Veterinary Faculty of UNMSM. After slaughter, the full carcasses and internal organs were completely dissected with each slice less than 0.5 cm thick. The total number of cysts was enumerated and their viability was assessed as described above. Information on breed, age and sex of these pigs was not available.

4.2.1.3 Heterologous infections

A total of 25 sera from pigs infected with *T. saginata asiatica* ($n = 6$), *T. hydatigena* ($n = 7$), *Trichinella spiralis* ($n = 5$) and *Trypanosoma congolense* ($n = 7$), collected from the serum bank of the Animal Health Department of the Institute of Tropical Medicine Antwerp (ITMA) were also included in the study. All samples were aliquoted (50 µl) and stored at -80°C prior to this study.

4.2.2 Sample fractionation

All binding and washing steps were performed using a bioprocessor device (Bio-Rad) and the Biomek 2000 workstation (Beckman Coulter, Fullerton, CA). Prior to fractionation, 20 µl of serum samples were mixed with 30 µl of U9 buffer (9 M urea, 2% CHAPS and 50 mM Tris-HCl pH 9) in a 96 well v-bottom plate. The samples were fractionated by pH using the ProteinChip serum fractionation kit (Bio-Rad). The kit consists of a 96-well filtration plate with Q HyperD F anion exchange beads that require rehydration and equilibration before use. The samples were eluted in a stepwise manner, by altering the pH of the wash buffer, until six fractions were collected (flow through + pH 9, pH 7, pH 5, pH 4, pH 3 and organic phase). Each of the six fractions was collected twice and the two collections were pooled. The pooled fractions were divided over 2 separate standard v-bottom 96-well plates, to reduce the number of freeze-thaw cycles. After fractionation, plates were stored at -20°C until use.

4.2.3 ProteinChip preparation

The fractionated serum samples were bound randomly on the chips to avoid introduction of systematic bias. Each chip included a quality control (QC) sample of pooled standard non-infected pig serum (originating from healthy pigs reared on a commercial farm in Belgium) and blank spots were included across the bioprocessor as negative controls.

4.2.3.1 WCX ProteinChip array preparation (CM10 ProteinChip)

Spots were equilibrated two times with 150 µl of CM binding/washing buffer (0.1 M sodium acetate, pH 4). Ten microlitres of fractionated serum sample was incubated in 90 µl of binding buffer (0.1 M sodium acetate) for 30 min on a shaker at room temperature. Afterwards, arrays were washed three times with 150 µl binding buffer and two times with de-ionized water to remove unbound serum proteins. After air-drying, 1 µl of energy absorbing matrix (saturated sinapinic acid (Sigma, St. Louis, MO) in an aqueous solution containing 50% acetonitrile and 0.5% trifluoroacetic acid) was added twice to each spot. The surface was allowed to air-dry between each application.

4.2.3.2 IMAC ProteinChip array preparation (IMAC30 ProteinChip)

Spots were first charged with 50 µl 0.1 M copper sulphate for 5 min followed by a first wash with 200 µl de-ionized water to remove the unbound metal. A second wash was done with 150 µl of neutralisation buffer (0.1 M sodium acetate, pH 4) and a last wash with de-ionized water. Each wash was performed by 5 min incubation at room temperature. Arrays were then incubated 2 times with 150 µl binding buffer (0.1 M sodium phosphate, 0.5 M NaCl pH 7) for 5 min. Ten microlitres of fractionated serum sample was spotted onto arrays with 90 µl of binding buffer for 30 min on a shaker at room temperature. After binding, washing and addition of energy absorbing matrix was done as described above.

4.2.4 Data acquisition and processing

ProteinChip arrays were analyzed in the PBS-IIc ProteinChip Reader (Bio-Rad) equipped with an autoloader to perform mass analysis. The m/z spectra were collected after determining the optimal laser settings for each fraction, distinguishing between low and high MW range. For each fraction 19 different settings were tested on pooled samples (laser intensity varied from 150 to 220 and detector voltage from 2,650 to 2,950). The best setting for each fraction was selected based on the S/N ratio, intensity and resolution of 20 manually selected peaks present in the different spectra.

The data were collected using an automated protocol. Each chip was analyzed in two consecutive runs: the first run for the low mass range, followed by a second run with higher intensity and sensitivity for the high mass range. Each spot was analyzed from position 20 to 80, delta 5, with 5 shots per position preceded with two warming shots (laser intensity 200 for the low mass range and 210 for the high mass range) and focus mass was set at the optimization centre. The optimization range was set at 2,000–20,000 Da for the low mass range and 20,000–50,000 Da for the high mass range.

4.2.5 Data analysis

The serum samples were classified in different groups to find distinguishing biomarkers. The first group consisted of the samples from the experimentally infected pigs prior to infection (control sera, "group C"), a second and third group consisted of the samples from these same pigs that developed viable cysts ("group V") or only degenerated cysts ("group D"), respectively. Serum samples from naturally infected animals with only degenerated cysts were designated as "group Df"; serum samples from animals with viable cysts as "group Vf". The last group consisted of serum samples from pigs with different parasitic infections (heterologous group). For each protein cluster P-values were calculated using

the Mann-Whitney U test for 2 sample groups and the Kruskal-Wallis H test for 3 sample groups.

The spectra were calibrated externally using the ProteinChip protein calibrant kit (Bio-Rad) containing 10 different calibrants with MW ranging from 5,733.6 Da (bovine insulin) to 147,300 Da (bovine IgG). The spectra were normalized to total ion current after baseline subtraction. The average (µ) of the normalization coefficients was calculated and spectra with normalization coefficients $\geq 2\mu$ were excluded from further analysis.

Analyses were performed in 2 steps. First, the Biomarker Wizard feature of the ProteinChip software (v3.2) and ProteinChip Data Manager Software (v2.1) (Bio-Rad) were used to create peak clusters and calculate P values for differences between different groups for each identified peak cluster. A peak cluster was recorded if a given peak was found in $\geq$ 10% of the spectra in one group. Automatic peak detection was performed using cut-off settings of $3 \times S/N$ for the first pass and $2 \times S/N$ for the second pass. The cluster mass window was set at 0.3% of the peak mass for the low mass range (2,500–100,000 Da) and at 2% of the peak mass for the high mass range (10,000–200,000 Da). As a quality control, peak clusters with P values < 0.05 were visually inspected and manually relabelled. After relabeling, the intensity values of the duplicates were averaged and exact P values for differences in average peak intensity between groups were calculated (second pass analysis). Reproducibility for QC samples was evaluated as CV ($CV = \sigma/\mu \times 100\%$ with σ the standard deviation and μ the mean) for m/z and peak intensities in intervals of 2,500–100,000 Da (laser intensity 200) and 10,000–200,000 Da (laser intensity 210) by automatic clustering of m/z values differing by a maximum of 0.3 and 2.0%, respectively, using the Biomarker Wizard software. Noise was calculated within the respective mass region and software settings were $S/N = 5$ and minimum peak threshold = 50%.

4.2.6 Protein purification and identification

Serum was fractionated using the ZOOM ® IEF Fractionator (Invitrogen) according to the manufacturer's instructions. Briefly, serum was first prepared in order to solubilize, denature and reduce serum proteins prior to IEF fractionation. The prepared sample was loaded into the assembled ZOOM ® IEF Fractionator and the protein sample was separated into well-resolved fractions based on the pI of each protein (pH range from 3–10). The fractions were then desalted using the methanol/chloroform precipitation method. The precipitated protein samples were dissolved in SDS sample buffer (containing 2-mercaptoethanol) and run on a 12% Bis-Tris gel according to the method of Laemmli (1970). Gels were fixed with fixer solution for 2 h and afterwards stained overnight with Coomassie to visualize the bands. Destaining with distilled water was performed until the bands were clearly visible. The Coomassie-stained protein bands

were excised from the gel: each band was further cut into cubes (approx. 1 mm) and placed into 1.5 ml eppendorf tubes. Trypsin digestion and liquid chromatography (LC)-MS/MS analysis were performed with an Agilent 1100 liquid chromatograph connected to a QSTAR mass spectrometer (Sciex-Applied Biosystems). Product ion spectra were automatically stored on all multiple-charged peptides recognized by the first quadrupole in the QSTAR. Database searches were performed using MASCOT search engine (`http://www.matrixscience.com`) using carbamidomethyl for Cys modification and oxidation of methionine as the other modification. Public domain databases (NCBI) were scanned for protein identification.

4.3 Results

4.3.1 Infection status of the animals

The infection status of the 18 experimentally infected piglets is summarized in Table 3.1 of the previous chapter (p.49). Examination of the carcasses showed that 6 animals had only degenerated cysts and 9 animals had mixed infections (viable + degenerated cysts). None of the animals had only viable cysts. Parasites did not establish in three animals; therefore only their pre-infection serum samples were used in this study. For two animals the pre-infection sera were not available and for another animal the serum collected at week 12 p.i. was missing. In total, there were 16 pre-infection sera (group C), 5 samples at week 12 p.i. from animals with only degenerated cysts (group D) and 9 samples at week 12 p.i. from animals with at least one viable cyst (group V) (Table 4.1). The animals were classified in the three groups solely based on the necropsy result.

From the 27 samples of the naturally infected Zambian pigs, there were 6 animals with only degenerated cysts, 8 animals with only viable cysts and 13 animals with mixed infections. The cyst burden varied from 1 to 2,932 cysts. From the 59 Peruvian samples, 15 animals harbored only degenerated cysts, 7 animals only viable cysts and 37 animals had mixed infections. The cyst burden varied from 1 to 5,336 cysts. Animals with at least one viable cyst were classified in group Vf (viable phenotype), regardless of the number of degenerated cysts present, since these animals can still actively transmit the disease. In summary, group Df (degenerated phenotype) comprised 21 samples and group Vf (viable phenotype) comprised 65 animals (Table 4.1).

Table 4.1: Summary of serum samples used in the study. No. control = number of samples from pigs prior to experimental infection; No. Degenerated = number of samples from pigs with only degenerated cysts; No. Viable = number of samples from pigs with only viable cysts; No. Mixed = number of samples from pigs with mixed infections (viable and degenerated cysts).

Origin	Total no. samples	No. Control	No. Degenerated	No. Viable	No. Mixed
Experimental infection	30	16	5	0	9
	Group:	C	D	V	
Field samples Zambia	27	–	6	8	13
Field samples Peru	59	–	15	7	37
	Total:		21	65	
	Group:		Df	Vf	

4.3.2 Discovery of Protein Biomarkers in the experimental samples

In a preliminary study (data not shown), a few samples of each group were bound on a CM10 ProteinChip array and all the 6 fractions were analyzed. The fractions yielding the most satisfactory results (i.e. the most differentiating biomarkers) were selected for further analysis: fractions 1 (pH9 and flow through), 4 (pH4) and 6 (organic) for analysis on CM10 ProteinChip arrays. In addition, the organic fraction was also selected for analysis on IMAC30 ProteinChip arrays.

Of the total number of 440 sample spectra collected, the normalization coefficients of 12 spectra did not pass the "twice the average rule" (normalization coefficient $\geq 2\mu$) and were rejected from further analysis.

4.3.2.1 First pass analysis

Viable versus degenerated EDM was done for *(1)* group D versus group C and *(2)* group V versus group C and P-values were calculated for the individual biomarkers. Significant biomarkers ($P < 0.05$) were selected for group D (degenerated phenotype) and group V (viable phenotype), respectively. Thereafter, the three groups (D, V, C) were taken together for EDM and P-value calculation. Biomarkers for degenerated and viable phenotype that remained significant were selected for second pass analysis (4.3.2.2).

Infected versus non-infected By performing EDM and P-value calculation of the three groups simultaneously, biomarkers for infected phenotype could be identified. A biomarker was specific for infected animals if there was a similar expression level in both group D and group V when compared to group C. At this stage, the results were compared to the results of the preliminary study to assess the robustness and reproducibility of the biomarkers: overall, results were comparable.

4.3.2.2 Second pass analysis

After manually relabeling the peaks, statistical analysis was done in the same way as in the first round of EDM: *(1)* group D versus group C, *(2)* group V versus group C, *(3)* three groups simultaneously. This resulted in 30 significant ($P < 0.05$) biomarkers (Table 4.2). Thirteen of these were biomarkers for the viable phenotype, 9 were biomarkers for the degenerated phenotype and 8 were biomarkers for the infected phenotype. Twenty six out of the 30 biomarkers (86.7%) had increased serum expression and 4 out of the 30 biomarkers (13.3%) had decreased serum expression. The biomarkers were checked for their specificity against the heterologous sample group by performing a separate round of EDM (results not shown). This yielded 15 markers potentially specific for cysticercosis: 3.60 kDa, 3.63 kDa, 7.26 kDa and 8.60 kDa in CM10 pH 9 fraction; 4.99 kDa, 12.3 kDa, 12.5 kDa, 12.7 kDa and 27.7 kDa in CM10 pH 4 fraction; 3.2 kDa, 5.57 kDa, 28.5 kDa and 55.9 kDa in CM10 organic fraction; 10.2 kDa and 10.6 kDa in IMAC organic fraction. The representative spectral views and gel views of three biomarkers (3.60 kDa, 3.63 kDa and 5.57 kDa) are presented in Figures 4.1 and 4.2.

4.3.2.3 Identification of protein biomarkers

Five biomarkers were identified (Table 4.2). The 3.63 kDa, 12.3 kDa and 15 kDa biomarkers were identified as C-terminal fragment of the alpha and beta chain of clusterin (accession no. Q29549), N-terminal fragment of vitronectin (accession no. P48819) and C-terminal fragment of apolipoprotein A-I (Apo A-I, accession no. P18648), respectively. In addition, the 22.5 kDa biomarker was shown to be the N-terminal fragment of lecithin-cholesterol acyltransferase (LCAT) with accession no. P30930, appearing in its dimerised form. All these biomarkers had an elevated serum expression in pigs infected with viable cysts (viable phenotype). Another biomarker, appearing at 10.3 kDa and with elevated serum expression in pigs infected with degenerated cysts, was identified as the N-terminal fragment of haptoglobin (accession no. Q8SPS7).

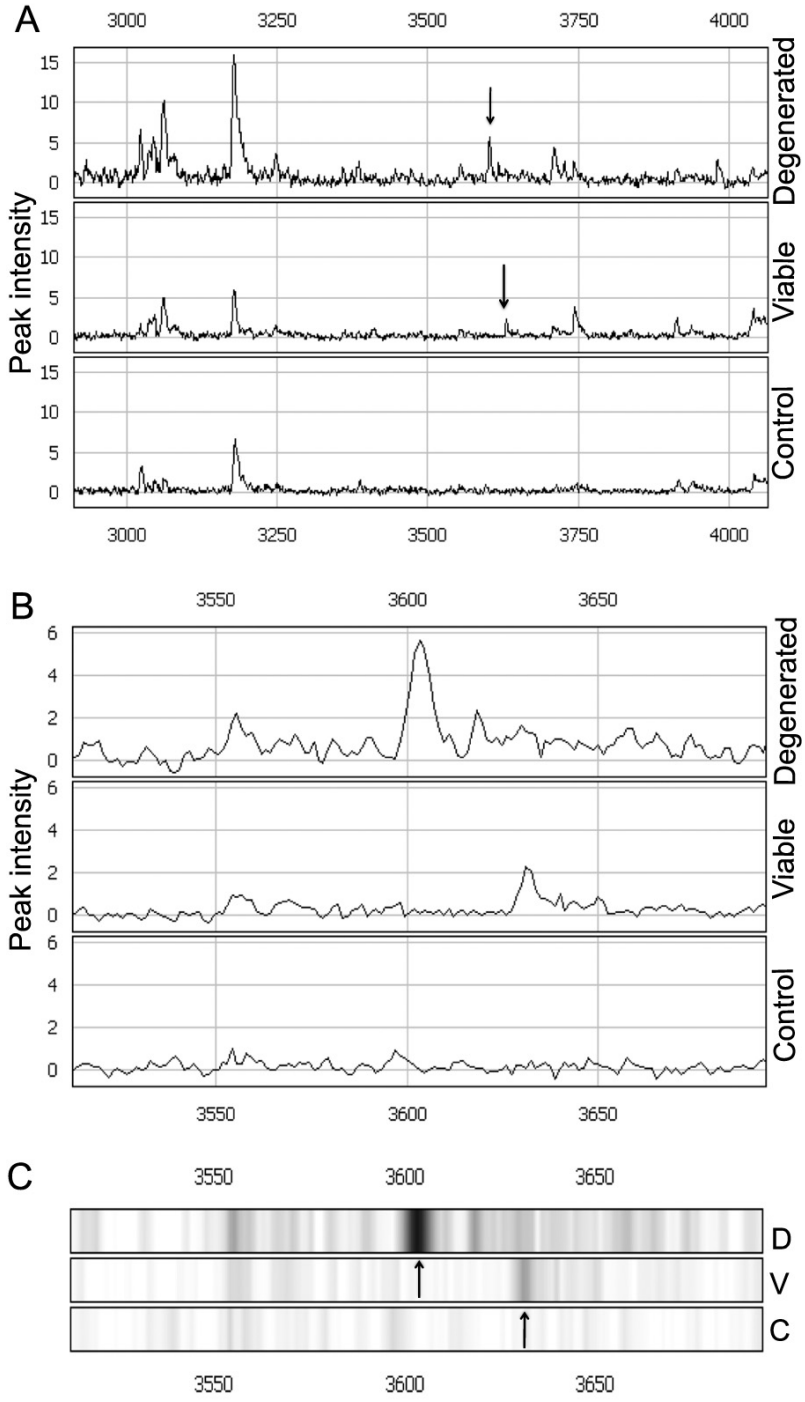

Figure 4.1: Example of the 3.60 kDa and 3.63 kDa protein biomarkers as discovered by SELDI-TOF analysis (CM10 ProteinChip Array) of serum samples from experimentally infected pigs. The 3.60 kDa biomarker is present in serum samples from pigs with only degenerated cysts at necropsy (degenerated phenotype). Note the absence of the biomarker in the pre-infection control samples and serum samples from pigs with viable cysts at necropsy. The 3.63 kDa biomarker is present in serum samples from pigs with viable cysts at necropsy (viable phenotype). *(A)* Spectral view of the protein profiles in the range 3–4 kDa. *(B)* Close-up spectral view (range 3,500–3,700 Da). *(C)* Gel view. D = sera from experimentally infected pigs with degenerated cysts; V = sera from experimentally infected pigs with viable cysts; C = pre-infection control sera.

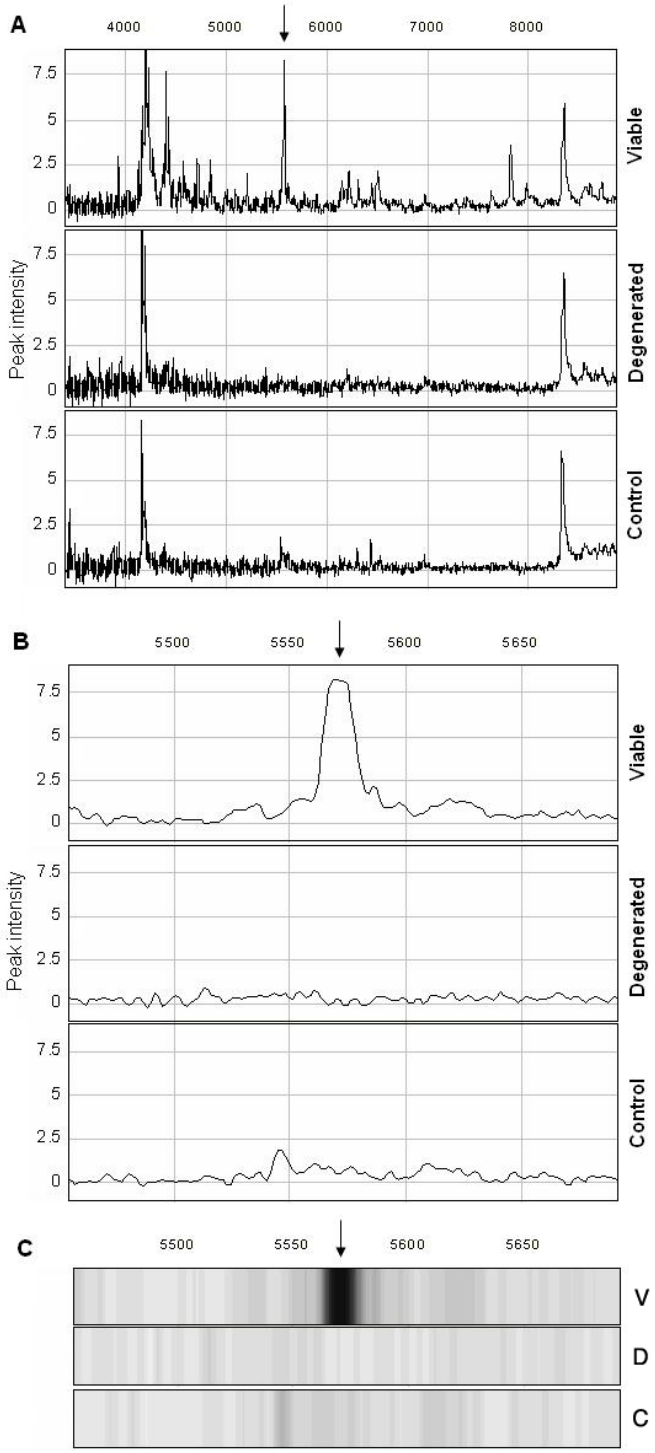

Figure 4.2: Example of the 5.57 kDa protein biomarker as discovered by SELDI-TOF analysis (CM10 ProteinChip Array) of serum samples from experimentally infected pigs. The biomarker is present (arrow) in serum samples from pigs with viable cysts at necropsy (viable phenotype) *(A)* Spectral view of the protein profiles in the range 3.5–9 kDa. *(B)* Close-up spectral view (range 5,450–5,700 Da). *(C)* Gel view. D = sera from experimentally infected pigs with degenerated cysts; V = sera from experimentally infected pigs with viable cysts; C = pre-infection control sera.

Table 4.2: Overview of significant biomarkers after SELDI-TOF analysis of serum samples from experimentally infected pigs.

MW (kDa)[a]	*P* value	Phenotype[b]	Chip type	Fraction	Biomarker identification
3.20 *	0.027	D +	CM10	Organic	
3.60 *	0.021	D +	CM10	pH 9	
3.63 *	0.009	V +	CM10	pH 9	Clusterin
4.03	0.030	V –	CM10	Organic	
4.99 *	0.004	I +	CM10	pH 4	
5.57 *	8.1×10^{-4}	V +	CM10	Organic	
7.26 *	0.004	V +	CM10	pH 9	
8.60 *	0.032	V +	CM10	pH 9	
9.24	0.013	I +	CM10	pH 4	
9.43	0.004	I +	CM10	pH 4	
9.46	0.018	V +	CM10	pH 4	
10.2 *	0.014	D +	IMAC30	Organic	
10.3	0.040	D +	CM10	Organic	Haptoglobin
10.5	0.038	D +	CM10	pH 9	
10.6	0.034	V +	CM10	pH 9	
10.6 *	0.016	D +	IMAC30	Organic	
12.3 *	0.003	V +	CM10	pH 4	Vitronectin
12.5 *	0.015	V –	IMAC30	Organic	
12.6	0.001	V –	CM10	pH 4	
12.7 *	0.017	I +	IMAC30	Organic	
12.8	0.004	I +	CM10	pH 4	
15.0	0.012	V +	CM10	pH 9	Apo A-I
16.1	4.5×10^{-4}	I +	CM10	pH 4	
19.9	0.047	D +	IMAC30	Organic	
22.5	0.021	V +	CM10	pH 4	LCAT
27.7 *	0.005	I +	CM10	pH 4	
27.9	0.035	V –	IMAC30	Organic	
28.5 *	0.010	D +	CM10	Organic	
41.6	0.002	I +	CM10	pH 4	
55.9 *	0.009	D +	CM10	Organic	

[a] * = biomarker specific for *T. solium* cysticercosis

[b] D= biomarker for degenerated phenotype; V = biomarker for viable phenotype; I = biomarkers for infected phenotype; + = upregulated biomarker; – = downregulated biomarker

4.3.3 Discovery of protein biomarkers in the field samples

Of the total number of 688 sample spectra collected, the normalization coefficients of 36 spectra did not pass the "twice the average rule" (normalization coefficient $\geq 2\mu$) and were rejected from further analysis. The average (inter-assay) CV value for peak intensity and mass to charge ratios were 43.2% and $< 0.40\%$, respectively.

4.3.3.1 First pass analysis

Viable versus degenerated EDM was performed for differentiation of group Df and group Vf in 2 different runs: *(1)* for the field samples from Peru and *(2)* for the field samples from Zambia. Only biomarkers that were significant ($P < 0.05$) in both runs were selected. Then, all the field samples were pooled in one group and again EDM analysis was done. Biomarkers for degenerated and viable phenotype that remained significant were selected for second pass analysis.

Infected versus non-infected Group C was included in the analysis to provide a reference expression level. By performing EDM and P-value calculation of the three groups simultaneously (group Df, group Vf and group C) biomarkers for the infected phenotype could be identified.

4.3.3.2 Second pass analysis

Significant peaks were manually relabelled in all spectra and another round of EDM was done. Again, statistical analysis was done and the different groups were compared: *(1)* group Df versus group C, *(2)* group Vf versus group C and *(3)* these three groups simultaneously. The biomarkers that passed the second round of EDM ($P < 0.05$) were compared to the biomarkers for the experimental samples. Analysis of the field samples resulted in 10 biomarkers that were significant ($P < 0.05$) in the samples originating from both Zambia and Peru (Table 4.3). Molecular weights ranged from 8.09 kDa to 158.1 kDa and in the pH 4 fraction (CM10) no significant biomarker was found. There were 3 biomarkers for the viable phenotype, 4 biomarkers for the degenerated phenotype and 3 biomarkers for the infected phenotype. Fifty percent had increased serum expression and 50% had decreased serum expression. Comparison with the significant biomarkers for the experimental samples showed that 3 biomarkers were significant in both sample sets: 8.60 kDa in the pH 9 fraction (CM10), 28.5 kDa and 55.8 kDa in the organic fraction (CM10). However, the peak profiles (relative normalized intensities for the different groups) were not consistent in the two sample sets (Figure 4.3). In the experimental sample group, the 8.6 kDa marker had a significant elevated serum expression in the samples of pigs in-

fected with viable cysts, whereas in the field sample group, it had a significant decreased serum expression in samples from pigs with degenerated cysts and pigs with viable cysts. The 28.5 kDa and 55.8 kDa markers had a significant elevated serum expression in the experimental samples of pigs infected with degenerated cysts, whereas in the field sample group, they had a significant decreased serum expression in samples from pigs with viable cysts. The other biomarkers from the experimental sample set were either absent or not significant in the field sample set (results not shown).

Table 4.3: Overview of significant biomarkers after SELDI-TOF analysis of 86 serum samples from naturally infected pigs in Zambia and Peru and comparison with biomarkers found after SELDI-TOF analysis of 30 serum samples from experimentally infected pigs.

			Field samples		Experimental samples	
MW (kDa)	Chip type	Fraction	*P* value	Phenotype[a]	*P* value	Phenotype[a]
8.09	CM10	Organic	0.002	D +	> 0.05	na
8.60	CM10	pH 9	0.003	I –	0.032	V +
14.0	IMAC30	Organic	0.004	D +	no cluster	na
14.04	IMAC30	Organic	0.036	D +	> 0.05	na
14.1	CM10	Organic	0.003	D +	> 0.05	na
16.2	CM10	pH 9	0.007	V –	> 0.05	na
28.5	CM10	Organic	0.048	V –	0.010	D +
54.3	CM10	pH 9	2.6×10^{-4}	I –	> 0.05	na
55.8	CM10	Organic	0.004	V –	0.009	D +
158.1	CM10	pH 9	1.2×10^{-4}	I +	> 0.05	na

[a] D= biomarker for degenerated phenotype; V = biomarker for viable phenotype; I = biomarker for infected phenotype; + = upregulated biomarker; – = downregulated biomarker; na = not applicable

4.4 Discussion

In this study we aimed to identify biomarkers in serum of pigs infected with *T. solium* that differentiate pigs with viable cysts (active disease) from pigs harboring only degenerated cysts (inactive disease) and secondly biomarkers that differentiate infected pigs from non-infected pigs. We used SELDI-TOF MS technology to profile the serum proteome.

The set up was as follows: *(1)* identification of potential biomarkers in a set of well defined serum samples from experimentally infected pigs (including the respective pre-

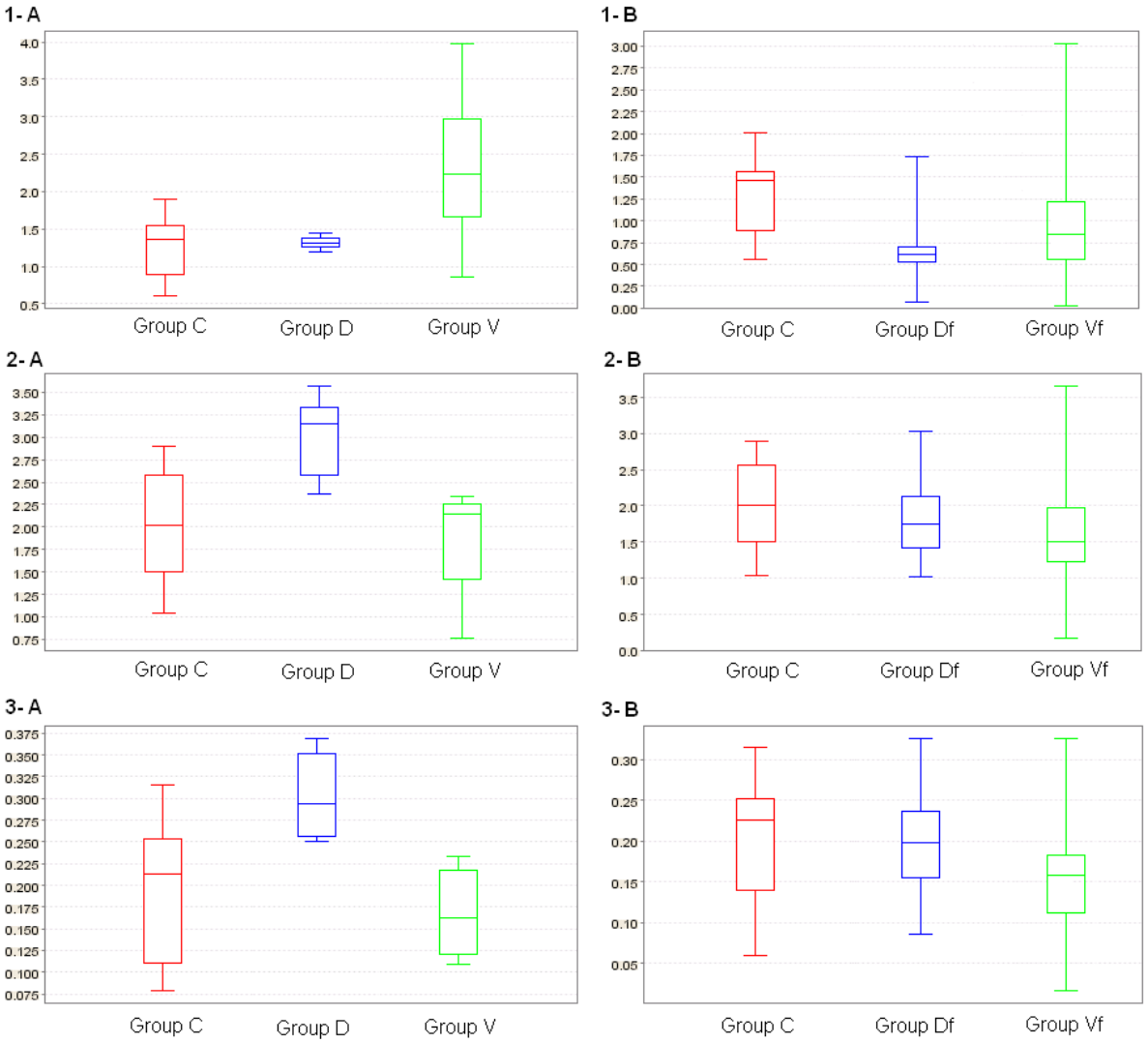

Figure 4.3: Box-and-whisker diagrams of the peak intensities of 3 biomarkers discovered by SELDI-TOF analysis (CM10 ProteinChip Array) of serum samples of pigs experimentally or naturally infected with *Taenia solium*. The left panels (*1A*, *2A*, *3A*) represent the experimental sample group (groups C, D and V), the right panels (*1B*, *2B*, *3B*) the field sample group (groups C, Df and Vf). *(1)* 8.6 kDa marker. In the experimental sample group, the 8.6 kDa marker has a significant elevated serum expression in the samples of pigs infected with viable cysts, whereas in the field sample group, it has a significant decreased serum expression in samples from pigs with degenerated cysts and pigs with viable cysts. *(2)* 28.5 kDa marker. In the experimental sample group, the 28.5 kDa marker has a significant elevated serum expression in the samples of pigs infected with degenerated cysts, whereas in the field sample group, it has a significant decreased serum expression in samples from pigs with viable cysts. *(3)* 55.8 kDa marker. In the experimental sample group, the 55.8 kDa marker has a significant elevated serum expression in the samples of pigs infected with degenerated cysts, whereas in the field sample group, it has a significant decreased serum expression in samples from pigs with viable cysts.

infection samples), *(2)* assessment of their specificity by analyzing serum samples from heterologous infections and *(3)* validation of the biomarkers by analyzing serum samples from naturally infected pigs. The different sample groups were processed randomly to avoid introduction of systematic bias.

In the experimental sample set 30 significant biomarkers were identified of which 15 biomarkers were potentially specific for porcine cysticercosis. However, only three of the biomarkers discovered in the experimental samples were significant in the field sample set (8.60 kDa in the pH 9 fraction (CM10), 28.5 kDa and 55.8 kDa in the organic fraction (CM10)). In addition, the peak profiles of these three biomarkers differed between the two sample sets. We were thus not able to validate the identified biomarkers.

Several technical and biological confounding factors may influence reproducibility of SELDI-TOF results: differential handling and/or processing of the samples, variations in mass spectrometer stability and protein chip performance, biological variability of the study subjects (e.g. sex, age, nutritional level and concurrent infections), inappropriate statistical design, among others. We tried to address these issues through our experimental set up, however some causes of variability could not be accounted for, e.g. the handling and storage of the field samples. Moreover, the baseline biological variability of samples collected in field conditions is a potential bottleneck when validating new diagnostic techniques. Various environmental, nutritional and physiological factors have an important impact on the pathogenesis of diseases. Especially in helminth infections, often characterized by down regulation of host immunity and harmonious host-parasite interplay (Maizels and Yazdanbakhsh, 2003), this baseline biological variability could have a major impact on the proteome and prevent extrapolation of serum profiling results between different sample groups.

A few studies applied SELDI-TOF technology to the study of biomarkers of parasitic diseases such as human African trypanosomiasis (HAT) (Papadopoulos et al., 2004) and fasciolosis in sheep (Rioux et al., 2007). The former study applied three different data-mining tools to identify proteomic signatures that could distinguish between HAT patients and controls without identifying individual biomarkers. They reported a sensitivity and specificity of 100% and 98%, respectively; however in this format the test is too expensive and impracticable for field use. Furthermore, these promising results still remain to be confirmed. The latter study aimed to identify diagnostic biomarkers for early parasite invasion. Two of the biomarkers were identified as transferrin and Apo A-IV. Up to now, these authors have not identified a biomarker that could be diagnostic of early infection or that may act as a marker of intensity of infection nor were they able to detect a serum biomarker corresponding to the main parasite biomarker in bile (isotypes of *Fasciola hepatica* cathepsin L).

In this study, five biomarkers discovered in the experimental sample group were identified, four of which (3.63 kDa biomarker (clusterin), 12.3 kDa biomarker (vitronectin), 15 kDa biomarker (Apo A-I) and 22.5 kDa biomarker (LCAT)) had an increased serum expression in animals infected with viable cysts, one identified biomarker (10.3kDa biomarker (haptoglobin)) had an increased serum expression in animals infected with degenerated cysts. Clusterin (complement cytolysis inhibitor or CP40) inhibits complement-mediated cell lysis (Berge et al., 1997) and enhances TGF-β-induced-transcriptional activity (Lee et al., 2008). TGF-β inhibits macrophage activation and can lead to the induction of Th2-type cells and immune suppression (Maizels and Yazdanbakhsh, 2003). Vitronectin (S-protein) also inhibits the membrane-damaging effect of the terminal cytolytic complement pathway (Johnson et al., 1994; Milis et al., 1993). Apo A-I, a negative acute-phase protein in pigs (Carpintero et al., 2005), is a major component of high density lipoprotein (HDL) in plasma and a potent activator of LCAT, a central enzyme in the extracellular metabolism of plasma lipoproteins involved in the synthesis of HDL (Hill and McQueen, 1997). HDL is known to have anti-inflammatory effects through inhibiting endothelial cell expression of cytokine-induced leukocyte adhesion molecules (Cockerill et al., 1995, 2001).

Taenia solium cysticercosis is characterized by a long lasting immune response occurring concomitantly to the presence of living parasites that develop immune evasion mechanisms to survive within an immunocompetent host (Correa et al., 1999a; Flisser et al., 2002). The different mechanisms are not yet clear; however inhibition of complement by paramyosin has already been observed (Laclette et al., 1992). The parasite also seems to mediate some kind of immune depression or a shift to a Th2-type cell response (Terrazas et al., 1998). Our results seem to confirm this state of immune depression in pigs infected with viable cysts on three levels: complement inhibition (clusterin and vitronectin), inhibition of leukocyte migration (Apo A-I and LCAT) and induction of Th2-type response and immune suppression through TGF-β (clusterin). However, considering the lack of correlation with the biomarkers in the field samples, this needs to be studied more thoroughly.

During the late phase of host-parasite immune interaction, the parasite is destroyed by an inflammatory reaction; the reasons for this are not well understood. In this respect, we observed the upregulation of a 10.3 kDa biomarker identified as N-terminal fragment of haptoglobin in animals with degenerated cysts. Haptoglobin is an important acute-phase protein in pigs and marker for inflammation (Chen et al., 2003; Parra et al., 2006). From the five identified biomarkers only the 3.63 kDa biomarker (clusterin) and the 12.3 kDa biomarker (vitronectin) seem to be specifically linked with cysticercosis infection. However, further studies are necessary to assess the direct effect of parasite establishment

on the upregulation of these two biomarkers. The differential expression profile of the other identified biomarkers (apo A-I, LCAT and haptoglobin) is most likely the result of aspecific epiphenomena related to the infection, since these biomarkers were not specific for cysticercosis after comparison with the heterologous samples (Table 4.2).

From this, it is clear that SELDI-TOF technology can provide useful insights in the biology and underlying mechanisms of host-parasite interactions in cysticercosis. However, for biomarkers to have diagnostic value, they not only should have discriminatory power between different classification groups (infections with viable or degenerated cysts), but also should be specific for a certain disease state, both under experimental and field conditions.

5

Nanobodies, a promising tool for species-specific diagnosis of *Taenia solium* cysticercosis

Adapted from:

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5.1 Introduction

Serodiagnosis of cysticercosis can be done by detecting antibodies or circulating parasite antigen. The current immunodiagnostic test that detects parasite antigen is a sandwich ELISA using monoclonal antibodies raised against ES products of the larval stage of *T. saginata*, the beef tapeworm (Harrison et al., 1989; Brandt et al., 1992; Van Kerckhoven et al., 1998). This assay only detects living cysts and is genus-specific, enabling diagnosis of infection with any *Taenia* species (Dorny et al., 2003). This is not a constraint for diagnosis in humans as they can only be infected with the larval stage of *T. solium*. However, pigs can also be infected with *T. hydatigena* (Dorny et al., 2004b) and *T. saginata asiatica* (Geerts et al., 1992), which are of no, or less importance for human health. Obviously, the antigen detection ELISA does not differentiate between these species in pigs. A specific serodiagnostic test in pigs is needed for epidemiological studies and monitoring of control programs.

Nanobodies (Nbs), camelid-derived single-domain antibody fragments (Hamers-Casterman et al., 1993; Arbabi-Ghahroudi et al., 1997), constitute an alternative to monoclonal antibodies. Nbs often recognize novel epitopes that are not readily accessible to conventional antibodies because of the larger antigen-binding site of the latter (Conrath et al., 2001b; Stijlemans et al., 2004; De Genst et al., 2006b). In addition, Nbs display a high affinity and specificity for their target antigens, are extremely stable (Muyldermans and Lauwereys, 1999; van der Linden et al., 1999; Dumoulin et al., 2002) and can be easily tailored in manifold constructs (Conrath et al., 2001a; Saerens et al., 2008a) making them excellent tools in diverse medical and biotechnological applications. In this chapter we report the generation, selection and characterization of Nbs that do not cross-react between *T. solium* and *T. hydatigena*. We provide evidence of their high potential as tool for species-specific diagnosis of *T. solium* cysticercosis in pigs.

5.2 Materials and methods

5.2.1 Antigens

A total antigen extract was prepared from viable *T. solium* cysts from naturally infected pigs. Cysticerci were recovered upon necropsy of the animals and washed in PBS. The cyst fluid from individual cysts was aspirated and pooled. The collected solution was centrifuged twice at 3,000 *g* for 30 min at 4°C. Protease inhibitors (Complete, Roche) were added to the final supernatant (TsAg) according to the manufacturer's recommendation. A similar procedure was used for the preparation of *T. hydatigena* antigen (ThAg).

An enriched fraction of *T. solium* antigen was prepared by partially purifying TsAg

by ion exchange chromatography on a Mono S cation exchange column (High S cartridge, BioRad) and Mono Q anion exchange column (High Q cartridge, BioRad) using a low-pressure chromatography system (ECONO system, BioRad). The homogeneity and apparent MW of the purified fractions were analyzed by SDS-PAGE according to the method of Laemmli (1970). Proteins were visualized by silver staining. Based on the appearance on one-dimensional (1D) gel, similar fractions were pooled and concentrated. The purified fraction used for immunization was designed as TsPur.

Taenia saginata somatic antigens (TsaAg), *T. crassiceps* cyst fluid (TcrAg) and *Trichinella spiralis* ES antigens (TspAg) were provided by the Animal Health Department, ITMA. All protein concentrations were determined by the method of Bradford (1976).

5.2.2 Serum samples

Taenia solium-positive serum samples were obtained from 24 heavily infected (tongue positive) pigs purchased at the Chibolya slaughter slab in Lusaka (Zambia). The pigs were humanely slaughtered and their carcasses were inspected for the presence of *T. solium* and *T. hydatigena* cysts. *Taenia hydatigena*-positive serum samples were obtained from 8 experimentally infected pigs; the samples were kindly provided by M. Lightowlers, University of Melbourne, Australia. Negative pig serum samples were obtained from the serum bank at ITMA.

5.2.3 Immunization of animals

Two adult dromedaries (*Camelus dromedarius*) kept at the Central Veterinary Research Laboratory (Dubai, U.A.E.) were immunized with TsPur and TsAg, respectively. The animals received six subcutaneous injections of 100 µg of protein at weekly intervals (Lauwereys et al., 1998), mixed with an equal volume of Gerbu adjuvant (Gerbu Biotechnik GmbH). Three days after the last injection, unclotted blood was collected and transported to the Brussels laboratory. Peripheral blood lymphocytes (PBLs) were isolated with Lymphoprep (Nycomed). PBLs were counted and aliquots of 5×10^6 cells were pelleted and stored at -80°C.

5.2.4 Nanobody library construction and selection of binders

The Nb libraries were constructed as described previously (Conrath et al., 2001b; Saerens et al., 2004). Basically, mRNA was isolated from the PBLs and cDNA was cloned by reverse transcriptase (RT)-PCR with a dN6 primer. All VH domains, including the VHH domains from Heavy-chain antibody IgG2 and IgG3 isotypes, were amplified with the

primers CALL001 and CALL002 (Conrath et al., 2001b). The VHH gene fragments (coding for the Nbs) were purified from agarose gel and re-amplified using nested primers containing the restriction sites for *Pst*I and *Not*I restriction enzymes. The final PCR products were cloned into the phagemid vector pHEN4 according to the methods of Saerens et al. (2004) and transformed in electro-competent *E. coli* TG1 cells. The Nb repertoire was expressed on phage after infection with M13K07 helper phages. Specific virions against TsAg were enriched by three consecutive rounds of in vitro selection on microtiter 96 well plates coated with antigen (10 µg/well). In order to obtain *T. solium*-specific binders not cross-reacting with *T. hydatigena*, the panning procedure was modified slightly. To remove the binding activity to cross-reactive ThAg, virions were pre-absorbed on ThAg (10 µg/well). Only these virions that did not bind to ThAg were used in the enrichment procedure with TsAg. The TsAg-bound phage particles were eluted with 100 mM triethylamine (pH 10.0). The eluate was neutralized with 1 M Tris-HCl (pH 7.4) and used to infect exponentially growing *E. coli* TG1 cells. After three rounds of panning, polyclonal phage ELISA was performed to monitor the success of selection. Pools of virions from each round were incubated on antigen-coated and non-coated wells. Binding was detected using an anti-M13-HRP conjugate (Amersham Biosciences). Monoclonal phage ELISA was used to identify individual positive clones, which were then sequenced to identify unique Nb genes. Expression in the periplasm and purification of Nb was performed as described previously (Conrath et al., 2001b). The Nbs were subsequently tested for antigen recognition in ELISA.

5.2.5 Nanobody protein production

The selected Nb clones were recloned into the expression vector pHEN6 (Conrath et al., 2001b), using restriction enzymes *Pst*I and *BstE*II. The plasmid constructs were transformed into *E. coli* WK6 cells. Production of recombinant Nbs and purification of the periplasmic extracts was done as described previously (Saerens et al., 2004). The final yield was determined from the UV absorption at 280 nm, and the theoretical mass extinction coefficient.

5.2.6 Binding specificity

To assess the specificities of the Nbs, microtiter plates (Nunc) were coated with different antigen preparations (TsAg, ThAg, TsaAg, TcrAg, TspAg) at a concentration of 5 µg/ml. Residual protein-binding sites were blocked with 2% skimmed milk in PBS. Nanobodies were biotinylated (Biotin Protein Labeling Kit, Roche) according to the manufacturer's recommendations. Biotinylated Nbs were added to each well (5 µg/ml). Detec-

tion was performed with streptavidin-HRP conjugate (Jackson) and *o*-phenylenediamine dihydrochloride (OPD, Dako).

5.2.7 Binding affinity

For affinity determination, different concentrations of the selected Nbs, ranging from 1 µM to 15.6 nM were added to a CM5 chip (BIAcore) to which 2000 RU of TsAg had been coupled. All measurements were performed using a flow rate of 30 µl/min in HBS buffer (10 mM Hepes pH 7.5, 150 mM NaCl, 3.5 mM EDTA and 0.005% Tween-20). Bound Nbs were eluted with 10 mM glycine-HCl pH 2.0. The kinetic and equilibrium parameters (k_{on}, k_{off} and K_D) were determined with the BIAevaluation software version 4.1 (BIAcore).

5.2.8 Western blotting and TsAg protein identification

TsAg was run on a 15% SDS-PAGE gel (Laemmli, 1970) and transferred to nitrocellulose membrane (Hybond). Blocking of the membrane strips was done overnight in PBS-Tween 0.5% + 5% skimmed milk at room temperature. All following incubations were done in PBS-Tween 0.5% + 2% skimmed milk during 1 h at 37°C. Biotinylated Nbs (20 µg per strip) were detected by streptavidin-HRP conjugate (1/5,000) and 3,3,5,5-tetramethylbenzidine (TMB, KPL) as substrate. TsAg was also transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore) after electrophoresis and stained with 0.5% Coomassie blue R250 in 40% methanol and 5% acetic acid. The bands on PVDF corresponding to the bands recognized by the Nbs were cut out and the eluted proteins were identified through N-terminal sequencing (Edman degradation). The resulting amino acid sequence was subjected to a search using the BLAST algorithm (Altschul et al., 1997).

To confirm the result of the TsAg protein identification, immunodetection was assessed for Ts14, Ts18var1, TsRS1 and TsRS2 (four synthetic proteins belonging to the 8 kDa diagnostic protein family (Hancock et al., 2003)) blotted on nitrocellulose membrane. Strips containing the synthetic proteins were incubated overnight at 4°C with 15 µg of biotinylated Nb diluted in PBS-Tween 0.5% + 5% skimmed milk. Detection of protein-associated Nb was done. Additional strips were incubated with serum from rabbits immunized against TsAg (provided by ITMA) and detected by goat-anti-rabbit-peroxidase conjugate and TMB substrate. The strips were kindly provided by Dr. Wilkins, Centers for Disease Control and Prevention (CDC), Atlanta, USA.

5.2.9 Epitope mapping

The complementation epitope groups of Nbs were investigated in competition ELISA. Non-biotinylated homologous and heterologous Nbs were diluted in a previously determined sub-saturating level dilution of biotinylated Nb, to analyze the binding-inhibition of the marked Nb (Harlow and Lane, 1988). Briefly, after coating of the microtiter plates with TsAg and blocking (see supra) a mixture of the same biotinylated Nb (1.25 µg/ml for Nbsol130, Nbsol68, Nbsol71 and Nbsol41; 5 µg/ml for Nbsol60, Nbsol62 and Nbsol111; 0.04 µg/ml for Nbsol52) and increasing concentrations (up to 200 µg/ml) of homologous or heterologous Nb were added. Streptavidin-HRP was used as conjugate and OPD as substrate. Decreasing OD when using increasing concentrations of a competing Nb indicate a competition for the same or overlapping epitope. The threshold for competition was set at a 50% reduction in OD when adding 200 µg/ml of competing Nb, compared with the OD of the biotinylated Nb in absence of competing Nb (OD = 100%).

To confirm the interaction observed in the competition ELISA between Nbsol60, Nbsol62 and Nbsol71 and Nbsol130 and Nbsol41, respectively, dilution series of biotinylated Nbsol60, Nbsol62 and Nbsol71 (starting from 20 µg/ml) were tested in an ELISA with and without the presence of either Nbsol130 or Nbsol41 at a constant concentration (20 µg/ml). After coating of the plates with TsAg and blocking, the Nbs were incubated for 1 h at RT. Detection was done with streptavidin-HRP and OPD as substrate.

5.2.10 Antigen capturing

Pairs of Nbs were tested in various combinations in sandwich ELISA to assess the capturing of antigens present in cyst fluid (TsAg and ThAg) or pooled serum samples (*T. solium*-positive, *T. hydatigena*-positive and negative serum). Briefly, plates were coated with the first (capturing) Nb at 10 µg/ml. After blocking and incubation of either cyst fluid or serum, captured antigens were detected by a second, biotinylated Nb (5 µg/ml) and streptavidin-HRP. Serum samples were pretreated with trichloroacetic acid (TCA) before incubation (De Jonge et al., 1987). Preliminary tests indicated that this approach produced better results than serum samples diluted in PBS (results not shown). To confirm that Nbs were able to capture serum antigen, an inhibition ELISA for the detection of antigen was performed as described by Harlow and Lane (Harlow and Lane, 1988). Briefly, after determining the optimal concentrations by titration, plates were coated with TsAg (1.25 µg/ml). Biotinylated Nb was added to the sample solution and pre-incubated for 1 h at RT. The sample solution consisted of either PBS, PBS spiked with TsAg, TCA treated negative serum, TCA treated negative serum spiked with TsAg or TCA treated positive serum. After blocking, the sample solution was added and bound Nbs were detected by

streptavidin-HRP conjugate.

5.2.11 Animal ethical approval

All animal treatment was according to the guidelines of the local Animal Care Committee and supervised by a veterinary surgeon.

5.3 Results

5.3.1 Selection of Ag-specific Nbs

The VHH gene fragments of the Heavy-chain antibodies from the immunized dromedaries, coding for the Nanobodies were cloned. Two different libraries of, respectively, 7.5×10^6 (for dromedary 1 immunized with TsPur) and 3.9×10^7 transformants (for dromedary 2 immunized with TsAg) were obtained. Within the first library, 100% of the clones contained a vector with a VHH gene insert of the appropriate size as determined by PCR. For the second library, 80% of the clones had an insert of proper size. The Nb repertoires of both libraries were expressed on phages and selection of phage particles expressing a specific antigen-binding Nb was performed. Three rounds of panning were performed on TsAg coated on microtiter plates after pre-adsorption on ThAg. A clear enrichment of specific phages during these consecutive rounds of panning was observed. After the third round of panning, 336 individual colonies were screened for antigen recognition in ELISA. Twenty-six clones were positive in this screening ELISA. The nucleotide sequence analysis of the Nbs revealed 17 distinct binders. A total of eight different TsAg binders (Nbsol) were selected for further work (Nbsol41, Nbsol52, Nbsol60, Nbsol62, Nbsol71, Nbsol68, Nbsol111, Nbsol130), based on their reactivity in the screening ELISA (results not shown). The deduced amino acid sequences of the Nbs are shown in Figure 5.1. All of the binders are derived from the Heavy-chain antibody specific VHH germline genes (Nguyen et al., 2000), as they contain the hallmark amino acid substitutions in frameworks 1 and 2. The disulfide bridge, frequently occurring between CDR1 and CDR3 in dromedary Nbs, was present in all clones except Nbsol68. In Nbsol41 and Nbsol130 the disulfide bridge occurred between CDR3 and FR2 (cysteine residue at position 50).

5.3.2 Production and purification of the different binders

The Nbs were produced as soluble protein after re-cloning into expression vector pHEN6 and transformation into *E. coli* WK6 cells. The single-domain antibody fragments, carrying a His_6 tag, are transported into the periplasm of *E. coli* and subsequently purified

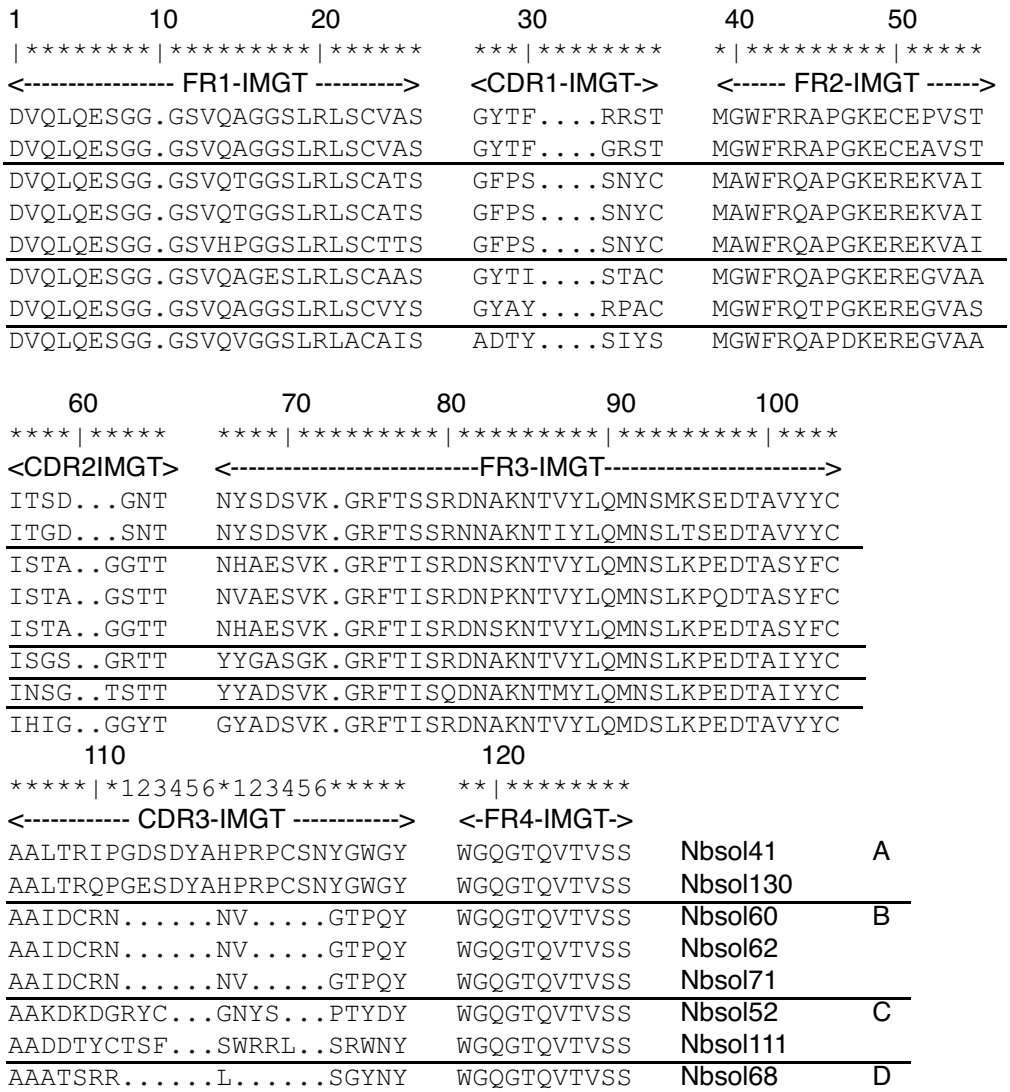

Figure 5.1: Amino acid sequence of the isolated Nanobodies from two dromedary immune libraries against *Taenia solium* antigen (TsAg), named Nbsol. The IMGT numbering and CDR designation are used (The International Immunogenetics Information System, `http://imgt.cines.fr`). A to D denote the different epitope-binding groups based on the results of competition ELISA.

from the periplasmic extract by immobilized-metal affinity chromatography. The yield of purified product varied from 0.1 to 12 mg/liter of culture, depending on the actual Nb.

5.3.3 Binding specificity

The result of the ELISA with heterologous antigens is shown in Figure 5.2. The Nbs were highly specific for *T. solium* antigen and no cross reactivity with *T. hydatigena*, *T. saginata*, *T. crassiceps* or *T. spiralis* was observed.

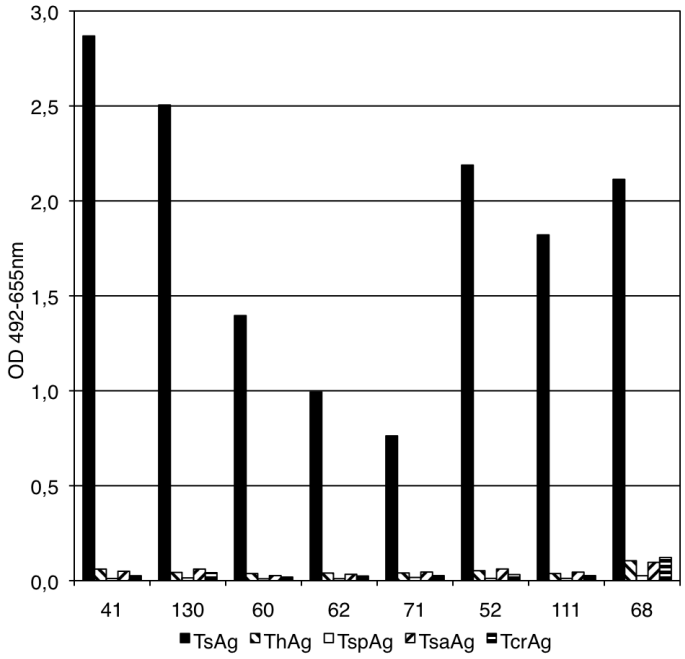

Figure 5.2: Specificity of Nanobodies raised against *Taenia solium* cyst fluid tested in ELISA with cyst fluid from *T. solium* (TsAg) and *Taenia hydatigena* (ThAg), *Trichinella spiralis* excretion-secretion antigens (TspAg), *Taenia saginata* somatic antigens (TsaAg) and *Taenia crassiceps* cyst fluid (TcrAg).

5.3.4 Binding affinity

The affinity of all Nbs for TsAg was determined by surface plasmon resonance on a BIAcore 3000. The association of each Nb with the antigen was recorded. Except for Nbsol68, the binding kinetics yielded k_{on} values in the range of $(7.9 \times 10^4 - 3.1 \times 10^6)$ $M^{-1}s^{-1}$ and k_{off} values of $(5.7 \times 10^{-4} - 1.2 \times 10^{-2})$ s^{-1} (Figure 5.3). From these kinetic rate constants corresponding K_D values ranging from 154 nM to 185 pM were calculated. Nbsol68 is removed from Figure 5.3 because of its high equilibrium dissociation constant ($K_D = 6.8\mu M$).

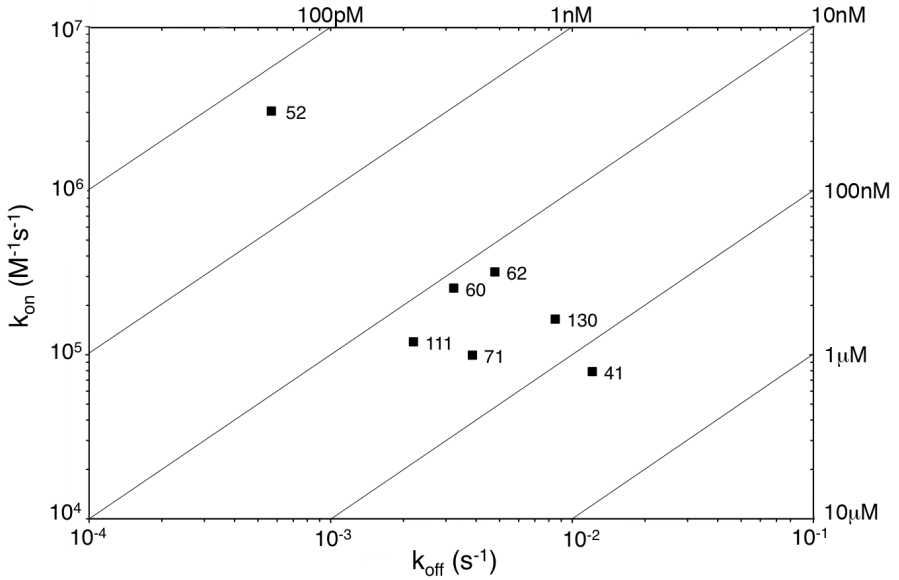

Figure 5.3: Rate plane with Isoaffinity Diagonals (RaPID) plot of the *Taenia solium* antigen (TsAg) binding Nanobodies (Nb). The kinetic rate values k_{on} and k_{off} for a particular Nb as determined by biosensor measurements are plotted on a two-dimensional diagram so that Nbs located on the same diagonal line have identical K_D values.

5.3.5 Western blot and protein identification

Figure 5.4A shows the results of the Western blot with TsAg on nitrocellulose membrane. All Nanobodies recognize the same protein bands. Nbsol60 and Nbsol62 only show a very faint reactivity with TsAg on Western blotting. After electrophoresis, blotting of TsAg on PVDF membrane and staining, the two protein bands between the 64.2 kDa and 48.8 kDa markers and 37.1 kDa and 25.9 kDa markers, respectively, were cut out of the membrane and sequenced (Figure 5.4B). Both the high MW band (± 50 kDa) and the low MW band (± 32kDa) contained the same N-terminal amino acid sequence (EKNKPKDVA). The resulting sequence showed a 100% identity with six glycoproteins (GenBank accession nos. ABI20731, AAM00204, AAF25005, AAM00206, AAM00208, AAX32918) all representing the 14 kDa diagnostic glycoprotein from the 8 kDa diagnostic protein family (Greene et al., 2000; Hancock et al., 2003; Lee et al., 2005). Figure 5.4C shows the results of Western blotting with the synthetic 8 kDa proteins. Nbsol130, Nbsol41, Nbsol52, Nbsol60, Nbsol62, Nbsol68 and Nbsol111 react with Ts18var1 protein. Only two Nbs, Nbsol130 and Nbsol41, recognized the Ts14 protein as well as TsRS2. None of the Nbs recognized TsRS1.

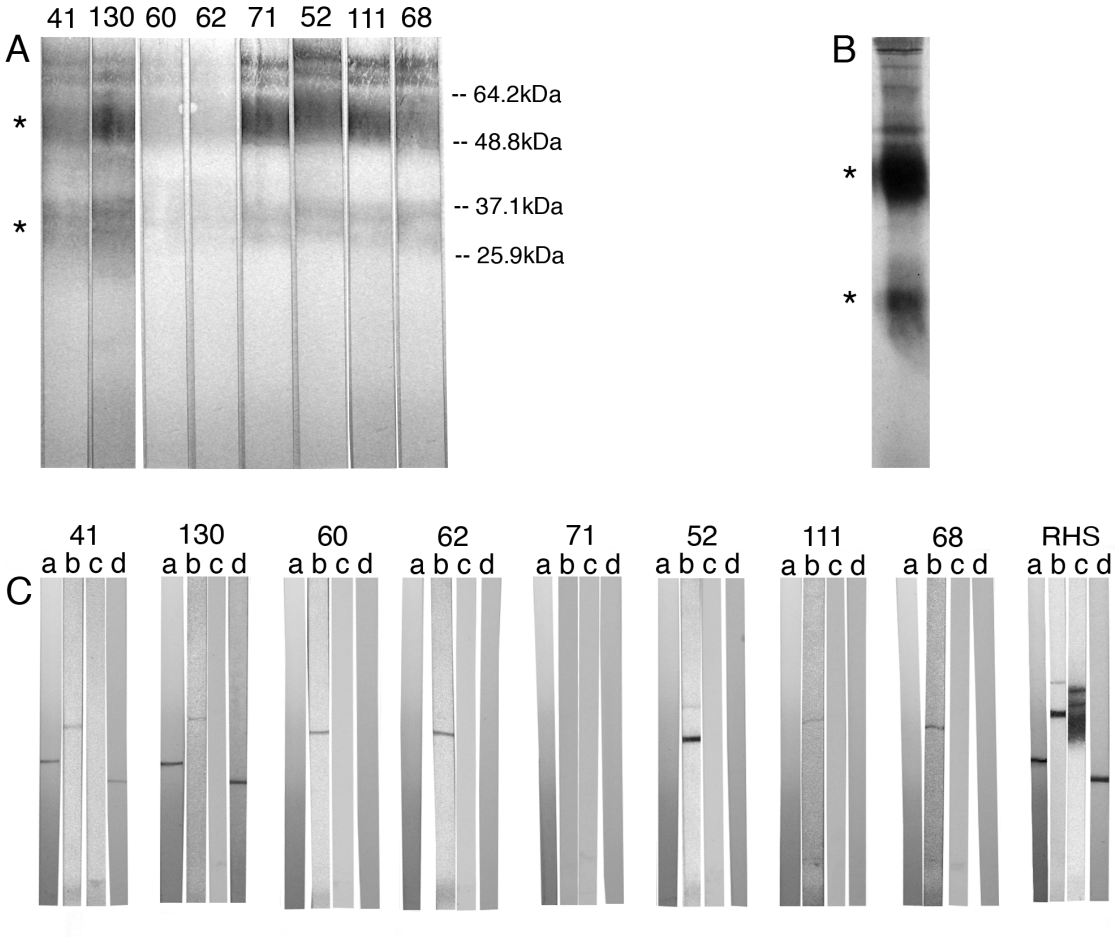

Figure 5.4: Western blotting with *Taenia solium*-specific Nanobodies (Nb). *(A)* Immunodetection of *T. solium* cyst fluid with Nbs after 15% SDS-PAGE and blotting on nitrocellulose membrane. Two major bands of approximately 50 and 32 kDa (marked with asterisks) were consistently recognized. Only Nbsol60 and Nbsol62 show a low reactivity with the antigen. The position of the MW markers is indicated. *(B)* Blotting of *T. solium* cyst fluid on polyvinylidene fluoride (PVDF) membrane and stained with Coomassie blue after 15% SDS-PAGE. The bands marked with an asterisk were cut out and identified by N-terminal sequencing, they both contained a 14 kDa glycoprotein. *(C)* Immunodetection of the 8 kDa diagnostic glycoproteins Ts14 (*a*), Ts18var1 (*b*), TsRS1 (*c*) and TsRS2 (*d*) with Nbs (RHS: rabbit hyperimmune serum raised against *T. solium* cyst fluid).

5.3.6 Epitope mapping

The results of the competition ELISAs are summarized in Table 5.1. Nbsol41 and Nbsol130 showed mutual competition to bind the epitopes on TsAg, which was expected based on the high sequence identity in their respective CDR regions. This also holds true for Nbsol60, Nbsol62 and Nbsol71: these Nbs have identical CDR sequences and also recognized the same epitope when tested in ELISA. Interestingly, the binding of Nbsol60, Nbsol62 and Nbsol71 to the epitope was enhanced when adding high concentrations of either Nbsol41 or Nbsol130 (Figure 5.5A). This phenomenon was confirmed by testing Nbsol60, Nbsol62 and Nbsol71 in ELISA in dilution series with or without adding a constant concentration of either Nbsol41 or Nbsol130 (Figure 5.5B). In the presence of one of the latter Nbs, a higher reactivity was noted for Nbsol60 and Nbsol62 and to a lesser extent Nbsol71. Nbsol52, Nbsol71 and Nbsol111 were able to inhibit the binding of Nbsol68 to the antigen (with biotinylated Nbsol68 as the detected Nb), but reversely there was no competition (with biotinylated Nbsol52, Nbsol71 or Nbsol111 as detected Nbs and Nbsol68 as competing Nb). This was also observed for Nbsol52 and Nbsol111: Nbsol52 was able to inhibit the binding of Nbsol111 to the antigen, but reversely there was no competition (with biotinylated Nbsol52 as detected Nb). Taken together, the Nbs can be categorized in four different epitope-binding groups: Nbsol41 and Nbsol130 bind to an identical epitope (group A); Nbsol60, Nbsol62 and Nbsol71 also share an identical epitope (group B), the epitopes for Nbsol52 and Nbsol111 are overlapping (group C) and Nbsol68 binds to a unique epitope (group D).

5.3.7 Antigen capture

All pairs of Nbs were tested two by two in a sandwich ELISA for detection of TsAg and ThAg. Eleven sandwich combinations were selected; on average, the OD of TsAg wells was 11-fold higher than the OD of ThAg wells (results not shown). Figure 5.6A shows the results of the four best performing combinations. No clear distinction was noticed between the different serum samples. Furthermore, there was a high background signal (not shown). To confirm that the Nbs were capable of capturing serum antigens, they were all tested in an inhibition ELISA with the biotinylated Nbs mixed in sample solutions containing either PBS, PBS spiked with TsAg, *T. solium*-negative serum, *T. solium*-negative serum spiked with TsAg, *T. solium*-positive serum and *T. hydatigena*-positive serum. The free Nb was captured on microtiter plate-coated TsAg and detected via its biotin (Figure 5.6B). Antigens present in the sample solution inhibit the binding of the Nbs to the antigen immobilized on the plate resulting in a lower OD compared with the PBS sample solution (0% inhibition). Under these conditions, Nbsol52 differentiated *T. solium*-

Table 5.1: Screening of Nanobodies for competition for *Taenia solium* cyst fluid antigens (TsAg) in indirect ELISAs.

	Competing Nanobody							
	Nbsol41	Nbsol52	Nbsol60	Nbsol62	Nbsol68	Nbsol 71	Nbsol 111	Nbsol 130
Nbsol 41-Biotin	+	-	-	-	-	-	-	+
Nbsol 130-Biotin	+	+	-	-	-	-	+	+
Nbsol 60-Biotin	e	-	+	+	-	+	-	e
Nbsol 62-Biotin	e	-	+	+	-	+	-	e
Nbsol 71-Biotin	e	+	+	+	-	+	-	e
Nbsol 52-Biotin	-	+	-	-	-	+	-	-
Nbsol 111-Biotin	-	+	-	-	-	+	+	-
Nbsol 68-Biotin	-	+	-	-	+	+	+	-

+ = competition; - = no competition; e = enhanced binding of biotinylated Nb.

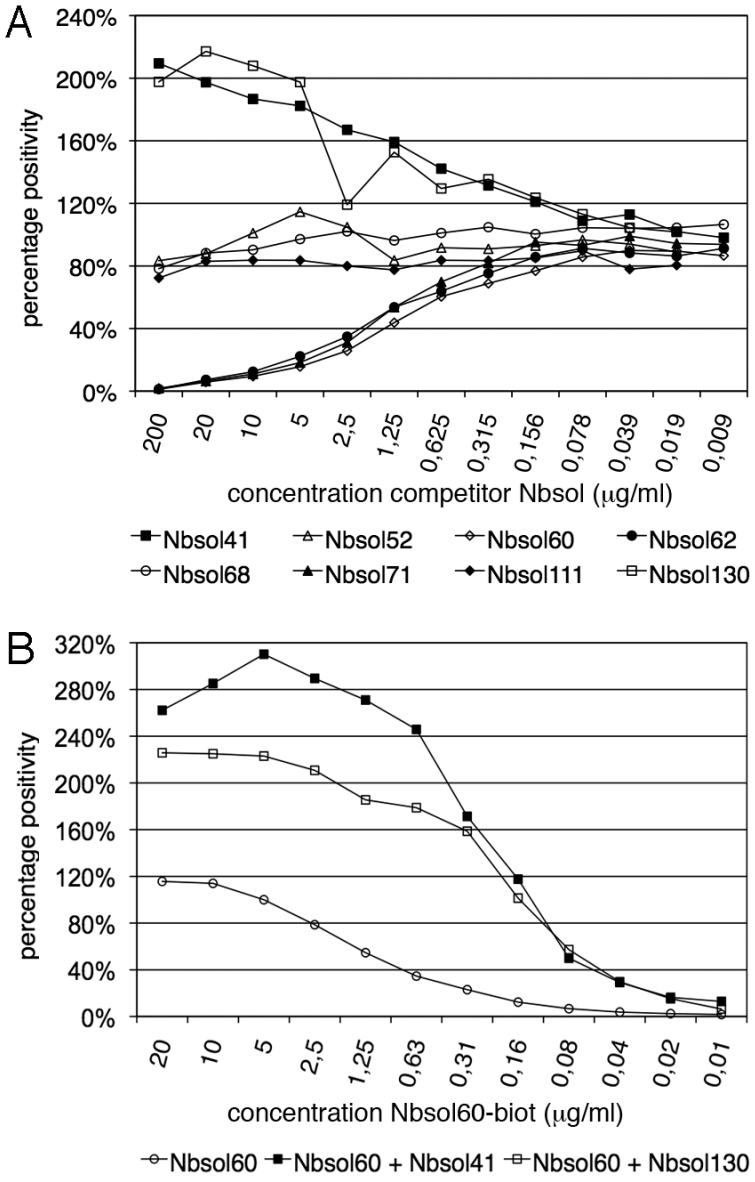

Figure 5.5: Epitope mapping of *Taenia solium*-specific Nanobodies (Nb). *(A)* Competition ELISA for the detection of *T. solium* cyst fluid using biotinylated Nbsol60 as detector antibody. Increasing concentrations of non-biotinylated competing Nb are added. OD is presented as percentage positivity ($OD_{Nbsol60(5\mu g/ml)} = 100\%$). *(B)* ELISA using a dilution series of biotinylated Nbsol60 as detector antibody. A constant concentration of either Nbsol130 or Nbsol41 is added to confirm the interaction between the Nbs: enhanced binding of Nbsol60 is noticed in presence of either Nbsol130 or Nbsol41. OD is presented as percentage positivity ($OD_{Nbsol60(5\mu g/ml)} = 100\%$).

positive samples from *T. solium*-negative and *T. hydatigena*-positive serum samples. The other Nbs gave similar results as shown for Nbsol41 in Figure 5.6B, whereby on average there was low binding inhibition in the TsAg-spiked PBS samples (average inhibition of 41%) and high binding inhibition in all of the serum samples, with no clear distinction between negative, positive or spiked serum samples.

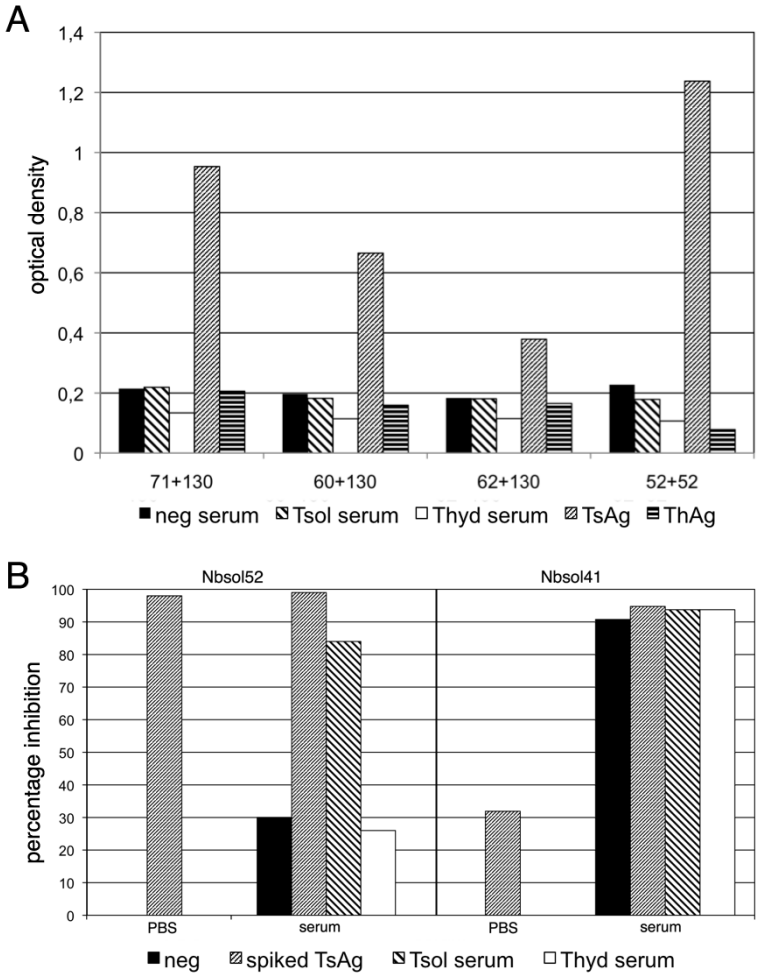

Figure 5.6: Antigen capturing by Nanobodies (Nb). *(A)* Sandwich ELISA with Nbs for the detection of *Taenia solium* (TsAg) and *Taenia hydatigena* (ThAg) cyst fluid and serum antigens (negative serum, serum from *T. solium* and *T. hydatigena*-infected pigs). The four best combinations are presented here, the first Nb noted is the capturing antibody, the second is the detector antibody. *(B)* Inhibition ELISA for the detection of TsAg in PBS and serum antigen (negative serum, negative serum spiked with TsAg, serum from *T. solium* and *T. hydatigena*-infected pigs).

5.4 Discussion

This study demonstrates the feasibility to isolate a panel of *T. solium*-specific Nbs from a dromedary immunized with *T. solium* cyst fluid. We have adapted the panning procedure to eliminate Nbs that cross react with *T. hydatigena*, without a priori knowledge of the proteins present in the crude antigen mix (Saerens et al., 2008b). Epitope mapping by competition ELISA showed that the Nbs are categorized in four complementation groups. Differences between Nbs from the same epitope group are most likely to be due to differences in affinity for the epitope. Interestingly, the prior binding of Nbsol130 and Nbsol41 (group A) to the antigen enhanced the subsequent association of Nbsol60, Nbsol62 and Nbsol71 (group B). Probably the Nbs of group A induced a conformational change within the antigen and/or fix a more appropriate epitope architecture for the group B Nbs (Saerens et al., 2004).

We identified the protein as *T. solium* 14 kDa diagnostic glycoprotein (Ts14), belonging to the 8 kDa glycoprotein family (Hancock et al., 2003). These 8 kDa antigens are the diagnostic proteins seen at 14, 18, and 21 kDa on the Western blot for cysticercosis (Tsang et al., 1989) and are also found in the bands at 24 and 39 to 42 kDa. Recent studies indicate that the 8 kDa proteins and other *T. solium* low-molecular-weight proteins are in fact part of an ES-type hydrophobic ligand-binding protein (Saghir et al., 2000; Lee et al., 2007). Western blot with the synthetic 8 kDa polypeptides did not fully confirm our initial result of protein identification (Ts14). Nbs reacted preferably with Ts18var1 (except for Nbsol71); only the group A Nbs (Nbsol41 and Nbsol130) also reacted with Ts14 and TsRS2. However, given the level of similarity within this family of proteins (Greene et al., 2000), it is possible that the Nbs reacted with both Ts14 and Ts18var1 in the first immunodetection blot assay. Possibly, we have sequenced Ts14 because it occurs more frequently in the larger heteromeric molecule as a subunit (Lee et al., 2005).

The ability of Nbsol52 to be used both as capturing and detecting Nbs in a sandwich ELISA indicates that Ts18var1 forms either homo- or heterodimers via disulfide bond formation through its cysteine residues (Greene et al., 2000). Not all Nbs were able to capture antigen in the sandwich ELISA. For Nbsol68, this can be explained by its low affinity for the antigen ($K_D = 6.8\mu M$). This also explains the observations in the competition ELISA where biotinylated Nbsol68 competes effectively with Nbsol52, Nbsol71 or Nbsol111 for the binding of the antigen but not the other way around. Despite its low affinity, Nbsol68 did perform well in both the direct ELISA and Western blot.

Preliminary results of the sandwich ELISA to detect antigens in serum from naturally infected pigs indicated a high background signal, preventing the assessment of the real reactivity. However, in the inhibition ELISA, Nbsol52 was capable of capturing antigens present in serum of *T. solium*-infected pigs. This can be explained by the fact that in

this test format capturing of the antigens is performed in solution. Also, Nbsol52 showed the highest affinity for the antigen ($K_D = 185pM$), determined by the BIAcore binding studies. The other Nbs were not able to capture the antigen in solution (low inhibition in the TsAg-spiked PBS sample). Furthermore, their binding to the microtiter plate bound-TsAg was inhibited by (aspecific?) proteins present in all of the serum samples. These results suggest that affinity for the epitope is more important than the actual epitope recognized. This is probably due to the complexity of the antigen that consists of a heteromerous association of different subunits (Lee et al., 2005).

Using purified polypeptides, such as the synthetic 8 kDa proteins (Hancock et al., 2003), for immunization and panning could result in the selection of more Nbs with higher affinities, thus improving the sensitivity of the assay.

This study demonstrates the feasibility of employing Nbs to develop an antigen detection assay specific for *T. solium* cysticercosis, after a further determination of the analytical sensitivity and test performance (diagnostic sensitivity and specificity). The introduction of Nbs, with their high (thermo-)stability and low production cost compared to conventional monoclonal antibodies (Frenken et al., 2000; Muyldermans, 2001), offers additional benefits to design an affordable field-assay for use in countries where cysticercosis is endemic.

6

General discussion, conclusions and recommendations

6.1 Introduction

The main objective of the present thesis was to study serological responses in pigs and to identify and develop tools for improved serodiagnosis of porcine cysticercosis. More specifically, the aim in chapter 3 on one hand was to link serological data (antibody and antigen responses) to parasitological findings in experimentally infected pigs in order to study the host-parasite relationship and on the other hand to study the relationship between number of cysts and titer of circulating antigen in order to develop a quantitative diagnostic test. In chapter 4, SELDI-TOF MS technology was applied to identify novel biomarkers in serum of infected pigs that can distinguish between active cysticercosis and inactive cysticercosis. Finally in chapter 5, Nanobodies specific for *T. solium* were produced that can be used for specific diagnosis of porcine cysticercosis. The objective of this chapter is to discuss the findings of the present thesis in a more general context of host-parasite immune interactions and implications for immunoassay development. The advantages and disadvantages of the applied methodology are discussed and further research priorities are identified.

6.2 Serological responses in experimentally infected pigs

In Chapter 3, circulating antibody and antigen kinetics were measured in experimentally infected pigs aged 1, 3 and 5 months at infection and linked with the parasitological outcome of infection. The simultaneous testing of circulating antigen and antibody resulted in some important observations: *(1)* circulating antigen is produced by cysts long before they are fully developed, *(2)* in some older animals with degenerated cysts at necropsy, the low levels of circulating antigen suggest that cysts were destroyed at very early stage during infection, *(3)* the rapid development of antibody response and subsequent abrogation of the infection in older animals is likely the result of a more efficient (innate) immune response compared to younger animals.

This age-dependent resistance has important repercussions for using vaccination as a strategy in control programs (Flisser et al., 2004; Gonzalez et al., 2005). Further research is required to determine the age at which piglets should be vaccinated. Up to now, most immunological research has been done on *T. crassiceps* in murine cysticercosis (Villa and Kuhn, 1996; Terrazas et al., 1998, 1999, 2005; Mooney et al., 2000; Toenjes and Kuhn, 2003), but more information is needed on both the mucosal and systemic immune response in pigs.

The use of both antigen and antibody detection allows a better understanding of

transmission dynamics and the modeling of the generated data. This can shed new light on incidence, frequency of exposure, establishment rate and immunity. These data should provide a solid basis for decision making on control measures to be taken.

In this respect, a recent community-based survey was performed by our group in Ecuador's southern Andean province of Loja, measuring antibody and antigen levels in approximately 800 inhabitants aged between 1 and 98 years (Praet et al., in preparation). The results showed that antibody and antigen profiles strongly depend on the age of the individuals. The number of antibody positives progressively increased till the age of 40 to become stable later. A simulation model taking into account a difference between primary and secondary humoral response induced by first or subsequent contact with the parasite could reproduce this progressive increase in younger individuals. They also noted a higher proportion of antigen-ELISA positives in individuals older than 60 years. In other words, viable cysts are more frequent in older individuals. This could be explained by the fact that a weaker immune system in the elderly facilitates the establishment and maintenance of viable cysts. At first sight, this is in disagreement with our observations in experimentally infected pigs. In field conditions however, viable cysts are often found in older pigs (Phiri et al., 2002). This could be explained by a less efficient immune response in these animals due to malnutrition or possibly the occurrence of subsequent infections. In the study in humans, no information was collected on individuals younger than 1 year old. Combining the results of the studies in pigs and humans, one could speculate that both very young and elderly individuals are more prone to infection with viable cysts due to the immature and senescent immune system, respectively. In any case, there seems to be a delicate balance between immune response and cyst development. Further studies are necessary to confirm this.

In the second part of the study, we observed a significant relationship between the number of cysts and the titer of circulating antigen in pigs. This is promising in view of the development of an assay to quantify the progress of an active *T. solium* infection. Such an assay would also be a useful tool in epidemiological studies to assess the infection burden and the risk of transmission of the disease. Handali et al. (2004) showed that antibody titers measured by the FAST-ELISA with sTs18VAR-1 seemed to correlate well with viability of the cysts. The use of specific antibody-detection assays combined with circulating antigen detection could improve our understanding of this relationship.

6.3 Biomarkers for active and inactive cysticercosis

In chapter 3, we reported different antibody and antigen kinetics in experimentally infected pigs linked to the parasitological outcome of infection. Several studies correlate

antibody titers with infection by viable cysts (de Aluja et al., 1996; Flisser et al., 2002; Handali et al., 2004). We, however, observed higher antibody titers in animals with a higher proportion of degenerated cysts. To elucidate these contradictory results, we further wanted to investigate these different phenotypic profiles. We used serum proteomic profiling by SELDI-TOF MS to identify biomarkers specific for these different disease states (active cysticercosis with viable cysts and inactive cysticercosis with degenerated cysts). Thirty biomarkers were identified in serum samples from experimentally infected pigs but none of these biomarkers could be validated by analyzing serum samples from naturally infected pigs.

Serum proteomic profiling is an emerging paradigm to generate a set of biomarkers diagnostic for a certain disease state. Petricoin et al. (2002a) published their seminal study using SELDI-TOF MS to profile the serum proteome of ovarian cancer patients and reported a set of 5 discriminatory biomarkers yielding a sensitivity of 100% and specificity of 95%. Since this first report, several published studies have applied similar technology to various cancers (Li et al., 2002; Adam et al., 2002; Qu et al., 2002; Petricoin et al., 2002b; Liu et al., 2005; Engwegen et al., 2006) and parasitic diseases (Papadopoulos et al., 2004; Rioux et al., 2007). The main advantage of SELDI-TOF technology is the capability of high throughput screening of complex samples in search for potential biomarkers. The main issue when setting up a SELDI-TOF experiment will be, however, to meet the necessary requirements with regard to the number of samples, choice of control samples, processing and handling of samples.

There has been more and more published criticism of SELDI-TOF MS methodology raising questions about sensitivity and especially reproducibility (Diamandis, 2003, 2004a,b; Baggerly et al., 2004b). Also, in some SELDI-TOF experiments, the presence of systematic bias distorted the results: discriminatory peaks were shown not to reflect the different disease stages but rather the run dates of the samples or the sample collection protocols (Sorace and Zhan, 2003; Baggerly et al., 2004a; Hu et al., 2005). Other authors also failed to confirm previous results with SELDI-TOF MS for the diagnosis of prostate cancer (Skytt et al., 2007). They state that several rather small case control studies have reported excellent validity for prostate cancer detection, but no studies have replicated these initial findings. Some authors claim that the Bio-Rad SELDI PBS II platform per se is not able to deliver the kind of reproducibility required for clinical testing and that the software possesses algorithmic weaknesses that can reduce the effective sensitivity of the instrument (Petricoin and Liotta, 2003; Coombes et al., 2005a). Researchers have been developing other methods for processing mass spectra (Coombes et al., 2003; Qu et al., 2003; Yasui et al., 2003a,b; Malyarenko et al., 2005; Morris et al., 2005; Coombes et al., 2005b), but these techniques are not so user-friendly and the results are often difficult to

interpret.

In chapter 4, five biomarkers in the experimental sample group were identified as clusterin, vitronectin, Apo A-I, LCAT and haptoglobin, respectively. Diamandis (2004b) listed the positively identified candidate biomarkers by mass spectrometry for various forms of cancer. Most identified proteins represent acute-phase reactants produced by the liver in response to inflammation. These proteins are present in extremely high abundance in serum and concentration differences between the disease states are extremely small and of doubtful clinical value. Deep proteome profiling (after depletion of high-abundance serum proteins) could proof to be a more valuable approach for detecting potential serum biomarkers (Au et al., 2007; Desrosiers et al., 2007).

One should bear in mind that a statistically significant biomarker is not equal to a clinically significant biomarker, i.e. suitable for use in a (SELDI-TOF derived) diagnostic assay. Considering the cost and reproducibility problems of this proteomic technology, a significant improvement in relation to established biomarkers is needed to justify the industrial development of this new generation of markers (Morra et al., 2007).

Proteomic profiling by SELDI-TOF MS could be a valuable starting point for immunological research and to investigate host-parasite interactions. To be applicable in a diagnostic setting, however, biomarkers need to be meticulously validated in different populations. In chapter 4 we observed a considerable discordance between experimental and field settings. The collection of sufficient samples that comply to the strict requirements for MS-based technology, could prove to be a serious bottleneck in biomarker discovery studies.

6.4 Specific diagnosis of porcine cysticercosis

Since serum proteomic profiling with SELDI-TOF MS did not result in cysticercosis-specific biomarkers that could be used for devising a new diagnostic assay, focus was shifted to improving the specificity of the current monoclonal antibody-based Ag-ELISA (Brandt et al., 1992; Dorny et al., 2002). Due to the genus-specificity, cross reactions with other taeniid spp. hamper the use of this test in pigs (Dorny et al., 2003).

In chapter 5, a panel of eight *T. solium* specific Nanobodies were identified, characterized and tested for specific diagnosis of porcine cysticercosis. One Nb in particular (Nbsol52), showed high potential for use in inhibition-ELISA.

A diagnostic assay that can specifically detect viable *T. solium* cysticerci in pigs would at present be a valuable tool for descriptive epidemiological research to identify regions of active transmission of the disease. Tongue inspection could also be used as a rapid assessment tool for this purpose, however, the sensitivity is by far inferior to serological

screening (Dorny et al., 2004b). Researchers are more and more shifting from descriptive research to implementation and evaluation of different intervention trials such as human mass chemotherapy, pig vaccination, pig cysticercosis treatment, infrastructure development, as well as health education campaigns (Sarti and Rajshekhar, 2003; Gonzalez et al., 2005; Ngowi et al., 2008). In this context, a specific antigen detection assay in pigs can contribute to the monitoring and evaluation of these different control strategies.

In the long run, a pen side test that is able to provide a relatively fast result for each animal individually, would be preferable. A test format such as lateral flow immunochromatographic assay would be most suited for this application. This way, pigs harboring viable cysts can be identified immediately and given anthelmintic treatment on the spot. Treating infected pigs with a single-dose of oxfendazole (30 mg/kg) has been proven to effectively kill cysts and clear the meat 12 weeks after therapy (Gonzalez et al., 1996, 1998); the drug has, however, little or no effect on brain cysts (Sikasunge et al., 2008b). Furthermore, treated animals seem to be protected from new infections for at least three months (Gonzalez et al., 2001). Given the relatively short life span of pigs, this could prove to be a sustainable control strategy in combination with other intervention measures. On the same account, the development of a bed side test for the diagnosis of human cysticercosis would be of great value for clinical case management in a hospital setting and for field based control programs.

A diagnostic test for *T. solium* cysticercosis would have to be suitable for use in endemic areas, these are often rural and remote areas in tropical conditions. This implies that the test should be cheap, easy to use and preferably cold chain independent. Nanobodies are most suitable for this purpose. For one they are very stable: three Nbs retained 100% activity after 1 week incubation at 37°C (Arbabi-Ghahroudi et al., 1997), other Nbs were still able to specifically bind antigen at temperatures as high as 90°C (van der Linden et al., 1999). Therefore it seems that Nbs will have a very good "shelf-life" and there will be no need for a cold chain. Nanobodies are efficiently expressed as soluble recombinant proteins. In high cell-density fermentation, a production level of over 1 g/l should be feasible (Muyldermans, 2001). As such, production costs will be much lower than for conventional monoclonal antibodies. Furthermore, Nbs can be tailored in different constructs and as such can be adapted to the required test format. Tailoring of the *T. solium* specific Nbs obtained in chapter 5 will be done to improve the assay. Generating bivalent constructs or Nbs linked to the Fc domain of IgG improves the immobilization of Nbs on hydrophobic ELISA plates (Conrath et al., 2001a; Saerens et al., 2005a). Other possibilities are the generation of pentavalent constructs or CDR grafting in an universal Nanobody scaffold (Zhang et al., 2004a; Saerens et al., 2005b).

In chapter 5, we have used crude *T. solium* cyst fluid to construct the dromedary

immune library and screen for *T. solium* specific Nbs. Another approach could be using either crude cysticercal ES antigens or purified recombinant or synthetic peptides for library construction and/or screening. We have already panned the dromedary 2 library using crude *T. solium* ES antigens but this did not result in selection of additional Nanobodies. Characterization and comparison of cysticercal ES proteins of different taeniids might lead to the identification of novel target antigens for the development of a specific antigen detection ELISA. To date, only a few studies have reported the characterization of ES antigens (Ko and Ng, 1998; Espindola et al., 2002; Baig et al., 2005), but to our knowledge no extensive proteomic studies have been carried out. The two currently used antigen detection ELISAs, the HP10 (Harrison et al., 1989) and the B158/B60 ELISAs (Draelants et al., 1995; Van Kerckhoven et al., 1998), detect a carbohydrate epitope and a partly protein/partly carbohydrate epitope, respectively, present on the surface and in the secretions of *T. saginata* cysticerci, but the actual protein has not been identified yet.

The *T. solium* Nbs recognized one or more synthetic peptides of the 8 kDa protein family (Hancock et al., 2003). These are well known proteins present in the diagnostic bands of the EITB Western blot for cysticercosis diagnosis (Tsang et al., 1989). As such, using these synthetic peptides for library construction and/or panning could result in the identification of additional Nanobodies.

6.5 Conclusions and recommendations

This study demonstrates the feasibility of *(1)* identifying serological markers that can differentiate between active and inactive cysticercosis and *(2)* developing a specific test for the diagnosis of *T. solium* cysticercosis. The first priority – well within reach now we have *T. solium* specific Nanobodies at our disposal – is the further development of a specific antigen-ELISA and adaptation to a pen side (or bed side) test.

The availability of an improved antigen detection assay provides clear prospects for epidemiological and immunological studies, follow-up of intervention studies and clinical monitoring in humans. In epidemiological studies, it can provide accurate information on sites of active transmission and risk factor assessment. Follow up of cyst longevity is possible in immunological studies and monitoring of control programs. In clinical cysticercosis cases in humans, it is a valuable decision making tool to start anthelmintic treatment and for follow-up of treatment efficacy.

However, more fundamental research is warranted to elucidate host-parasite interactions and identify good target antigens for diagnosis. Work is in progress to sequence the complete *T. solium* genome. Up to now, the complete mitochondrial genome has been sequenced (Nakao et al., 2003), 24,790 expressed sequence tags (EST), 177 mRNA

and 162 genomic nucleotide sequences are identified [1]. Once the full genome has been sequenced, this will provide valuable baseline data for conducting further studies. A proteomics study comparing ES products of *T. solium*, *T. hydatigena*, *T. saginata* could lead to the identification of specific diagnostic antigens.

There is also a need to further elucidate the immune response in *Taenia* infections, not only in the murine model of cysticercosis, but also in porcine and human cysticercosis. The experimental infection model developed by the Cysticercosis Working Group in Peru provides interesting possibilities for this purpose. Studying the immune response in pigs could possibly serve as a valuable model for human cysticercosis. Indeed, several studies indicate that the pig is suitable as animal model for immunology, despite the anatomical particularities of the porcine immune system (Duchene et al., 2008; Herbert et al., 2008; Rothkotter, 2008).

[1]Genome project data available from: `http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj`

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Summary

Taenia solium taeniosis/cysticercosis is an important (re)emerging zoonotic helminth infection in developing countries. The general introduction of **chapter 1** outlines briefly the life cycle of the parasite, impact of cysticercosis and its control. The remaining part of this chapter presents an update of the data available in the literature regarding diagnosis of cysticercosis and taeniosis. Focus is put on immunodiagnostic tools. Measuring antibody or antigen responses provides different information on the course of infection. In general, antibody detection assays only reflect exposure to the parasite whereas antigen detection assays indicate the presence of living parasites. The first objective of this thesis was to link serological data (antibody and antigen responses) to parasitological findings in experimentally infected pigs in order to study the host-parasite relationship (**objective 1**).

The current monoclonal antibody based antigen-ELISA detects circulating parasite antigens, which indicate the presence of living cysts. However, no information is available on whether this test has potential for quantitating the number of viable cysts present or whether it is merely a qualitative measurement. Hence, the second objective was to study the relationship between number of cysts and titer of circulating antigen in pigs in order to develop a quantitative diagnostic test (**objective 2**).

Diagnostic tests for porcine cysticercosis were recently validated based on a Bayesian approach. In a recent report by FAO, WHO and OIE, the development of more sensitive and specific diagnostic tests for use in pigs was stated as one of the research priorities for taeniosis/cysticercosis. For one, there is no test available that can distinguish between infections with viable cysts (active cysticercosis) and infections with degenerated cysts (inactive cysticercosis). Therefore, the third objective of this thesis was to identify novel biomarkers in serum of infected pigs that can distinguish between active and inactive cysticercosis, using surface enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF MS) (**objective 3**).

Another gap in the diagnosis of porcine cysticercosis is the fact that the use of the antigen-ELISA in pigs is hampered by cross reactions with other taeniid species. Hence, the last objective was to produce Nanobodies (camelid derived single domain antibody fragments) specific for *T. solium* that can be used for diagnosis of porcine cysticercosis (**objective 4**).

Chapter 2 gives a short overview of the two techniques used in the experimental work of this thesis (objectives 3 and 4, respectively). In the first part the general methodology of SELDI-TOF MS is presented along with examples of applications for disease biomarker discovery. Furthermore, critical reviews raising concerns regarding the reliability of the SELDI-TOF MS approach for biomarker discovery are discussed.

The second part of this chapter discusses the properties of Nanobodies compared to conventional antibody fragments, and the production and selection of antigen-specific Nanobodies. Finally, a short overview of biotechnological and therapeutic applications is given.

In **chapter 3**, antibody and antigen levels were determined in experimentally infected pigs and linked to the parasitological outcome of infection. In the experimental model, 3 groups of pigs aged respectively 1, 3 and 5 months at infection were infected with a full proglottid. The 1-month old animals developed mainly viable cysts, the 5-month old animals mainly degenerated cysts, whereas the 3-month old animals showed an intermediate profile. All animals harboring viable cysts at necropsy had high antigen levels, whereas animals harboring no cysts or only degenerated cysts, had low or nil antigen levels. Antigen levels increased more rapidly in 1-month old animals harboring viable cysts than in 5-month old animals harboring viable cysts. Also, the antigen levels reached were higher in the animals infected at 1 month of age compared with animals infected at 3 and 5 months of age. Serum antibody levels appeared to follow a reverse kinetics compared with circulating antigen. Antibody titers were highest in animals infected at 5 months of age. In the animals infected at 1 and 3 months of age, antibody levels increased slowly and remained substantially lower compared with the older age group. These results indicate the presence of an age-dependent immune response: an efficient antibody response in older animals prevents the establishment of fully developed viable cysts whereas in younger animals the immune system cannot adequately react to the infection resulting in the establishment of viable cysts (accompanied by high circulating antigen levels).

In the second part of this chapter, a quantitative ELISA for measuring the concentration of circulating antigen was constructed using a reference standard curve of serial dilutions of ES products of *T. saginata*. A significant correlation between the number of viable cysts and the concentration of circulating antigen was found. This result is promising in view of the development of an assay to quantify the progress of an active *T. solium* infection.

In **chapter 4**, serum samples from the same experimentally infected pigs were analyzed by SELDI-TOF MS to identify biomarkers that can distinguish between infections with viable cysts and infections with degenerated cysts. Thirty discriminating biomarkers were found: 13 specific for the viable phenotype, 9 specific for the degenerated phenotype and 8 specific for the infected phenotype (either viable or degenerated cysts). Five biomarkers were identified as clusterin, lecithin-cholesterol acyltransferase (LCAT), vitronectin,

haptoglobin and apolipoprotein A-I. The possible function of the identified biomarkers is discussed with regard to host-parasite immune interactions.

An attempt was made to validate the biomarkers by analyzing serum samples from naturally infected pigs. Only 3 of the biomarkers were also significant in the field samples; however, the peak profiles were not consistent among the two sample sets. Thus, it was not possible to validate the biomarkers. Possible explanations for this event are presented in the discussion.

In **chapter 5**, Nanobodies were cloned following immunization of 2 dromedaries with *T. solium* cyst fluid and 8 *T. solium* specific Nanobodies were selected after phage display and biopanning. Their binding characteristics and potential for the diagnosis of porcine cysticercosis were investigated.

The Nanobodies were highly specific for *T. solium* and no cross reactions with *T. hydatigena*, *T. saginata*, *T. crassiceps* and *T. spiralis* were observed. Affinity measurements by BIAcore binding studies resulted in equilibrium dissociation constants (K_D) in the picomolar range for Nbsol52, in the micromolar range for Nbsol68 and in the nanomolar range for the 6 remaining Nanobodies. Western blotting of *T. solium* cyst fluid and immunodetection with the Nanobodies was performed. After transfer to a PVDF membrane and N-terminal sequencing of the proteins in the bands corresponding to the bands recognized by the Nanobodies, the target antigen was identified as 14 kDa diagnostic glycoprotein. Immunodetection was also assessed of the synthetic Ts14, Ts18var, TsRS1 and TsRS2 peptides. The Nanobodies preferably reacted with Ts18var1, only 2 Nanobodies also reacted with Ts14 and TsRS2. The complementation epitope groups of Nanobodies were investigated in competition ELISA. This way, the Nanobodies could be categorized in four different epitope-binding groups. Furthermore, the prior binding of the group A Nanobodies to the antigen seemed to induce a conformational change within the antigen and/or fix a more appropriate epitope architecture for the group B Nanobodies. Antigen capturing was assessed by testing the Nanobodies in various combinations in sandwich ELISA. The Nanobodies were able to distinguish between *T. solium* and *T. hydatigena* cyst fluid, however, there was no clear distinction between serum samples from *T. solium* infected pigs, *T. hydatigena* infected pigs and negative pigs. Next, the Nanobodies were tested in inhibition ELISA for the detection of circulating antigen. One Nanobody (Nbsol52) was able to differentiate between the different serum samples.

These results indicate the high potential of the selected Nanobodies for species-specific diagnosis of *T. solium* cysticercosis, after further assay optimization and validation.

Finally in **chapter 6** the obtained results are put into a broader perspective, and

the advantages and disadvantages of the applied methodology are discussed. The general conclusions and recommendations for future work are presented.

The results of the present thesis demonstrate the feasibility to identify serological markers that can lead to improved diagnosis of porcine cysticercosis. To further elucidate host-parasite interactions and identify alternative target antigens for diagnosis, more fundamental research should be conducted. Therefore, the immune response in *Taenia* infections should be investigated, as well as comparative mapping of the parasite's proteome.

Samenvatting

Taenia solium taeniose/cysticercose is een belangrijke opkomende zoönotische worminfectie in ontwikkelingslanden. In de algemene inleiding van **hoofdstuk 1** worden kort de cyclus van de parasiet, impact en controle van cysticercose toegelicht. Het overig deel van dit hoofdstuk verschaft een update van de informatie over de diagnose van cysticercose en taeniose beschikbaar in de literatuur, met nadruk op de immunodiagnostische methoden. Detectie van antistoffen of antigeen levert verschillende informatie over het verloop van een infectie. In het algemeen weerspiegelt antistofdetectie enkel voorafgaand contact met de parasiet. Antigeendetectie tests daarentegen geven een indicatie over de aanwezigheid van levende parasieten. De eerste doelstelling van deze thesis betracht om serologische gegevens (antistof- en antigeenrespons) te linken aan parasitologische bevindingen in experimenteel geïnfecteerde varkens om op deze manier de interactie tussen gastheer en parasiet te bestuderen (**doelstelling 1**).

De huidige antigeen-ELISA gebaseerd op monoklonale antistoffen detecteert circulerende parasietantigenen die duiden op de aanwezigheid van levende cysten. Het is echter niet bekend of deze test ook potentieel biedt om het aantal levende cysten te kwantificeren of enkel een kwalitatief resultaat geeft. Daarom was de tweede doelstelling het bestuderen van de relatie tussen het aantal cysten en de titer van circulerend antigeen in varkens met het oog op de ontwikkeling van een kwantitatieve diagnostische test (**doelstelling 2**).

Diagnostische tests voor porciene cysticercose zijn recentelijk gevalideerd door middel van Bayesiaanse analyse. In een recent rapport van de FAO, WGO en OIE werd de ontwikkeling van sensitievere en specifiekere diagnostische testen voor varkens naar voor geschoven als één van de onderzoeksprioriteiten voor taeniose/cysticercose. Er is om te beginnen geen test beschikbaar die het onderscheid kan maken tussen infecties met levende cysten (actieve cysticercose) en infecties met gedegenereerde cysten (inactieve cysticercose). De derde doelstelling van deze thesis was dan ook om in serum van geïnfecteerde varkens nieuwe biomerkers te identificeren die een onderscheid kunnen maken tussen actieve en inactieve cysticercose. Hiervoor werd gebruikt gemaakt van "surface enhanced laser desorption and ionization time-of-flight" massa spectrometrie (SELDI-TOF MS) (**doelstelling 3**).

Een andere tekortkoming in de diagnose van porciene cysticercose is het feit dat het gebruik van de antigeen-ELISA in varkens gehinderd wordt door de aanwezigheid van kruisreacties met andere *Taenia* species. De laatste doelstelling was dan ook het produceren van "Nanobodies" (enkel domein kameelantilichaamfragmenten) specifiek voor *T. solium* die gebruikt kunnen worden voor diagnose van porciene cysticercose (**doelstelling 4**).

Hoofdstuk 2 geeft een kort overzicht van de twee technieken gebruikt in het experimenteel werk van deze thesis (doelstellingen 3 en 4, respectievelijk). In het eerste gedeelte

wordt de algemene methodologie van SELDI-TOF MS aangehaald, alsook voorbeelden van toepassingen op het vlak van ziekte-specifieke biomerkeridentificatie. Bovendien worden enkele kritische besprekingen onder de loupe genomen die hun bezorgdheid uiten over de betrouwbaarheid van de SELDI-TOF MS technologie voor de ontdekking van biomerkers.

Het tweede gedeelte van dit hoofdstuk handelt over de eigenschappen van Nanobodies in vergelijking met conventionele antilichaamfragmenten en de productie en selectie van antigeenspecifieke Nanobodies. Tenslotte volgt een kort overzicht van verschillende biotechnologische en therapeutische toepassingen.

In **hoofdstuk 3** werden in experimenteel geïnfecteerde varkens de antistof- en antigeengehaltes bepaald en gelinked aan de parasitologische uitkomst van de infectie. In het experimenteel model werden 3 groepen varkens van respectievelijk 1, 3 en 5 maanden oud geïnfecteerd met een volledige proglottide. De 1 maand oude dieren ontwikkelden voornamelijk levende cysten, de 5 maand oude dieren vooral gedegenereerde cysten, terwijl de 3 maand oude dieren een intermediair profiel vertoonden. Alle dieren die op het moment van de lijkschouwing levende cysten hadden, vertoonden een hoge antigeenrespons. De dieren die geen of enkel gedegenereerde cysten hadden, vertoonden geen of slechts een lage antigeenrespons. De antigeengehaltes namen sneller toe in de 1 maand oude dieren met levende cysten dan in de 5 maand oude dieren met levende cysten. De antigeengehaltes waren ook hoger in de dieren geïnfecteerd op de leeftijd van 1 maand vergeleken met de dieren geïnfecteerd op 3 en 5 maanden leeftijd. Het gehalte aan serumantistoffen leek een omgekeerde kinetiek te vertonen vergeleken met circulerend antigeen. De antistoftiters waren het hoogst in de 5 maand oude dieren. In de 1 en 3 maand oude dieren namen de antistofgehaltes slechts traag toe en bleven aanzienlijk lager vergeleken met de oudere dieren. Deze resultaten wijzen op een leeftijdsafhankelijke immuunrespons: een efficiënte antistofrespons in de oudere dieren verhindert de vorming van volledig ontwikkelde levende cysten. In jongere dieren daarentegen kan het immuunsysteem niet adequaat reageren op de infectie met als gevolg de ontwikkeling van levende cysten (vergezeld van hoge gehaltes circulerend antigeen).

In het tweede deel van dit hoofdstuk werd een kwantitatieve ELISA ontwikkeld voor de bepaling van de concentraties circulerend antigeen. De test werd ontwikkeld aan de hand van een standaardcurve van een seriële verdunning van *T. saginata* excretie secretie produkten. Er werd een significante correlatie gevonden tussen het aantal levende cysten en de concentratie van circulerend antigeen. Dit resultaat is veelbelovend met het oog op de ontwikkeling van een kwantitatieve test voor de opvolging van een actieve *T. solium* infectie.

In **hoofdstuk 4** werden dezelfde serumstalen van de experimenteel geïnfecteerde varkens geanalyseerd d.m.v. SELDI-TOF MS om biomerkers te ontdekken die het onderscheid kunnen maken tussen infecties met levende cysten en infecties met gedegenereerde cysten. Er werden zo 30 biomerkers gevonden: 13 specifiek voor het fenotype "levend", 9 specifiek voor het fenotype "gedegenereerd" en 8 specifiek voor het fenotype "geïnfecteerd" (geïnfecteerd met levende of gedegenereerde cysten). Vijf biomerkers werden geïdentificeerd als clusterine, lecithine-cholesterol acyltransferase, vitronectine, haptoglobine en apolipoproteïne A-I. De mogelijke functie van de geïdentificeerde biomerkers wordt besproken met betrekking tot gastheer-parasiet immuuninteracties.

Er werd gepoogd om de biomerkers te valideren door analyse van serumstalen van natuurlijk geïnfecteerde dieren. Slechts 3 biomerkers waren ook significant in deze groep serumstalen. De piekprofielen waren echter niet consistent in de twee groepen. Validatie van de biomerkers was aldus niet mogelijk. Mogelijke verklaringen hiervoor worden geopperd in de discussie.

In **hoofdstuk 5** werden Nanobodies gekloond na immunisatie van 2 dromedarissen met *T. solium* cystevloeistof. Na faagdisplay en biopanning werden er acht *T. solium* specifieke Nanobodies geselecteerd. Hun bindingseigenschappen en potentieel voor diagnose van porciene cysticercose werden onderzocht.

De Nanobodies waren zeer specifiek voor *T. solium* en er werden geen kruisreacties waargenomen met *T. hydatigena*, *T. saginata*, *T. crassiceps* en *T. spiralis*. Affiniteitsmetingen d.m.v. BIAcore bindingstudies resulteerden in evenwichtsdissociatieconstanten (K_D) in de grootte-orde van picomolair voor Nbsol52, micromolair voor Nbsol68 en nanomolair voor de overige zes Nanobodies. Er werd een Western blot uitgevoerd met *T. solium* cystevloeistof gevolgd door immunodetectie met de Nanobodies. Na transfer naar een PVDF membraan en N-terminale sequenering van de proteïnen in de banden corresponderend met de banden herkend door de Nanobodies, werd het herkende antigeen geïdentificeerd als 14 kDa diagnostisch glycoproteïne. Er werd tevens immunodetectie van de synthetische peptiden Ts14, Ts18var1, TsRS1 en TsRS2 uitgevoerd. De Nanobodies reageerden bij voorkeur met Ts18var1, enkel 2 Nanobodies herkenden ook Ts14 en TsRS2. De complementaire epitoopgroepen van de Nanobodies werden onderzocht in een competitie ELISA. Op deze manier konden de Nanobodies onderverdeeld worden in 4 verschillende epitoopgroepen. Bovendien induceerde de voorafgaande binding aan het antigen van de groep A Nanobodies schijnbaar een conformatieverandering in het antigen en/of veroorzaakte dit een betere epitooparchitectuur voor de groep B Nanobodies. De mogelijkheid om met de Nanobodies antigeen te capteren werd onderzocht door deze in verschillende combinaties te testen in een sandwich ELISA. De Nanobodies waren in

staat om een onderscheid te maken tussen cystevloeistof van *T. solium* en *T. hydatigena*. Het was echter niet mogelijk om op deze manier een duidelijk onderscheid te bekomen tussen serumstalen van varkens geïnfecteerd met *T. solium* of *T. hydatigena* en negatieve varkens. De Nanobodies werden vervolgens getest in een inhibitie ELISA voor antigeendetectie. Eén Nanobody (Nbsol52) was in staat om te differentiëren tussen de verschillende serumstalen.

Deze resultaten wijzen op het grote potentieel van de geselecteerde Nanobodies voor de specifieke diagnose van *T. solium* cysticercose, na verdere aanpassing en validatie van de test.

Tot slot worden in **hoofdstuk 6** de behaalde resultaten in een breder perspectief geplaatst. De voor- en nadelen van de toegepaste methodologie, de algemene conclusies en de aanbevelingen worden besproken.

De resultaten van deze thesis tonen aan dat het mogelijk is om serologische merkers te identificeren die gebruikt kunnen worden voor een verbeterde diagnose van porciene cysticercose. Om de gastheer-parasiet interacties verder uit te diepen en nieuwe diagnostische antigenen te identificeren, is meer fundamenteel onderzoek nodig. De immuunrespons tegen *Taenia* infecties dient daarom verder bestudeerd te worden, evenals vergelijkend onderzoek van het proteoom van de parasiet.