Development and validation of immunochromatographic test (ICT) for diagnosis of animal trypanosomosis

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家畜トリパノソーマ病診断用イムノクロマトグラフィー法の開発と評価に関する研究

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Contents

Abbreviations

B *B. bigemina*: *Babesia bigemina*

B. bovis: *Babesia bovis*

BLAST: basic local alignment search tool

BSA: bovine serum albumin

C CATT: card agglutination test for trypanosomosis

D DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphate

E *E. coli*: *Escherichia coli*

ELISA: enzyme-linked immunosorbent assay

F FAO: Food and Agriculture Organization

I ICT: immunochromatographic test

IFAT: indirect fluorescent antibody test

IgG: immunoglobulin G

IgM: immunoglobulin M

IPTG: isopropyl β-D-1-thiogalactopyranoside

L LAMP: loop-mediated isothermal amplification

O OD: optical density

OIE: Office International Des Epizooties

P PBS: phosphate buffered saline

PBS-T: phosphate buffered saline containing 0.05% Tween 20

PCR: polymerase chain reaction

S SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOB: super optimal broth

T *T. b. brucei*: *Trypanosoma brucei brucei*

T. cruzi: *Trypanosoma cruzi*

T. congolense: *Trypanosoma congolense*

T. equiperdum: *Trypanosoma equiperdum*

T. evansi: *Trypanosoma evansi*

T. orientalis: *Theileria orientalis*

T. simiae: *Trypanosoma simiae*

T. suis: *Trypanosoma suis*

T. theileri: *Trypanosoma theileri*

T. vivax: *Trypanosoma vivax*

Unit abbreviations

B bp: base pair

C ^{o}C: degree Celsius

H h: hour

K kD: kilo dalton

M µl: microliter

mg: milligram

min: minute

ml: milliliter

mM: millimolar

N ng: nanogram

nm: nanometer

P pmol: picomol

S sec: second

General introduction

1. Taxonomy and pathogenicity of trypanosome

Trypanosome is a protozoan parasite living in blood circulation of the host and cause disease to both human and animals. According to the classification of Levine *et al.* (1980), it belongs to the subkingdom PROTOZOA, phylum SARCOMATIGOPHORA, class ZOOMASTIGOPHOREA, order KINETOPLASTIDA, family TRYPANOSOMATIDAE, and genus *TRYPANOSOMA*. Species of trypanosome infecting mammals fall into two distinct groups: (A) the Stercoraria (subgenera *Schizotrypanum*, *Megatrypanum* and *Herpetosoma*), in which animal infective metacyclic form (MCF) trypanosomes are typically produced in the hindgut and are then passed on by contaminative transmission from the posterior; and (B) the Salivaria (subgenera *Duttonella*, *Nannomonas* and *Trypanozoon*) in which transmission occurs by the anterior station and is inoculative.

The clinical and pathological responses to trypanosome infection in domestic animals have been well studied (Maudlin *et al.*, 2004). Many of the clinical and pathological manifestations of the trypanosomosis are common to domestic animals, irrespective of the species of trypanosome involved. None the less, the range and severity of the pathological effects are influenced by a variety of factors. Distinct pathological changes may be caused by the different livestock-infective trypanosome species. The range and severity of the pathological effects of the parasites are influenced by a variety of factors. Pathology in tissue is associated with the relative ability of the parasites to invade extravascular spaces and organs. For example, whereas *T. congolense* remains confined to the vascular system,

trypanosome species of the *Trypanozoon* group (*T. b. brucei*, *T. evansi* and *T. equiperdum*) and *T. vivax* are distributed in both the circulation and in the tissue. Furthermore, there is remarkable intra-species variation in the pathogenicity of different parasite stocks, especially stocks isolated from distinct geographical regions. Some East African isolates of *T. vivax* may cause an acute hemorrhagic disease in cattle, in contrast to a milder non-hemorrhagic disease that result from infection with most West African *T. vivax* isolates. A number of host factors also contribute to determining the severity of disease. For example, the wildlife of Africa is generally more resistant than the domestic ruminants and often serves as a reservoir for human- and livestock-infective trypanosomes. The physiological statuses of the host, as well as nutritional and environmental factors, also play important roles in modulating the severity of trypanosomosis.

There are at least three identified animal syndromes caused by pathogenic trypanosomes names nagana (animal African trypanosomosis), surra and dourine. Nagana is a disease caused by *T. congolense*, *T. vivax*, *T. simiae*, *T. b. brucei* or *T. suis* to ruminants, camels, equines, swine and carnivores in Africa. In South America, trypanosomosis due to infection with *T. vivax* is predominantly a disease of cattle but sheep, goats, horses and water buffaloes can also be infected. In nagana, incubation period is usually 1-3 weeks, depending on the virulence of the infecting trypanosome, the infective dose and the immune status of the host. The early acute phase of the disease is characterized by the continuous presence of trypanosomes in the blood (10^3-10^8/ml). Remittent fever is parallel with parasitemia waves. With the onset of parasitemia, anemia develops. Lymph nodes and spleen are enlarged. Weakness, lethargy and loss of condition are patent; abortion and

reduced milk production are common. Death of the infected animal may occur in the first few weeks or months as a result of this acute disease. In some cases, the clinical condition stabilizes after 6-8 weeks and a slow recovery process begins. More frequently the animals enter into the chronic phage. Stunting, wasting and infertility are characteristics of cattle suffering from chronic trypanosomosis syndrome. The chronic phase may last for months or years and is most often terminated by death.

The second type of animal trypanosomosis is surra. Surra is caused by *T. evansi* which is widely distributed in Asia, South America and Africa. The disease can be acute in young animals and pregnant females, which die within few weeks, but the usual form in endemic areas is chronic one that lasts for years and leads to cachexia and death. Some individuals that survive chronic infection for years may eventually self-cure. Clinical signs of surra are intermittent fever, anemia, emaciation, edema, conjunctivitis and lacrymation, enlargement of the lymph nodes and spleen, impaired motor function and abortions. In camels, *T. evansi* infection is sometimes complicated by pulmonary infection. Cattle are often asymptomatic carriers in South America. Water buffaloes develop acute or sub-acute infections that lead to death within a few weeks in 5-15% of the cases.

Dourine is the last type of animal trypanosomosis. It is a venereally-transmitted disease caused by *T. equiperdum* that executively affects equines and has a wide geographically distribution: North Africa, the Middle East, Eastern Europe, South America, central and south east Asia. Horses are highly susceptible to *T. equiperdum* and usually die at the end of a chronic disease that may last for 1-2 years. Occasionally, acute forms that lead to death in 2-3 months. Donkeys and mules develop a mild syndrome or remain

asymptomatic. The incubation period in horses ranges from 1 week up to 6 months. There are typically three clinical phases. The early signs consist of edema of the genital area and genital organs, which may spread to the ventral abdominal wall. The second phase begins 30-40 days after infection and is characterized by urticarial plaques on the skin and enlargement of the lymph nodes. This coincides with the appearance of general signs, namely fever, anemia, weight loss and possibly abortion. Later in infection, the neurological signs worsen, leading to a paralytic syndrome. Animals that die from dourine are usually severely emaciated and anemic.

2. Disease impact

Animal trypanosomosis currently distributes worldwide, suggesting its huge global impact. Recently, the amount of information on the impact of the disease has increased enormously. There are the results of numerous studies trying to quantify the impact of the disease on cattle production parameters in village and pastoral settings, and a number of carefully structured studies have examined the effects on agriculture development. Basically, the impact of animal trypanosomosis in livestock can be divided into two aspects: direct and indirect impacts.

Direct impacts of the disease in livestock are measured by mortality, calving rates and other measurements such as milk and meat production, weight loss and infertility. According to Kristjason *et al.* (1999), the disease costs livestock producers and customers an estimated $1,340 million annually in Africa. Swallow (2000) and Shaw (2009) reported calf mortality rates were 6-10% higher in infected animal populations, but the difference

could be up to 20%. Mortality rates in older animals were 2-8% higher and annual calving rates were typically 7% lower in infected populations. The effect on milk yields ranged from a reduction of 2-26%. In the Philippines, impact of surra in water buffaloes was computed by Dargantes *et al.* (2009). The authors reported that all the surveyed areas were dominated by female buffaloes (69%). In the main reproductive years (4-10) female removal/mortality was <1% and 10% for low and high risk areas, respectively. The annual calving rate of those areas was 15% and 47%, respectively. As a result, in the high risk areas, surra has imposed significant financial losses.

Indirect effects of the disease in livestock include use of animals in the agricultural sector choice of breed, herd size and structure and migration. Despite the presence of trypanosomosis, work animals have become widely used in sub-Saharan Africa, notably in West Africa, Ethiopia and southern Africa. Among the villages in Ethiopia, the proportion of work oxen among cattle in the endemic area was far lower than that in the non-endemic area (just under 30% as against nearly 60%) (Jemal *et al.*, 1995). In the unprotected village, a third of adult males was replaced annually, reflecting the far higher mortality. In the endemic areas, different categories of cattle show different susceptibility to trypanosomes. The indigenous or local breeds are usually trypanotolerant, however, less productive than the trypanosusceptible breeds. Either breed would be costly to the farmers. The impact of such a potential increase in cattle population would be also on crop production, via the use of animal fraction and manure. Therefore, Swallow (1997) suggested that a 1% increase in livestock numbers would increase agricultural output including that from livestock by 0.2%.

On the other hand, decrease of animal population due to trypanosomosis would harmful to the agriculture.

3. Diagnosis importance

Diagnosis plays an important role in control of animal trypanosomosis. At the rural level, confirmation of diagnosis is often retrospective; but blood samples taken at the time of treatment should be used to monitor the incidence of infection. On larger farms, routine sampling for diagnosis is needed to assist disease management and decisions. At district or provincial level, diagnosis is essential for disease surveillance and for monitoring the progress of control program. At each level, the appropriate diagnostic method must be used (FAO, http://www.fao.org/docrep/006/x0413e/x0413e02.htm). However, the diagnosis of trypanosomosis is notoriously difficult due to nonpathognomonic clinical signs, the intermittent and usually low parasitemia. According to OIE, diagnosis of the disease is based on the demonstration of the parasites in the blood, supplemented by hematological, biochemical and serological tests. Although the clinical signs are indicative, they are not sufficiently pathognomonic, and diagnosis must be confirmed by laboratory methods which are classified into two categories: identification of the pathogen and serological tests.

Identification of the pathogen includes parasitological tests, antigen detection enzyme-linked immunosorbent assay (ELISA) and molecular tests such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP). In acute phase or when parasitemia is high in blood circulation of the animals, the examination of wet blood films or stained blood smears and lymph node material should reveal trypanosomes.

However, in chronic cases, such as the carrier state, the examination of thick blood smears, as well as parasite concentration and experimental animal inoculation are recommended. The ELISA, PCR and LAMP have been introduced and evaluated on a relatively large scale, are considerably sensitive and specific. Sensitivity of PCR detection methods reach 10 parasites/ml of blood (Claes *et al.*, 2004; Desquesnes *et al.*, 2002; Desquesnes *et al.*, 2001), and that of loop-mediated isothermal amplification assays is 0.1 parasite/ml blood (Ngaira *et al.*, 2003; Reid and Copeman, 2002).

Serological tests determine the specific antibody to trypanosomes. In the laboratory, indirect fluorescent antibody test (IFAT) and antibody detection ELISA can be employed. For field use, card agglutination test for trypanosomosis (CATT) and latex test are available (Natulya, 1994; Hilali *et al.*, 2004; OIE, 2012). Although a range of serological tests has been applied to the diagnosis of animal trypanosomosis, a major problem arises from the lack of definiteness whereby even if anti-trypanosomal antibodies are detected; it cannot distinguish current from past infection. Moreover cross reactions may occur between some trypanosome species. The CATT adopted to assist diagnosis of West African human sleeping sickness relies on the presence of anti-trypanosomal antibody to agglutinate intact, stained and fixed trypanosomes. Whilst, with some modifications, this system has been found useful in the diagnosis of *T. evansi* infections in water buffalo, the non-specific agglutination caused by dust, and the evaporation in a hot, dry climate render this method unsuitable for field diagnosis of *T. evansi* infections in camels. The most successful serological adjuncts to diagnosis of trypanosomosis are the IFAT and ELISA. The ELISA compares favorably with the IFAT and has been found to give results which correlate with

the local history of treatments. However, each test system requires expensive equipment (fluorescent microscope or microplate reader) and at best the result of a single determination is only indicative of exposure to the pathogen. These tests do nevertheless have a role in surveillance of the disease, either before or after a control operation, when a population of animals may be screened.

4. Objectives of the present study

The present study aimed to develop an effective, accurate and rapid serological diagnostic test for detection of animal trypanosomosis. For that purpose, firstly I attempted to identify and produce a novel diagnostic antigen. Secondly, the antigen was evaluated for its sensitivity and specificity in ELISA, and then used to develop an immunochromatographic test. Finally, the newly developed test was assessed and validated using both experimental and field-derived samples.

Chapter 1

Recombinant TeGM6-4r as potential diagnostic antigen for animal trypanosomosis

1-1. Introduction

Diagnosis of animal trypanosomosis mainly relies on the identification of the parasites (OIE, 2012). However, sensitivity of parasitological diagnosis is limited due to low parasitemia in infected animals. Molecular diagnostic assays have been developed, but most of them have not been validated yet (Desquesnes and Davila, 2002; Thekisoe *et al*., 2005). Therefore, serological diagnosis is still retained a common method.

OIE recommends use of trypanosome lysate antigen in ELISA for serological diagnosis of surra (OIE, 2012). However, quality of the lysate antigen is difficult to standardize due to variable protocols and techniques used in different laboratories (Reid and Copeman, 2002; OIE, 2012). It also shows high cross-reactivity in the diagnostic tests, leading to the increase of false-positive results. On the other hand, recombinant antigen is significantly beneficial, more specific, time-efficient and no-animal requirement. Several recombinant antigens of *Trypanosoma* spp. were introduced. The recombinant variable surface glycoprotein RoTat1.2 was successfully expressed by insect cells, showing no differences to the native antigen in serological diagnostic tests of *T. evansi* infection in dromedary camels (Lejon *et al.*, 2005). Nevertheless, it failed to detect *T. evansi* type B which lacks or does not express RoTat1.2 (Ngaira *et al.*, 2005; Tran *et al.*, 2009). Another

promising recombinant protein is the invariant surface glycoprotein 75 (ISG75) presenting in approximately 5 x 10^4 molecules on trypanosome cell surface (Tran *et al.*, 2009). Furthermore, tandem repeat (TR) proteins have been recently proposed by Goto *et al.*, 2010 and Nguyen *et al.*, 2012.

Tandem Repeat (TR) proteins of trypanosomatid parasites are often targets of B cell responses (Goto *et al.*, 2007; Kemp *et al.*, 1987; Reeder and Brown, 1996). A number of *Leishmania* species and *T. cruzi* TR proteins have been characterized and demonstrated their immunological dominance (Goto *et al.*, 2010; Goto *et al.*, 2008; Goto *et al.*, 2007). For instance, rK39 of *Leishmania* and B13, CRA, TcD and TcE of *T. cruzi* have been employed effectively in serodiagnosis of the diseases (Hernandez *et al.*, 2010; Ozerdem *et al.*, 2009; Sundar *et al.*, 1998). Likewise, *T. brucei* is also rich in proteins with large TR domains, and recently identified TR proteins showed reactivity to sera of trypanosome-infected mice (Goto *et al.*, 2007; Goto *et al.*, 2011). Nguyen *et al.*, 2012 also reported that recombinant TbbGM6 from *T. brucei* was highly antigenic to water buffalo sera experimentally infected with *T. evansi*. GM6 is a cytoskeletal protein, located at the connection site between the microtubules of the membrane skeleton and the flagellum of the parasite. The protein is recognized in the early phase of infection when many parasites are destroyed by the host immune response, therefore, having good qualities of a diagnostic antigen (Muller *et al.*, 1992; Imboden *et al.*, 1995). Since GM6 is highly conserved among the trypanosomes, it may be used as a potential diagnostic marker for general animal trypanosomosis. In order to improve the antigenicity of the recombinant protein, I produced GM6 which consisted of four rather than two TR domains in the previous study (Thuy *et al.*,

2012). GM6 derived from *T. evansi* genomic data and consisted of four repeats was named TeGM6-4r. In the present study, antigenicity of newly expressed TeGM6-4r was evaluated using 15 serum samples from 3 water buffaloes (WB) experimentally infected with *T. evansi*.

1-2. Materials and methods

Cloning and sequencing of the TeGM6-4r. The gene encoded TeGM6-4r was amplified by conventional PCR using genomic DNA from *T. evansi* Tansui strain and the primer set 5'-GGA TCC ATG GAG CTT GCT AAA-3' and 5'-GAA TTC CTA ATG TGA ATG CTC-3' (underlined nucleotides are restriction sites of *Bam* HI and *Eco* RI). The PCR mixture (50 μl) contained 1.5 mM $MgCl_2$, 2 mM of each dNTP, 5 pmol of each primer and 1 unit of *Taq* DNA polymerase (Invitrogen Japan, Tokyo). Reactions were conducted for 30 cycles, at 94 ^{o}C for 30 sec (denaturation), 54^{o}C for 30 sec (annealing), and 7 ^{o}C for 1.5 min (extension). PCR products containing various numbers of repeat units were separated by agarose gel electrophoresis. DNA fragments consisting of 4-repeat domains were extracted from the gel (Japan-QIAGEN K.K., Tokyo) and ligated into the vector pCR2.1, then transformed into *E. coli* DH5α following the TA cloning procedure (Japan-QIAGEN K.K., Tokyo). After digestion with *Eco* RI restriction enzyme, direct sequencing of the inserts was carried out using the PCR primers, BigDye Terminator Ready Mix (Applied Biosystems, Life Technologies, Carlsbad, CA) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA). Nucleotide and amino acid sequences were identified and analyzed using Genetyx version 8.0 (Genetyx Co., Tokyo)

and BLAST (http://blast.ncbi.nlm.nih.gov/).

Expression of recombinant TeGM6-4r. The expression procedure of the recombinant tandem repeat protein has been previously described by Goto *et al.* (2006). In brief, the gene fragment encoding TeGM6-4r was inserted into pET-28a vector (EMD Biosciences, San Diego, CA), then transformed into *E. coli* BL 21. The transformed *E. coli* BL 21 was cultured in SOB medium (BD, Sparks, MD) to an OD_{600} of 0.4-0.6. The expression of the recombinant protein was initialized by adding 1 mM isopropyl-thio-β-galactosidase (IPTG) and maintained for 3 h. The recombinant TeGM6-4r was purified in soluble form using Ni-NTA agarose (Japan-QIAGEN K.K., Tokyo) in accordance with the manufacturer's instructions. The integrity and purity of the protein were evaluated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., Waltham, MA). Purified recombinant protein was stored until use at -80 ^{o}C.

Enzyme-linked immunosorbent assay (ELISA). Recombinant TR antigens were screened by ELISA of which procedures were recommended in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2008). Each microplate well (Maxisorp, Thermo Fisher Scientific, Nalgene-Nunc, Rochester, NY) was coated 200 ng of the TR antigen and incubated for 4 h at room temperature. Then antigen-coated wells were washed five times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), once with PBS and incubated with blocking solution (PBS-T containing 1% bovine

serum albumin (BSA)) for overnight at 4 ^{o}C. Serum samples diluted at 200 times with PBS-T containing 0.1% BSA were applied (100 µl) into each well and incubated at room temperature for 2 h. According to the manufacturer's instructions, horseradish peroxidase-conjugated protein G (Invitrogen Japan, Tokyo) and tetramethylbenzidine (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) were utilized for detection of antigen-antibody reaction. Finally, 50 µl of stop solution (1M phosphoric acid) was added and the absorbance was read at 450 nm by MTP-500 microplate reader (Corona Electric, Japan).

Water buffaloes and serum samples. Fifteen serum samples used in the study were from three experimental water buffaloes. The three water buffaloes detected negative through microhematocrit method and CATT/*T. evansi* (ITM, Belgium) were experimentally infected with *T. evansi* cpogz strain. Each water buffaloes was inoculated intravenously with 1 x 10^4 parasites. Sera were collected from the jugular vein by using heparinized syringe at five time points, namely 22 days before infection (pre), 8, 15, 22 and 48 days post-infection (DPI). Parasitemia was examined by microscopy (wet blood film method) each time point after serum collection. All water buffaloes were handled in accordance with Animal Welfare guidelines in Sun Yat-Sen (Zhongshan) University, China.

1-3. Results

Expression of recombinant TeGM6-4r. TR recombinant TeGM6-4r was successfully produced from genomic DNA of *T. evansi*. The gene encoding a single repeat GM6 domain was 204 bp; and that of four repeat domains was 816 bp in size respectively

(Fig. 1A). Recombinant TeGM6-4r consisted of four repeat units and six histidine tag derived from the cloning vector pET28. The protein was estimated to be 32.04 kD and pure as shown in SDS/PAGE image (Fig. 1B). The GM6 homologs among other trypanosomes and *Leishmania* were shown in Figure 2. *T. evansi* GM6 is highly identical to *T. brucei* and *T. gambiense* (100 to 97%. identity) and share from 64 to 52% similarity with *T. congolense*, *T. vivax* and *T. cruzi*. In comparison with *Leishmania*, the divergence level was 100%.

Immunoantigenicity of recombinant TeGM6-4r to water buffalo sera. Antigenicity of TeGM6-4r was compared with TbbGM6-2r in ELISA. The antigens were coated on the ELISA plate with the same amount (200 ng/well) and their immunoreactivity to *T. evansi* infected water buffalo sera was evaluated. The TeGM6-4r and TbbGM6-2r/ELISA demonstrated similar pattern. The cut-off values (Mean + 3SD) were calculated using sera at 22 pre, and 5 PI as negative controls. The values were 0.31 and 0.32 in TeGM6-4r and TbbGM6-2r/ELISA respectively. In both ELISAs, all the three water buffaloes became positive from day 15 PI; the OD values of WB sera increased and reached the highest on day 48 PI. In TeGM6-4r/ELISA, OD values of positive sera were significantly higher ($P>0.05$) (Fig. 3).

1-4. Discussion

The new recombinant protein TeGM6-4r was successfully produced by bacterial expression system, showing high similarity in amino acid sequence with GM6 from other

Trypanosoma species. The protein is most identical to TbbGM6 (100%) and less to TcrGM6 (52%). This suggests that utilization of only single recombinant antigen TeGM6-4r may be able to detect several species of trypanosomes.

TbbGM6-2r and TeGM6-4r are identical in amino acid sequence as described previously, but different only in the number of repetitiveness. The result of this study showed that TeGM6-4r had higher immuno-reactivity to WB sera (positive controls) than TbbGM6-2r. This result is similar to the reports on TR protein of *T. cruzi* FRA and several TR proteins of *Leishmania* that the copy number of the repeat affects the affinity of binding between antigens and antibodies (Valiente-Gabioud *et al.*, 2010; Goto *et al.*, 2010). In ELISA, OD value of TR antigen increases with increasing number of repeat unit. This was explained by the increase of the epitope located in the repeat unit which provides more binding sites for the antibodies. According to Valiente-Gabioud *et al.* (2010), the optimal repeat number of FRA was four because antigen coating and antigen-antibody reactivity inside the ELISA well would be interfered when protein size became bigger. Since *T. cruzi* FRA and GM6 are similar in size, I believe that increasing the repetitiveness of TeGM6 by four is most suitable to improve the antigenicity of the antigen. Taken together, TeGM6-4r demonstrated higher immuno-reactivity than TbbGM6-2r. The new antigen could distinguish negative and positive sera better and can be a novel antigen candidate for detection of *Trypanosome* spp.

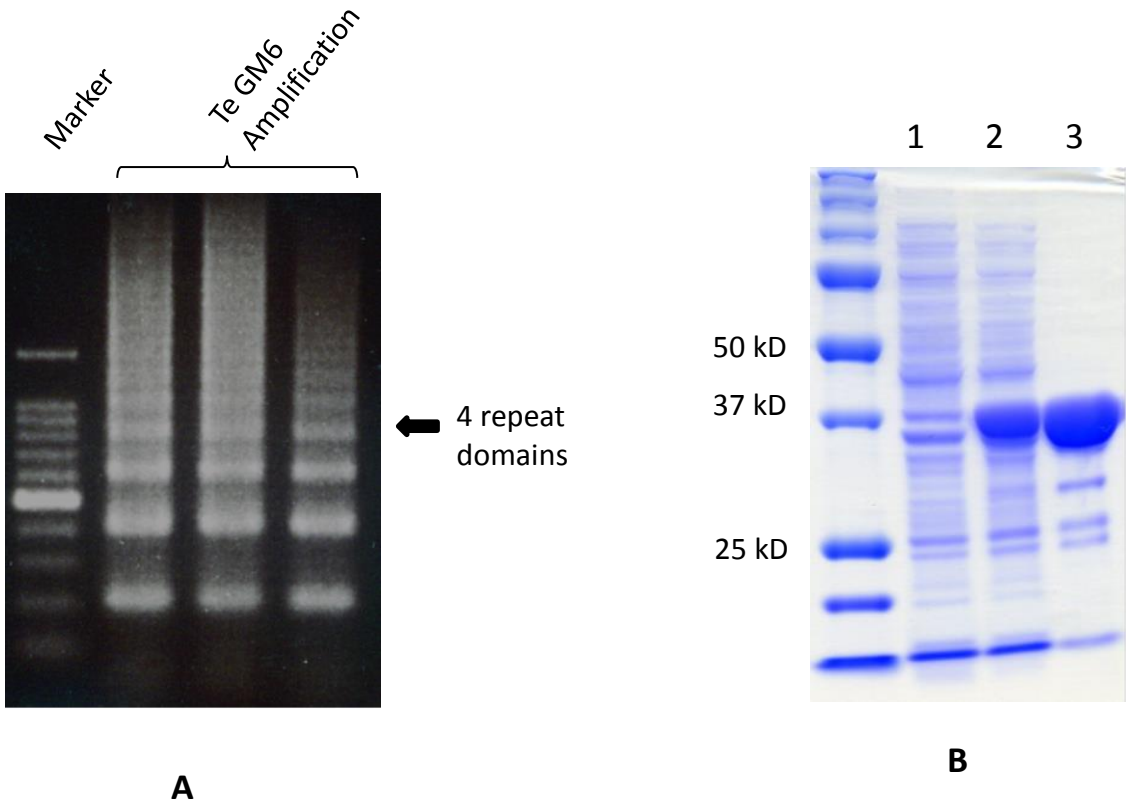

Figure 1: Expression of recombinant protein TeGM4-4r

A. PCR amplification of GM6 from genomic DNA of *T. evansi*. The PCR products show different bands in gel electrophoresis corresponding to the number of repeat domains. One repeat domain is 204 bp.

B. Image of recombinant TeGM6-4r in SDS-PAGE during protein expression. 1. before induction; 2. after induction; 3. purified protein.

Percent Identity

Divergence

	1	2	3	4	5	6	7		
1		100	97.1	63.8 .	54.5	52.2	42.6	1	Te.GM6
2	0		97.1	63.8	54.5	52.2	42.6	2	Tbb.GM6
3	3.0	3.0		63.8	54.5	52.2	42.6	3	Tbg.GM6
4	49.2	49.2	49.2		50.0	50.7	41.2	4	Tco.GM6
5	68.5	68.5	68.5	79.9		54.5	43.1	5	Tv.GM6
6	74.2	74.2	74.2	77.9	68.5		58.8	6	Tcr.GM6
7	100	100	100	100	100	59.0		7	Li.GM6
	1	2	3	4	5	6	7		

Figure 2. GM6 homologs among *Trypanosoma* spp. and *Leishmania* sp.

Te: *T. evansi*, Tbb: *T. brucei*. *brucei*, Tbg: *T. b. gambiense*, Tco: *T. congolense*, Tv: *T*. *vivax*; Tcr: *T. cruzi* and Li: *Leishmania*

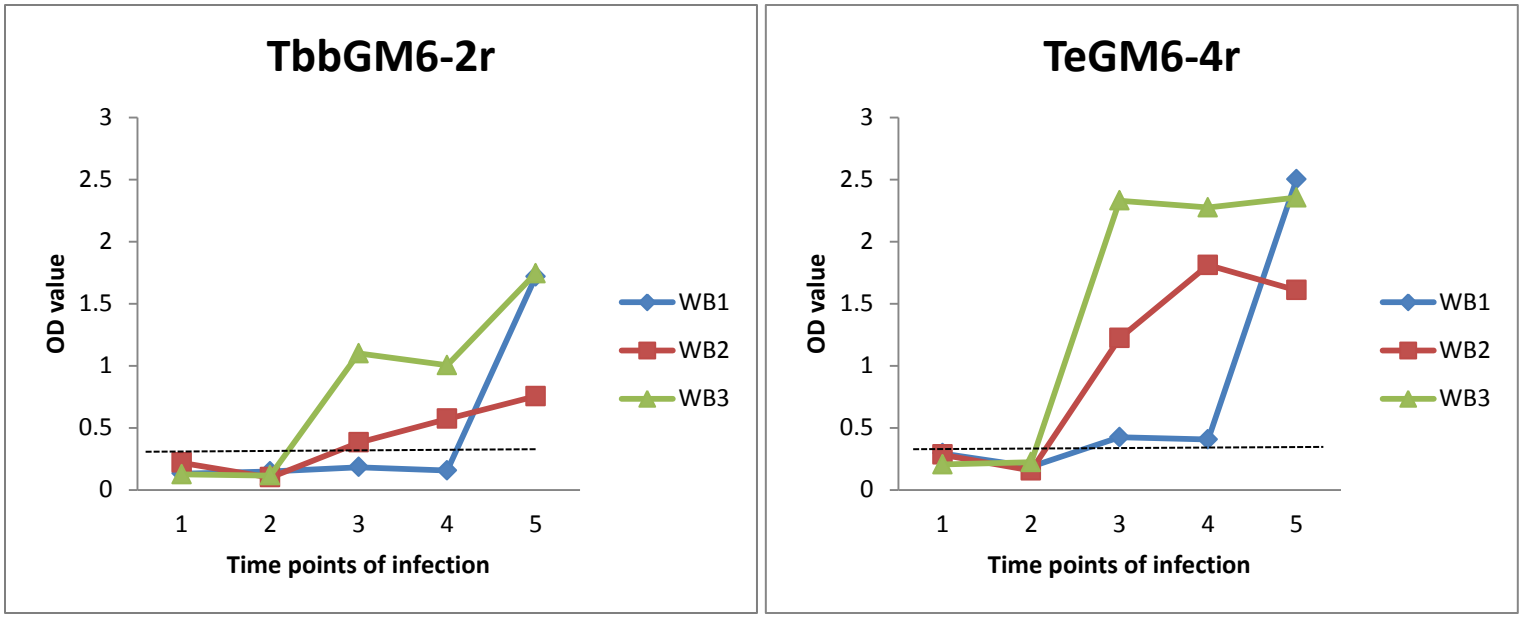

Figure 3. Immunoreactivity of TbbGM6-2r and TeGM6-4r to water buffalo sera in ELISA

WB 1, 2 and 3: water buffalo number 1, 2 and 3. WBs were experimentally infected with *T. evansi*. Time points of infection 1, 2, 3, 4 and 5: 22 days before infection, 8, 15, 22 and 48 days after infection. TeGM4-4r which consists of 4 repeats shows stronger immunoreactivity to WB sera than TbbGM6-2r with only two repeat domains. The ELISA cut-off values are indicated by the broken lines.

Chapter 2

Diagnostic value of the recombinant tandem repeat antigen TeGM6-4r for surra in water buffaloes

2-1. Introduction

Serodiagnosis of *T. evansi* infection or surra mostly relies on the lysate antigen of the parasite. Although lysate antigen production has been standardized; it is usually difficult to get the standard protocol accepted and strictly followed by laboratory workers (Reid and Copeman, 2002; OIE, 2012). Furthermore, the lysate antigen shows cross-reactivity in diagnostic tests, leading to false-positive results. On the other hand, recombinant antigens can be developed to be highly specific and have the additional advantage that consumption of laboratory animals is reduced. Several recombinant antigens of *T. evansi* were introduced including the variable surface glycoprotein, RoTat 1.2 (Lejon *et al.*, 2005), the invariant surface glycoprotein, ISG 75 (Tran *et al.*, 2009) and tandem repeat (TR) protein GM6 (Thuy *et al.*, 2012). TR protein GM6 is a cytoskeletal protein, located at the connection site between the microtubules of the membrane skeleton and the flagellum of the parasite. The protein is recognized in the early phase of infection when many parasites are destroyed by the host immune response (Muller *et al.*, 1992; Imboden *et al.*, 1995). I reported that recombinant TbbGM6 from *T. brucei* was highly antigenic to water buffalo sera that had been experimentally infected with *T. evansi* and that it held qualities that would make it useful as a diagnostic antigen (Thuy *et al.*, 2012). To continue from my

findings, it was necessary to further evaluate and validate GM6 for its future application to the serodiagnosis of *T. evansi* infection in animals, especially water buffaloes. The world population of water buffaloes is estimated at 168 million, 161 million of which are in Asia (Michelizzi *et al.*, 2010), where surra is endemic and remains a major constraint for water buffalo productivity (Luckins, 1988; Davison *et al.*, 2000; Villareal *et al.*, 2013). The present study, which involved a survey on surra among water buffaloes that was conducted in five provinces in Northern Vietnam utilizing TeGM6-4r-based ELISA, proposed the recombinant TR antigen "TeGM6-4r" as a novel candidate diagnostic antigen for surra in water buffaloes. The results from the survey are useful for gaining a greater understanding of the occurrence of surra and preventing epidemics in the study areas.

2-2. Materials and methods

Cloning and sequencing of the TeGM6-4r. The method was described in the Materials and Methods of chapter 1.

Expression of recombinant TeGM6-4r. The method was described in the Materials and Methods of chapter 1.

Preparation of trypanosome cell lysate antigen. Trypanosome cell lysate antigen was produced from the *T. evansi* Tansui strain, which was propagated using HMI-9 medium as previously described (Hirumi *et al.*, 1997). The preparation procedure was described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012).

Positive and negative sera. Twenty positive and 8 negative serum samples were obtained from Vietnamese water buffaloes. Positive serum samples were collected at day 87 or 94 from 12 water buffaloes that had been experimentally infected with *T. evansi* (field isolate from buffalo in Ha Tay province, Vietnam in 1998). The surra status of each animal was confirmed by microscopic examination of thin blood smear and buffy coat, mouse inoculation test and CATT/*T. evansi* (Institute of Tropical Medicine, Antwerp, Belgium) according to the OIE manual for surra diagnosis (OIE, 2012). In addition to the 8 negative control sera from healthy water buffaloes, the study also used serum samples collected from Japanese cattle experimentally infected with *Theileria orientalis* (n=10), *Babesia bovis* (n=3), *Babesia. bigemina* (n=7) and *Trypanosoma theileri* (n=59) as negative controls infected with non-related hemoprotozoan parasites. The infections were confirmed through observation of parasitemia and specific-antibody detection in cattle blood and sera. Handling of the experimental animals strictly accorded to the guidelines on Animal Experimentation of Department of Animal Health of Vietnam and the ethics committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (Approval number: 24-48).

Field survey. A field survey was conducted in Northern Vietnam, where many serious surra outbreaks occurred during the 1990s and where the disease remains endemic. Five provinces were chosen at random from areas with high water buffalo populations that were located at least 100km from each other. Figure 4 illustrates the locations and names of 5 the surveyed provinces in the map of Vietnam. Ha Giang, Cao Bang, Thai Nguyen, and Quang Ninh province belong to the North East region, while Son La province is located in the North West region. The above areas have the highest population density of water buffaloes in the country, where the animals serve the Red River Delta as a supply of draft power, meat, and other buffalo products. At present, there are no intensive prevention methods for surra in place in any of the 5 surveyed provinces. Infected animals are routinely treated with Berenil® (Intervet, Germany) or Trypamidium® (Merial, France). Blood collection was carried out from March 2012 to March 2013. In each province, one village was assigned as a sampling site where the farmers were asked to bring their animals for blood collection. Animals of all ages and sexes were included. Ten ml of blood was extracted from the jugular vein into a plastic tube for serum preparation at ambient temperature. Serum samples were transferred to the National Institute for Veterinary Research, Hanoi, Vietnam and stored at -30^{o}C. Sample size was calculated using software sample size calculations (http://epitools.ausvet.com.au/content.php?page=SampleSize) with the expected prevalence of 20% according to Holland et al., 2004. In total, 484 serum samples were collected during the survey.

CATT/*T. evansi*. CATT/*T. evansi* was conducted according to the instructions of the

manufacturer (Institute of Tropical Medicine, Antwerp, Belgium) and OIE manual for surra diagnosis (OIE, 2012).

ELISA. The study used trypanosome cell lysate antigen and TeGM6-4r as ELISA antigens in order to detect specific antibody in the serum samples. The protocol has previously been described in the Materials and Methods of chapter 1. In brief, 1 µg of the lysate antigen or 200 ng of TeGM6-4r diluted in coating buffer (50 mM carbonate bicarbonate buffer pH 9.6) was coated on Maxisorp 96-well plates (Nalgene-Nunc, NY) for 4 h then blocked overnight with 1% BSA. After washing with PBS-T, serum samples diluted 200 times in dilution buffer (PBS-T contained 0.1% BSA) were added to each well and incubated at room temperature for 2 h. The plates were washed with PBS-T before adding secondary antibody (horseradish peroxidase-conjugated protein G, Invitrogen, Tokyo, Japan). Washing was then performed. Tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was applied and the reaction was stopped using 1 M phosphoric acid. The absorbance was read at 450 nm with an MTP-500 microplate reader (Corona Electric, Japan).

Adsorption of sera with *E. coli* lysate. Adsorption of serum samples with *E. coli* lysate antigen followed the method of Laal *et al*., 1997. Briefly, the *E. coli* BL21 was cultured overnight and used for preparation of lysate antigen. Each well of the Maxisorp ELISA plates was coated with 100 µg of the *E. coli* lysate antigen, blocked with 5% BSA in PBS for 90 min and washed with PBS-T. The serum samples were added into the ELISA

plates that had been coated as described above and incubated for 90 min, then transferred to other coated plates. In total, eight cycles of the adsorption in the ELISA plates were performed. The absorbed serum samples were collected and used within 1 week.

Data analysis. Data were analyzed using Graphpad (http://graphpad.com/quickcalcs/). Paired Student's *t* test was used to compare the OD value of serum samples pre-absorption with *E. coli* lysate with those after the adsorption. Fisher's exact test was performed to compare seroprevalence of surra in different provinces. $P < 0.05$ indicated statistical significance.

2-3. Results

Sensitivity and specificity of TeGM6-4r-based ELISA. The recombinant tandem repeat protein "TeGM6-4r" was successfully produced using the *E. coli* expression system. TeGM6-4r contains a 6 x histidine tag and 4 repeat domains and molecular mass is estimated to be 32 kD. The single-repeat domain was comprised of 68 amino acids, of which the sequence alignment has been previously reported (Thuy *et al.*, 2012). Trypanosome cell lysate was utilized as a reference antigen for the evaluation of recombinant TeGM6-4r. Results of the lysate antigen and TeGM6-4r in the detection of positive and negative controls from water buffaloes are shown in Fig. 5**A**. Cutoff values (mean + 3SD) for lysate antigen-based ELISA and TeGM6-4r-based ELISA were calculated from OD values of 8 negative controls, giving 0.33 and 0.35, respectively. Negative controls were all negative in each of the tests; however, 4/20 positive controls were found

to be negative (false negative) by the recombinant protein. Sensitivity and specificity of the lysate antigen was all 100% and that of TeGM6-4r were 80% and 100% respectively. TeGM6-4r-based ELISA did not react with *Theileria orientalis*, *B. bovis* or *B. bigemina*, but 11 out of 59 (18.6%) *T. theileri* infected samples were positively detected. In contrast, the lysate antigen-based ELISA showed high cross-reaction with 9 out of 10 (90%) *Theileria*-infected samples, 3 out of 10 (30%) *Babesia*-infected samples and 4 out of 59 (6.8%) *T. theileri*-infected samples (Fig. 5**B**). Another reference test, CATT/*T. evansi*, showed only cross-reaction with 3 out of 59 (5.1%) *T. theileri*-infected samples.

To determine whether anti-*E. coli* antibodies could yield false positives between the recombinant protein with *T. theileri* infected cattle sera, the serum samples were adsorbed with *E. coli* lysate before testing in ELISA. The OD (mean + SD) for the pre-adsorbed sera was 0.24 + 0.12; and post-adsorbed sera had an OD of 0.22 + 0.17. There was no difference in the reactivity of sera ($P > 0.05$), demonstrated that cross-reaction between TeGM6-4r with *T. theileri* infected sera was not due to *E. coli*-antibodies.

Seroprevalence of surra among water buffaloes in Northern Vietnam determined by CATT/*T. evansi* and TeGM6-4r-based ELISA. Since CATT/*T. evansi* is the standardized, widely evaluated and recommended field test by OIE, I utilized it as a reference antibody detection test to evaluate TeGM6-4r-based ELISA. In this study, 484 field samples from water buffalo in Northern Vietnam were screened by CATT/*T. evansi* and TeGM6-4r-based ELISA. Seroprevalence of surra was determined and indicated in Table 1. CATT/*T. evansi* detected 131/484 (27.1%) positives. Ha Giang and Thai Nguyen

province had the highest prevalence of surra (39.8% and 35.7%). The prevalence in the other three provinces was not significantly different (P>0.05), ranged from 17.4-22.0%. In case of TeGM6-4r-based ELISA, 260 (53.7%) serum samples tested were found to be positive. The prevalence in Thai Nguyen (67.3%) and Son La province (36.8%) differed significantly from the average (53.7%). In the three other provinces there was no significant difference in surra prevalence (P>0.05). In comparison with CATT/*T. evansi*, TeGM6-4r-based ELISA detected more positive samples. Among those, 113 samples were true positives and 147 were false positives, giving 86.3% sensitivity and 58.3% specificity.

2-4. Discussion

The serodiagnostic value of the tandem repeat domain of GM6 for surra was first reported in my previous study (Thuy *et al.*, 2012). However, without adequate numbers of positive and negative controls, its immunodiagnostic potential was not completely evaluated. The present study overcomes this limitation and its results show TeGM6-4r to be an effective and useful tool for the serodiagnosis of surra in water buffaloes. In distinguishing infected and non-infected serum samples, TeGM6-4r exhibited moderate sensitivity (80%) in ELISA. The 4 false-negative serum samples with TeGM6-4r also had low OD values in the lysate antigen. While the recombinant antigen was 100% specific with *Theileria* or *Babesia* infected serum samples, its specificity with *T. theileri* was only 81.4%. Even the reference test showed cross-reaction with *T. theileri* infected sera (6.8% in lysate antigen-based ELISA and 5.1% in CATT/*T. evansi*). The prior adsorption of the sera with *E. coli* lysate before testing revealed that the cross-reaction was not due to anti-*E. coli*

antibodies present in cattle reacting with *E. coli* BL 21-derived protein expressed in the purified recombinant TeGM6-4r. According to Muller *et al*., 1992, GM6 is well conserved between different species of salivarian trypanosomes (including *T. b. brucei* and *T. congolense*) but less so with the stercorarian (including *T. cruzi* and *T. theileri*). Nevertheless, further study is needed to better understand whether *T. theileri* possessed any similar antigenic structures. In my study, lysate antigen had 100% sensitivity but showed strong cross-reaction with *Theileri* and some *Babesia* infected sera, different from the report of Reid and Copeman (2002) on high specificity but low to moderate sensitivity of lysate antigen. This indicates that antigenicity of lysate antigen might be inconsistent due to differences in laboratory protocols and also differences in interpretation of data.

In utilizing TeGM6-4r for my water buffalo surra survey in Northern Vietnam, I found that the disease was still widely endemic in the region. Seroprevalence was 27% detected by CATT/*T. evansi*. Anti-TeGM6-4r antibodies were detected in 53% of the animals. Previously, the prevalence of 15-21% was reported by Thu *et al.* (1998), and was of 16-22% in the surveys of Verloo *et al.* (2000) and Holland *et al.* (2004). The high seroprevalence illustrates the widespread occurrence of *T. evansi* in Northern Vietnam at the present. Due to the geographical characteristics, the surveyed areas have limited land for farming with mostly forests, open highlands, and bushes. The hot and humid conditions in these areas during summer provide an ideal environment for insect vectors to develop, reproduce, and transmit diseases. Winter, in contrast, is cool and dry. During the winter season, buffalo have to work under harsh conditions, such as insufficient food supply, and the previously infected buffalo are likely to develop clinical symptoms of diseases.

Consequently, outbreaks of surra and other infectious diseases usually occur during winter. Among the surveyed provinces, Thai Nguyen province had higher seroprevalence (35.7% by CATT/*T. evansi* and 67.3% by Te-GM6-4r/ELISA). I found this to be consistent with surra disease dynamics associated with the movement of animals from highlands to the plains. It has been reported that buffaloes transported to plain areas suffer from stress and are thus more susceptible to the disease. Although a high presence of anti-*T. evansi* antibodies was detected in my study, it does not necessarily reflect a high infection rate of surra. In the areas, drug administration is provided by local veterinarians and antibodies remain in the blood circulation of the animals long after successful treatment. Moreover, high seroprevalence especially determined by TeGM6-4r may be largely due to *T. theileri* infection since the recombinant antigen is only 81.4 % specific to *T. theileri* infected sera. The non-pathogenic infection caused by *T. theileri* had been reported in buffaloes (Rodrigues *et al.*, 2003). In Vietnam, Sivakumar *et al*. (2013) found the parasite in 10% of the cattle but the seroprevalence in water buffaloes is still unknown. In screening of field-derived serum samples, TeGM6-4r demonstrated sensitivity of 86.3% and specificity of 58.3% comparing to the reference test CATT/*T. evansi*. Low specificity is best explained by its cross-reactivity with *T. theileri*. Prevalence of *T. theileri* infection in Northern Vietnamese water buffaloes should be confirmed in the future. Taken together, the results of the study demonstrated that TeGM6-4r-based ELISA might be an effective and useful tool for assessing seroprevalence of surra in water buffaloes.

Surra control is of great concern in order to protect the worldwide livestock production. Vaccination against the disease is unavailable; moreover, the insect vectors and

animal reservoirs are still abundant. As a result, control programs mostly depend on accurate detection and treatment of infected cases. I suggest that the considerable sensitivity of TeGM6-4r would make it a useful antigen for the diagnosis of surra and that it would be of benefit to future surveillance programs. However, utilization of the tandem repeat antigen may not be only restricted in ELISA, a laboratory test but also potential for development of a field test such as an immunochromatographic device. The results from the study also suggest that surveillance programs for surra in water buffaloes need to be carried out regularly, not only in Vietnam but also in other water buffalo-producing countries.

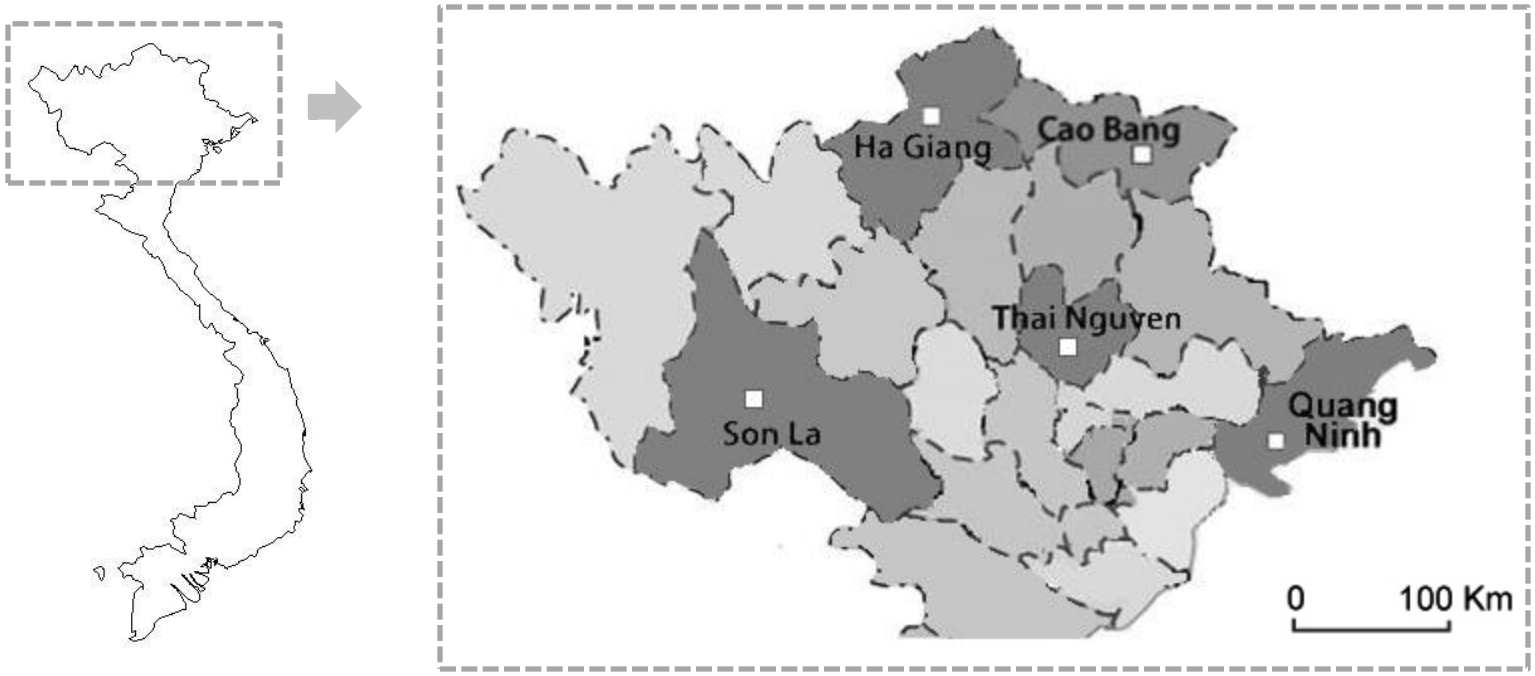

Figure 4. Map of Vietnam showing location of the five provinces surveyed in the study during the period 2012-2013.

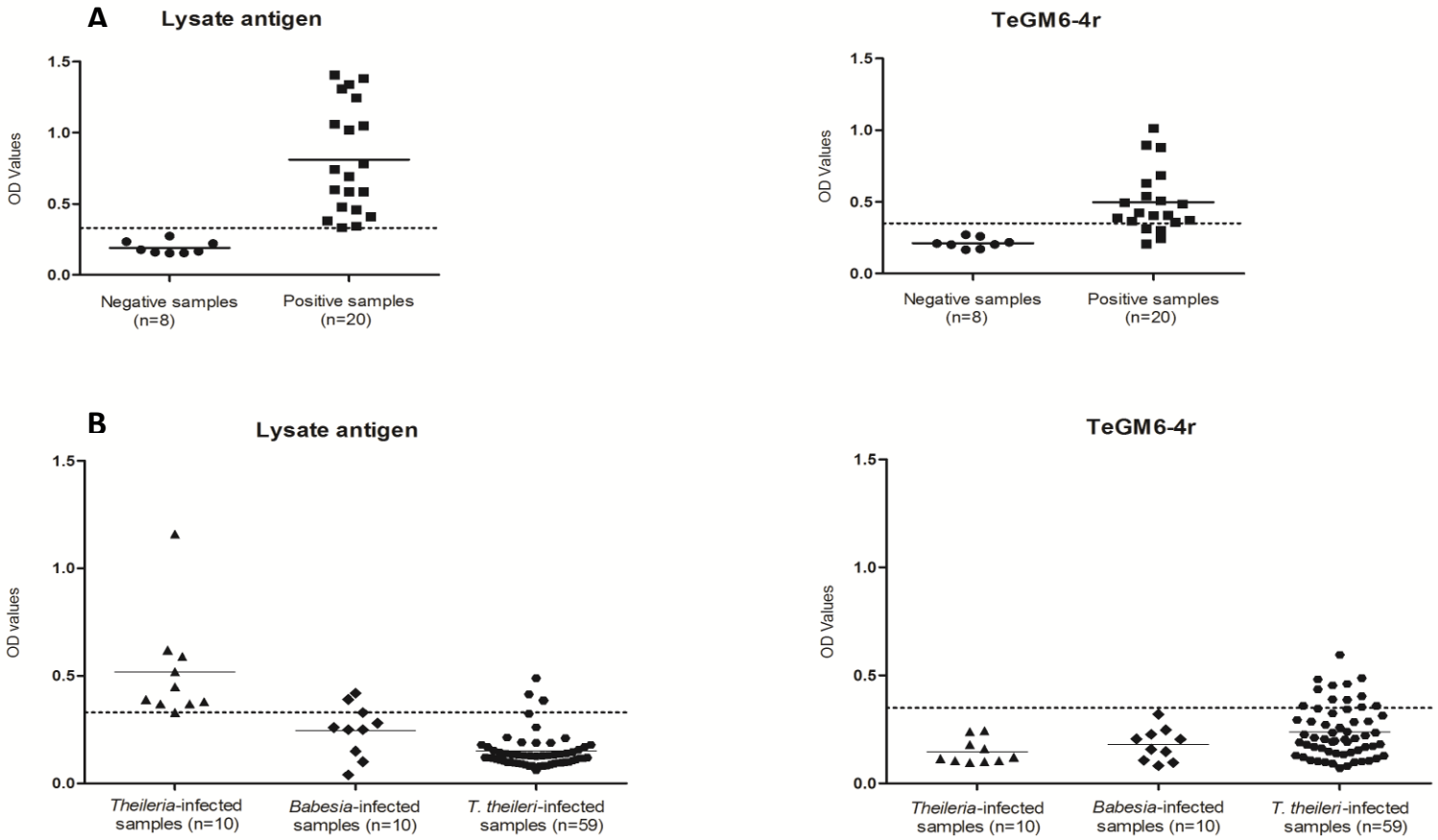

Figure 5. Evaluation of lysate antigen and TeGM6-4r in ELISA using confirmed positive and negative water buffalo sera (**A**). Cutoff values of the tests were 0.33 and 0.35 respectively (indicated by broken lines). Samples from Japanese cattle experimentally infected with each of the parasites *Theileria orientalis*, *Babesia bovis and B. bigemina* and *Trypanosoma theileri* (**B**).

Table 1. Seroprevalence of surra among water buffalo (n=484) in different provinces in Northern Vietnam, determined by using CATT/*T. evansi* and TeGM6-4r based ELISA. * shows significant difference of the prevalence to the average prevalence

Provinces	Number of serum samples	CATT/*T. evansi*			TeGM6-4r/ELISA		
		Number of positive samples	Seroprevalence (%)	P value[1]	Number of positive samples	Seroprevalence (%)	P value[1]
Cao Bang	118	26	22.0	0.29	72	61.0	0.18
Ha Giang	93	37	39.8	0.02*	44	47.3	0.26
Thai Nguyen	98	35	35.7	0.09	66	67.3	0.005*
Son La	106	21	19.8	0.14	39	36.8	0.001*
Quang Ninh	69	12	17.4	0.11	39	56.5	0.69
Total	484	131	27.1		260	53.7	

Chapter 3

A TeGM6-4r antigen based Immunochromatographic Test (ICT) for detection of animal trypanosome infections

3-1. Introduction

Animal trypanosomosis has been considered as one of the biggest constraints for worldwide livestock production. According to Kristjanson *et al.*, 1999, in Africa only, animal trypanosomosis costs livestock producers and consumers an estimated $1,340 million annually from the direct losses such as milk and meat reduction. There are also a number of 'indirect' losses that the presence of insect vectors (tsetse flies) affects people's production methods by limiting their use of draft power, making it difficult to upgrade livestock breeds while considering between trypanotorelrant and trypanosusceptible cattle as the breed of choice, avoiding grazing or settling in certain areas (Shaw, 2009).

To date, animal trypanosomosis is widely distributed in Asia, South America and Africa. Although several outbreaks of the disease have been reported in some European countries (Desquesnes *et al*., 2008; Tamarit *et al*., 2011), the highly endemic regions are still located in the developing countries, specifically in the rural areas. Those areas are usually far from the research center or a diagnostic laboratory which makes the diagnosis of the diseases a difficult task. Currently, many diagnostic methods for trypanosomosis have been developed and validated, including parasitological, serological and molecular tests

(OIE, 2012). However, the CATT is the only widely applicable tool in the field. Despite its efficiency and usefulness, CATT has been known for some acknowledged limitations including varying of specificity and sensitivity, its inability to detect *T. evansi* strain B which does not express the variable surface antigen RoTat 1.2 and the only specialized manufacturer and distributer had limited the availability of the test in the local market especially in the developing countries (Njiru *et al*., 2010; Sullivan *et al*., 2013). Therefore development of a new field test is significant to supplement the current CATT and provide more tests of choice for the detection of animal trypanosomosis.

Immunochromatographic test (ICT) utilizes the concept of chromatography and has special design for single diagnosis outside the laboratory. Essential in the current ICT is the movement of a liquid sample passing various zones along the test trip where molecules have been attached that exert specific interaction with the analyte (Paek *et al.*, 2000; Posthuma-Trumpie *et al*., 2009). Results usually come within 10-20 min. The first commercially successful ICT was pregnancy test which rapidly detects human chorionic gonadotropin in urine by simply adding urine to the test strip. Nowadays, with better methods and technology presented, new generation of ICT are widely used for qualitative and semi-quantitative detection on both human medical and veterinary purposes. Based on the typical format of ICT, this study aimed to develop ICT for *Trypanosoma* spp.-antibody detection using the tandem repeat antigen GM6. The antigen is highly conserved among the *Trypanosoma* species and exhibited strong antigenicity to *T. evansi* and *T. congolense*-infected water buffalo and cattle serum samples (Goto *et al.*, 2011; Thuy *et al.*, 2012). Different from the previous studies using the antigen consisted of two repeat domains, I

expressed new recombinant antigen GM6 which derived from *T. evansi* and consisted of four repeat domains, named TeGM6-4r in this study. The TeGM6-4r based ICT was produced and evaluated using 15 positive and negative controls from three water buffaloes experimentally infected with *T. evansi*, and 437 field samples from Tanzania and Uganda cattle. Performance of the test was also compared with the standard test lysate antigen-based ELISA as recommended by OIE.

3-2. Materials and methods

Production of TeGM6-4r. TeGM6-4r amplification and sequencing followed the methods that described previously in the Materials and Methods of chapter 1 and 2.

For protein expression, the gene fragment encoding the TeGM6-4r was inserted into the pGEX-6P-1 (GE Healthcare Bio-Sciences AB, Sweden), followed by transformation into *E. coli* BL 21. The transformed *E. coli* BL 21 was cultured in SOB medium (BD, Sparks, MD) to an OD_{600} of 0.4-0.6. The expression of the recombinant protein was induced by adding 0.5 mM IPTG and cultivated for 3 hrs. The recombinant TeGM6-4r was purified in soluble form using glutathione Sepharose 4B and cleaved from GST-tag by PreScission Protease according to the manufacturer's instructions (GE Healthcare Bio-Sciences AB, Sweden). Integrity and purity of the protein were evaluated by SDS-PAGE (12% gel). Protein concentration was determined by BCA assay (Thermo Fisher Scientific Inc., Waltham, MA). The purified recombinant protein was stored at -80^{o}C until use.

Production of lysate antigens. Bloodstream forms of *T. b. brucei* (GUTat3.1) and procyclic forms of *T. congolense* (IL3000) were maintained in National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine in *in vitro* culture medium HMI-9 and TVM-1(Hirumi *et al.*, 1980), and utilized as the sources of trypanosome lysate antigens. Preparation of *T. b. brucei* and *T. congolense* lysate antigens was described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2012.

ELISA. ELISA was performed following the protocol described previously in the Materials and Methods of chapter 1 and 2.

Production of anti-TeGM6-4r polyclonal antibody. Each BALB/c mouse was immunized with 100 µl emulsion of purified recombinant TeGM6-4r (100 µg) and TiterMax® Gold adjuvant (Sigma-Aldrich, Norcross, GA). After two weeks, antibody titer was determined using TeGM6-4r-based ELISA. The mice which produced high antibody titer were sacrificed for blood collection and serum extraction. Sera were then subjected to polyclonal antibody purification using MAbTrap kit (Sigma-Aldrich, Norcross, GA) following the instruction of the manufacturer. The concentration of the polyclonal antibody was determined by BCA assay. Anti-sera were stored at -80^{o}C until use.

Construction of TeGM6-4r-based ICT. TeGM6-4r-based ICT was designed for antibody detection. All of the membranes used for ICT (glass fiber, absorbance and

nitrocellulose membranes) were purchased from EMD Millipore Corporation (Billerica, MA). The recombinant TeGM6-4r was conjugated with gold colloid 20 nm (British Biocell International, UK) by the following procedure: 100 µl of TeGM6-4r (at the concentration of 300 µg/ml) was gently mixed with 1 ml of gold colloid for initial binding, incubated for 10 min and added with 0.05% polyethylene glycol (PEG) 20,000 and 1% BSA. The complex gold colloid-labeled TeGM6-4r was washed twice with blocking solution which contained 0.05% PEG 20,000 and 0.5% BSA. After homogenization by ultra-sonication (Bransonic, Danbury, CT 06813, USA), the concentration of the conjugate were determined by reading OD_{520}. The final concentration was adjusted at OD_{520} 1.5 with the dilution buffer (10 mM Tris-HCl pH 8.2 and 5% sucrose). Finally, the conjugate solution was absorbed into the glass fiber strip and dried in the dryer (VD-500R, TAITEC, Japan) for 3 h.

To create the test line and control line on the ICT strip, TeGM6-4r (0.5 mg/ml) and anti-TeGM6-4r polyclonal antibody (1 mg/ml) were jetted on the nitrocellulose membrane by XYZ Dispensing Platform (Bio DOT, Irvine, CA). The membrane was then blocked with 0.5% casein and dried overnight at room temperature. Subsequently, all of the ICT components were assembled manually and cut into 3 mm strips by CM 4000 (Bio DOT, Irvine, CA). For running ICT, 10 µl of serum sample was diluted 5 times with PBS and loaded on the sample pad. The result showed in 10-20 min. ICT was determined positive if it displayed both test and control lines, and negative if only the control line developed.

Serum samples. Negative and positive controls were obtained from three water buffaloes experimentally infected with *T. evansi* in China. Sera were collected at 5 time

points during the infection; namely 22 days before infection (pre), 8, 15, 22 and 48 days post-infection (DPI). Sera pre and 8 DPI were confirmed negative and the rest (15, 22 and 48 DPI) were positive by parasitological tests and ELISA. The total 437 field samples were collected from cattle in Uganda and Tazania from May to July in 2006. Handling of the experimental animals was according to the guidelines on Animal Experimentation of the ethics committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (Approved number: 24-48), and in accordance with Animal Welfare guidelines in Sun Yat-Sen (Zhongshan) University, China

Data analysis. Data was analyzed by using the software Graphpad (http://graphpad.com/quickcalcs/). Calculation and interpretations of kappa value followed the methods of Viera. A. J *et al*., 2005.

3-3. Results

Detection of *T. evansi* antibodies in experimentally infected water buffaloes byTeGM6-4r/ICT. Recombinant antigen TeGM6-4r was successfully produced as the non-tagged protein fragment which was isolated and characterized from *T. evansi*, and estimated to be 28.9 kD (Fig 6.). The protein fragment contained 4 repeat units. Each repeat unit was constructed from 68 amino acids. Database searching with GM6 amino acid sequence revealed the similarity with the antigen from other trypanosomes. GM6 from *T. evansi* shares 100% identity with *T. b. brucei*, 63.8% with *T. congolense* and 54.5% with *T. vivax* derived GM6. Since GM6 is highly conserved among the *Trypanosoma* spp., only *T.*

evansi-derived GM6 was chosen as diagnostic antigen for trypanosomosis. Antigenicity of the newly expressed TeGM6-4r was compared with the GM6 that consisted of only two repeat domains which produced from the previous study in ELISA, using 15 positive and negative controls from WB experimentally infected with *T. evansi*. The result showed that OD value (mean ± SD) of WB sera in TeGM6-4r/ELISA was 1.08 ± 0.95; and that of two-repeat-unit GM6/ELISA was 0.56 ± 0.57. Immunoreactivity of TeGM6-4r was significantly increased (P value = 0.001; paired Student's *t* test).

Subsequently, TeGM6-4r based ICT was produced. In preliminary evaluation of the test, TeGM6-4r/ICT was performed using positive and negative controls from WB experimentally infected with *T. evansi*. All of the WB negative sera (at pre. and 8 DPI) were negative and positive sera (15, 22, and 48 DPI) were positive in ICT (Fig. 7A). Sera at 22 DPI were in red color due to hemolysis; however, the test lines were still visible. TeGM6-4r/ICT was strongly in agreement with TeGM6-4r/ELISA. In TeGM6-4r/ELISA, OD values of pre. and 8 DPI was lower than the cutoff value (0.3), increased from 15 DPI and considerably high at 48 DPI (Fig. 7B). To determine the sensitivity of ICT in comparison with ELISA, positive sample was diluted at serial dilutions and tested with the two tests. The results are shown in Figure 8. ICT could detect the positive sample at 32 times dilution; whereas that of ELISA was 512.

Detection of *T. congolense* and *T. vivax* infections in field-derived serum samples by TeGM6-4r/ICT. Field samples (n=437) were collected from Tanzania and Uganda cattle from May to July in 2006. The samples were previously screened by microscopic

examination of the buffy coat which detected 15 *T. congolense*, 4 *T. vivax*, and 5 mixed *T. congolense* and *T. vivax* positive. Since *T. vivax* crude antigen was unavailable, *T. b. brucei* and *T. congolense* crude antigen-based ELISAs were utilized as reference tests (as recommended by OIE). In the first ELISA, I used HRP-conjugated protein G as the secondary antibody to detect IgG in the serum samples. The *T. b. brucei* and *T. congolense* crude antigen and TeGM6-4r based tests detected 272, 262 and 235 positive samples respectively. The TeGM6-4r/ICT detected 228 positive samples. However, there were 23 serum samples positive in ICT but negative in *T. b. brucei* crude antigen based ELISA; and those in case of *T. congolense* crude antigen and TeGM6/ELISA were 24 and 32 sera. Therefore, the second ELISA was performed using HRP-conjugated bovine IgM antibody to detect IgM in the serum samples. The result showed that among the ICT positive but ELISA negative samples, 21/23, 14/24 and 32/32 sera became positive in *T. b. brucei* and *T. congolense* crude antigen and TeGM6-4r based ELISA respectively. Taken together, TeGM6-4r/ICT demonstrated 76.5% sensitivity, 93.4% specificity and kappa value 0.64 with *T. b. brucei* crude antigen/ELISA and those with *T. congolense* crude antigen/ELISA were 79.6%, 98.7% and kappa value 0.72 respectively (Table 2). The test showed substantial agreement (kappa value > 0.61) with crude antigen-based ELISA in detection of trypanosomes in field samples. All the positive samples by microscopic examination of the buffy coat were also positive in TeGM6-4r/ICT. In comparison with TeGM6-4r/ELISA, TeGM6-4r/ICT had relatively lower sensitivity, but higher specificity. Kappa value between the two tests was 0.78 indicating substantial agreement.

3-4. Discussion

Trypanosoma evansi infection is widely distributed in the world and is particularly severe in camels and water buffaloes. Using sera of experimentally infected sera, the TeGM6-4r/ICT could distinguish 100% of the positive and negative samples suggesting that the test is an accurate diagnostic tool. My study demonstrated that increase of the repeat domains of GM6 by four significantly increased the immunoreactivity of the antigen. The result was consistent with the study of Valiente-Gabioud *et al.*, 2011. The TeGM6-4r/ICT was in substantial agreement with TeGM6-4r/ELISA. Although the detection level of ICT was lower than ELISA, using higher concentration of serum samples for testing in ICT (5 times dilution in this study) might partly overcome the problem. Improvement of the sensitivity of the immunochromatographic test is also a great concern. Several techniques have been proposed recently; however, few of them were neither greatly effective nor convenient for use under field conditions (Fu *et al.*, 2011; Tanaka *et al.*, 2006). Future worked is still needed for further evaluation of the test using higher number of serum samples.

In comparison to CATT/*T. evansi*, the advantage of TeGM6-4r/ICT is that it can detect other species of trypanosomes including *T. b. brucei*, *T. congolense* and *T. vivax*. When screening field-derived cattle sera samples, the ICT detection performance was equivalent to parasitological method, that is, 24 Uganda and Tanzania cattle which were confirmed *T. congolense* and/or *T. vivax* positive by microscopic examination of the buffy coat were also positive by ICT. Within comparison to other serological tests, ICT showed substantial agreement with *T. b. brucei* and *T. congolense* lysate antigen/ELISAs (kappa value 0.64 and

0.72). ICT was able to detect both IgG and IgM in the serum samples; however, conventional ELISA is mostly just IgG detection. Utilizing the same antigen, TeGM6-4r/ICT and TeGM6-4r/ELISA also had substantial agreement (kappa value 0.78). The agreement level did not reach the ideal perfect kappa value of 1 which is well explained by the number of serum samples that showed different results in the two tests. The 34/249 positive sera by ELISA were negative in ICT indicating that ICT was less sensitive than ELISA as discussed previously. There were 254/437 (58.1%) positive samples in both *T. b. brucei* and *T. congolense* lysate antigen/ELISAs. Microscopic examination of the buffy coat detected only five samples as positive for both *T. congolense* and *T. vivax* infections. Therefore, high percentage of co-infection detected by ELISA might due to cross-reaction between the two crude antigens. Another reason could be that ELISA detects persistent antibodies even in the absence of visible parasites.

This is the first time an ICT is developed and evaluated for detection of animal trypanosomosis. The test utilized tandem repeat antigen TeGM6-4r and the recombinant DNA technology, therefore was able to be produced with high quality and large quantities. Although sensitivity of TeGM6-4r/ICT was relatively lower than TeGM6-4r/ELISA, it could detect both IgG and IgM in the serum samples which more advantageous than ELISA. While CATT/*T. evansi* is only available for *T. evansi* detection, ICT is able to recognize *T. evansi*, *T. congolense* and *T. vivax*. Since the TeGM6-4r antigen shares high similarity (63.8%) with *T. b. gambiense* and *T. b. rhodesiense*, it may also have diagnostic potential for human African trypanosomosis. Further studies and extensive evaluation of TeGM6-4r/ICT are required to improve the performance of the test. Together with CATT/*T.*

evansi, TeGM6-4r/ICT would be a good diagnostic tool for both research purposes and on-site diagnosis of animal trypanosomosis.

In conclusion, the results of my study indicated that TeGM6-4r-based ICT is capable of detecting circulating antibody to trypanosomes in water buffaloes and cattle. The test is relatively sensitive, reliable and comparable to trypanosome lysate antigen-based ELISAs, the reference tests which are recommended by OIE. Moreover, it is suitable for field use, and is simple to carry out and interpret.

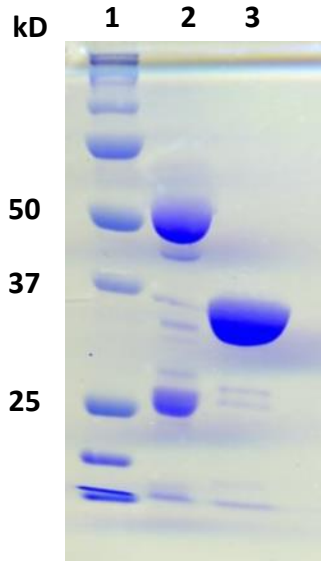

Figure 6. The SDS-PAGE image of recombinant TeGM6-4r.

Lane 1: Protein size marker. Lane 2: GST-tagged recombinant TeGM6-4r which was estimated to be 54.8 kDa including GST about 25.9 kDa in size. Lane 3: Non-tagged recombinant TeGM6-4r which was estimated to be 28.9 kDa.

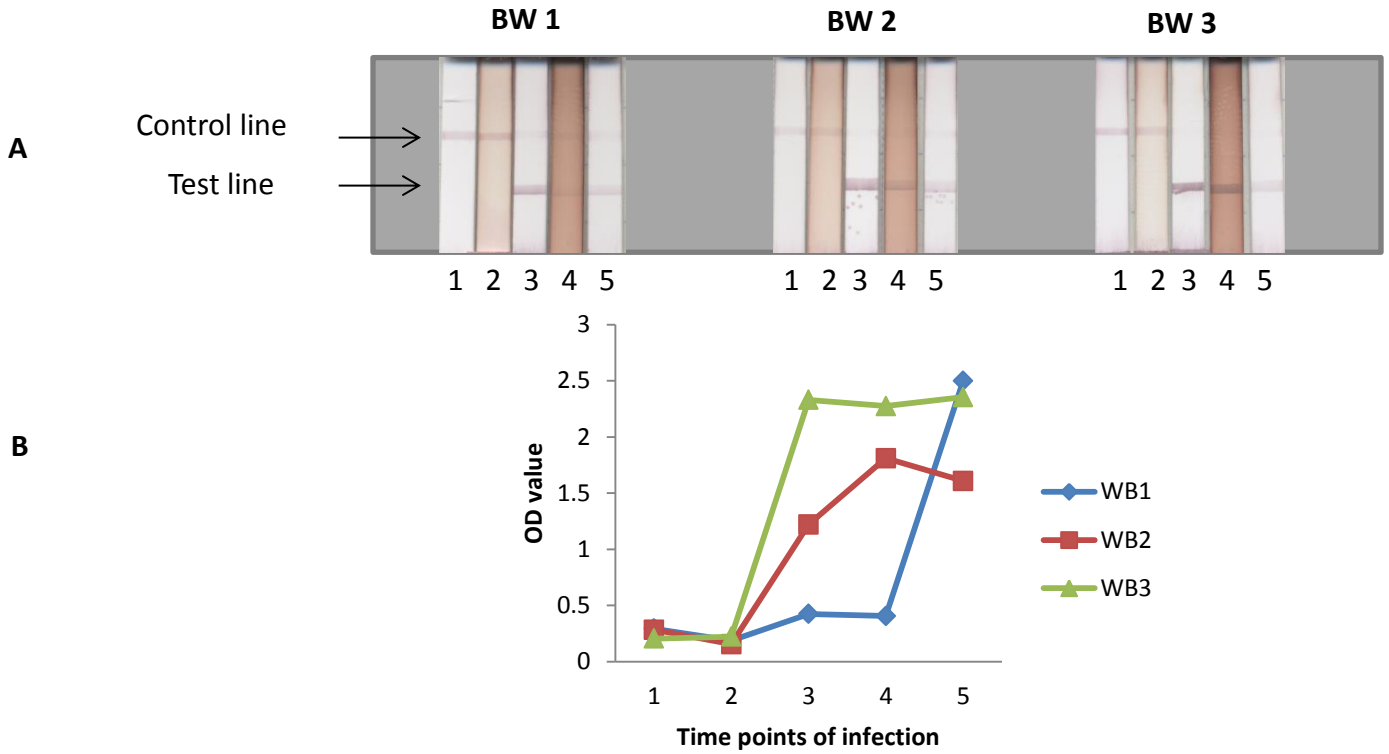

Figure 7. Recombinant TeGM6-4r-based ICT (A) and ELISA (B) using positive and negative controls from water buffalo experimentally infected with *T. evansi*. The two tests were in agreement in distinguishing negative controls (1 and 2) and positive controls (3, 4 and 5). Due to hemolysis in the sera 4, the background of ICT trips showed brown color. WB1, 2 and 3 indicate water buffalo No. 1, 2 and 3, respectively. The number 1, 2, 3, 4 and 5 indicate serum samples collected at day 22 before infection, day 8, 15, 22 and 48 post-infection, respectively. Cutoff value of ELISA was 0.3 as indicated by the broken line.

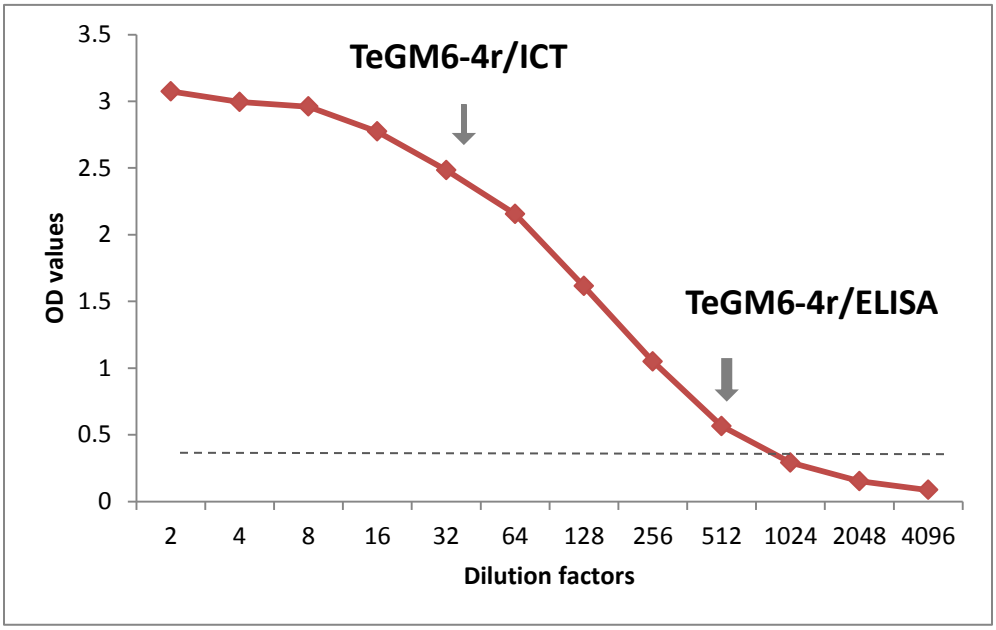

Figure 8. Sensitivity of TeGM6-4r based ICT and ELISA.

Cutoff value of ELISA was 0.3 as indicated by the broken line. The ICT could detect positive sample at dilution factor of 32, while that of both ELISAs was 512.

Table 2. Statistical analysis of recombinant TeGM6-4r based ICT and crude antigen ELISAs. Serum samples were collected from Tanzania and Uganda cattle (n = 437) from May to July in 2006.

Tests	Number of positive samples	Number of negative samples	Sensitivity (%)	Specificity (%)	Kappa values	Sensitivity (%)	Specificity (%)	Kappa values
			Versus Tbb CA/ELISA			Versus Tco CA/ELISA		
Tbb CA/ELISA	293	144						
Tco CA/ELISA	276	161	87.7	89.8	0.76			
TeGM6-4r/ELISA	267	170	76.3	83.8	0.58	79.3	76.9	0.58
TeGM6-4r/ICT	228	209	76.5	93.4	0.64	79.6	98.7	0.72

Chapter 4

Application of serological assays, ELISA, CATT and ICT for diagnosis of animal trypanosomiasis in South Africa

4-1. Introduction

Nagana is the form of African animal trypanosomosis caused by *T. b. brucei, T. congolense* and *T. vivax* in domestic animals including cattle, dogs, donkeys, goats, horses and sheep (Namangala *et al.*, 2013; Simo *et al*., 2013; Sow *et al*., 2013). These trypanosomes are mainly transmitted to their host by arthropod vectors of the genus *Glossina* which are commonly known as tsetse flies **(**Kappmeier *et al*., 1998; Gillingwater *et al*., 2010; Motloang *et al*., 2012). Dourine which is caused by *T. equiperdum* (Li *et al*., 2005) is another form of African animal trypanosomiasis known to exist in South Africa.

Historically, in South Africa four tsetse species have been in existence namely, *G. morsitans morsitans, G. pallidipes, G. brevipalpis* and *G. austeni* (Motloang *et al*., 2012). The *G. m. morsitans* completely disappeared from South Africa after the rinderpest epizootic in 1897 and *G. pallidipes* was totally eliminated in an extensive air spraying and animal dipping in insecticides during 1950s (Kappmeier *et al*., 1998). To date, only *G. brevipalpis* and *G. austeni* remain and are confined to the indigenous forests, river beds, protected nature reserves and game parks located in KwaZulu-Natal Province (Van Den Bossche *et al*., 2006; Motloang *et al*., 2012). Recent prevalence studies of Nagana in South

Africa reported that species occurring in the vector as well as livestock are *T. congolense* and *T. vivax* in the north eastern KwaZulu-Natal province of South Africa (Mamabolo *et al*., 2009; Gillingwater *et al*., 2010; Motloang *et al*., 2012).

The 1990 Nagana outbreak in north-eastern KwaZulu-Natal lead to implementation of emergency control measures (Kappmeier *et al*., 1998), but it indicated the fact that this disease was neglected in South Africa, as a result it highlighted the need for frequent epidemiological surveys to monitor the prevalence of trypanosome infections in South Africa. All prevalence and epidemiological studies of Nagana in South Africa have been determined by use of microscopic and PCR based diagnostic techniques (Van den Bossche *et al*., 2006; Latif *et al*., 2009; Mamabolo *et al*., 2009; Gillingwater *et al*., 2010; Motloang *et al*., 2012).

Serological assays are useful tools for large scale epidemiological surveillance. Antibody detection ELISA utilizing trypanosome crude antigen is regarded as a conventional and standard method for animal trypanosomiasis diagnosis according to World Organization for Animal Health (OIE, 2012). Another available method, CATT/*T. evansi* has been standardized and recommended for detection of *T. evansi* infections and is also suitable for application in field conditions (Verloo *et al.*, 2000; OIE, 2012). Besides the conventional tests, the development of recombinant technology has introduced a number of new methods. This technology has led to development of several recombinant antigen assays including the TeGM6-4r based serological tests (Goto *et al*., 2011; Thuy *et al*., 2012). TeGM6-4r based ELISA and immunochromatographic test (ICT) which have been developed recently are capable of detecting antibodies to animal trypanosomes

effectively. This study was therefore aimed at evaluating the application of crude and recombinant ELISA, CATT and ICT in serodiagnosis of animal trypanosomiasis in livestock in Umkhanyakude District of KwaZulu-Natal Province, South Africa.

4-2. Materials and methods

Sample collection and harvesting of serum. Blood collection was carried out in 3 local municipalities (Hlabisa [28.1333° S, 31.8667° E], Mtubatuba [28.4167° S, 32.1833° E], and The Big 5 false bay [28.0189° S, 32.2675° E]) of the Umkhanyakude district (Fig. 9) located in the north eastern part of KwaZulu-Natal Province between March and May 2013. In the sampling areas, the farming system is called rural communal farming which is not for commercial purposes but for family consumption and a sign of wealth. Sample animals were from communal farmers for which owners did not have information on the age of animals. The cattle breed was Nguni; and there was no information on goat and sheep breeds. Blood was collected from cattle, goats and sheep into silicone coated vaccutainers which allowed clotting of the blood from which serum was harvested accordingly. A total of 231 serum samples were obtained from 9 sheep, 99 goats and 123 cattle. Low numbers of sheep because sheep is not preferred domestic livestock in KwaZulu-Natal province. The serum samples were stored at -20^{o} C until use.

Crude antigen ELISA. The ELISA assays were performed utilizing the *T. b. brucei* crude antigen (TbbCA), *T. congolense* crude antigen (TcoCA) or TeGM6-4r. Trypanosome

crude antigens were prepared according to OIE manual for terrestrial animals (OIE, 2012) from *T. b. brucei* blood stream forms and *T. congolense* procyclic forms which were propagated in HMI-9 and TVM-1 *in vitro* cultures as described previously (Hirumi and Hirumi, 1989, 1991; Hirumi *et al*., 1997).

Recombinant antigen (TeGM6-4r) ELISA. The recombinant antigen TeGM6-4r was expressed using bacterial expression system as described by Thuy *et al*., (2012). ELISA was conducted following the protocol described previously in the Materials and Methods of chapter 1 and 2. There were some minor modifications, whereby, stop solution was omitted but 5 min after adding the substrate the reaction was read directly at 620 nm with a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, South Africa).

CATT/*T. evansi* and ICT. The tests were conducted according to instructions from the manufacturer and OIE manual (OIE, 2012). The protocol was described previously in the Materials and Methods of chapter 2 and 3.

4-3. Results

The results showed that serum samples from cattle, goats and sheep demonstrated immuno-reactivity in ELISA assays (Fig. 10). The average OD values of bovine, caprine and ovine sera in TbbCA-ELISA, TcoCA-ELISA and TeGM6-4r/ELISA were not

statistically different (P>0.05). The ELISAs showed similar pattern in detection of trypanosome antibodies in the sera. However, in case of TeGM6-4r ELISA, one ovine serum and one bovine serum had significantly higher OD values (P<0.05) indicating high expression level of specific antibody to TeGM6-4r. To calculate ELISA cut off value, five ovine samples, ten caprine and ten bovine with OD values less than the OD average and were negative by ICT and CATT/*T. evansi* were chosen as negative controls for each ELISA test. Table 3 shows the number of positive serum samples detected by ELISA assays, ICT and CATT/*T. evansi.* The TeGM6-4r ELISA detected the highest number of trypanosome positive samples at 29% (67/231). The two crude antigen based ELISA assays (TbbCA and TcoCA) both detected 27.3% (63/231) trypanosome positive samples (Fig. 11). In comparison to crude antigen based ELISAs, the TeGM6-4r ELISA demonstrated high sensitivity and specificity ranging from 73.0 to 79.4% and from 87.5 to 89.9%, respectively with kappa value of 0.6 - 0.7 indicating substantial agreement (Viera and Garrett, 2005).

For ICT and CATT/*T. evansi* assays, 19.9% (46/231) and 16% (37/231) of samples were seropositive for trypanosome infections (Fig. 11), respectively. The ICT had a relatively low sensitivity (30.2 - 36.5%), but moderate specificity (83.9-86.3%) with kappa value of 0.2 - 0.3 (low agreement). ICT was highly in agreement with PCR. Among the samples positive in ICT, two sheep, none goat and 34 cattle were also detected trypanosomosis-positive by PCR method in a study conducted in the laboratory of Zoology and Entomology of University of Free State, South Africa (data not shown). Among 37 samples positive in CATT/*T. evansi,* 11 were also positive by ELISA assays and 13 were positive in ICT. Other serological techniques applied in this study are not species specific,

however, the CATT/*T. evansi* is supposed to be only specific to *T. evansi*. Since *T. evansi* does not exist in South Africa, I suspect that CATT/*T. evansi* is cross reacting with *T. b. brucei* or possibly with *T. theileri*. The *T. equiperdum* is present in South Africa but is known to be restricted to equines only, hence it cannot be suspected as the one cross reacting with the CATT/*T. evansi* in this case. Positive samples detected by CATT/*T. evansi* need to be further confirmed by other antigen detection and DNA based tests.

Seroprevalence of animal trypanosome infection was variably detected in different sero-diagnostic tests applied in this study (Fig. 11). The prevalence in ovine, caprine and bovines was 0 - 44.4%; 0 - 9.1%, and 19.9 - 29.0% respectively. There were no positively detected ovine samples by CATT/*T. evansi*, whilst none of the caprine samples were positively detected by ICT. Similar to previous animal trypanosomiases prevalence reports in South Africa, the disease is more prevalent in cattle (Van den Bossche *et al*., 2006; Latif *et al*., 2009; Mamabolo *et al*., 2009; Gillingwater *et al*., 2010; Motloang *et al*., 2012). Considering the high number of samples collected in this study, goats appear to be less susceptible to animal trypanosome infections or rather less preferable host for the trypanosome vector as compared to cattle. Goats are considered as important reservoirs for trypanosomes, therefore they should be taken into consideration in all programs aimed at controlling the disease (Gutierrez *et al*., 2006).

4-4. Discussion

Historically, South Africa occupies an important position as far as description of animal African trypanosomosis is concerned because David Bruce discovered the parasite from animals in South Africas' Zululand in 1895 (Bruce 1895), which resulted in formulation of control strategies continentally. Reports on animal trypanosome prevalence and epidemiological studies in South Africa are based on microscopic and PCR based diagnostics (Van den Bossche *et al.* 2006; Latif *et al*., 2009; Mamabolo *et al.* 2009; Gillingwater *et al*., 2010; Motloang 2012), surprisingly sero-diagnostic assays have not been exploited for animal trypanosomiasis in South Africa. This study evaluated the application of various serological assays for diagnosis of animal trypanosomiasis in South Africa.

In conclusion, this is the first time a variety of serodiagnostic tests has been evaluated for diagnosis of animal trypanosomiasis in South Africa. The results of the study showed that ELISA assays utilizing crude and recombinant (TeGM6-4r) trypanosome antigens were highly sensitive and efficient for application in sero-diagnosis of trypanosome infections. Since production of recombinant antigen is more advantageous, TeGM6-4r ELISA can be a useful and reliable supplementary diagnostic technique to microscopy and PCR for animal trypanosomiasis in South Africa. The TeGM6-4r ICT was less sensitive than ELISA however is relatively specific, simple and rapid. Remarkably, the ICT results were highly in agreement with the results obtained from PCR method, which means ICT was able to detect the truly active infections. With further improvement on sensitivity the trypanosome ICT has the potential for use in both research and on-site diagnosis in trypanosome endemic countries.

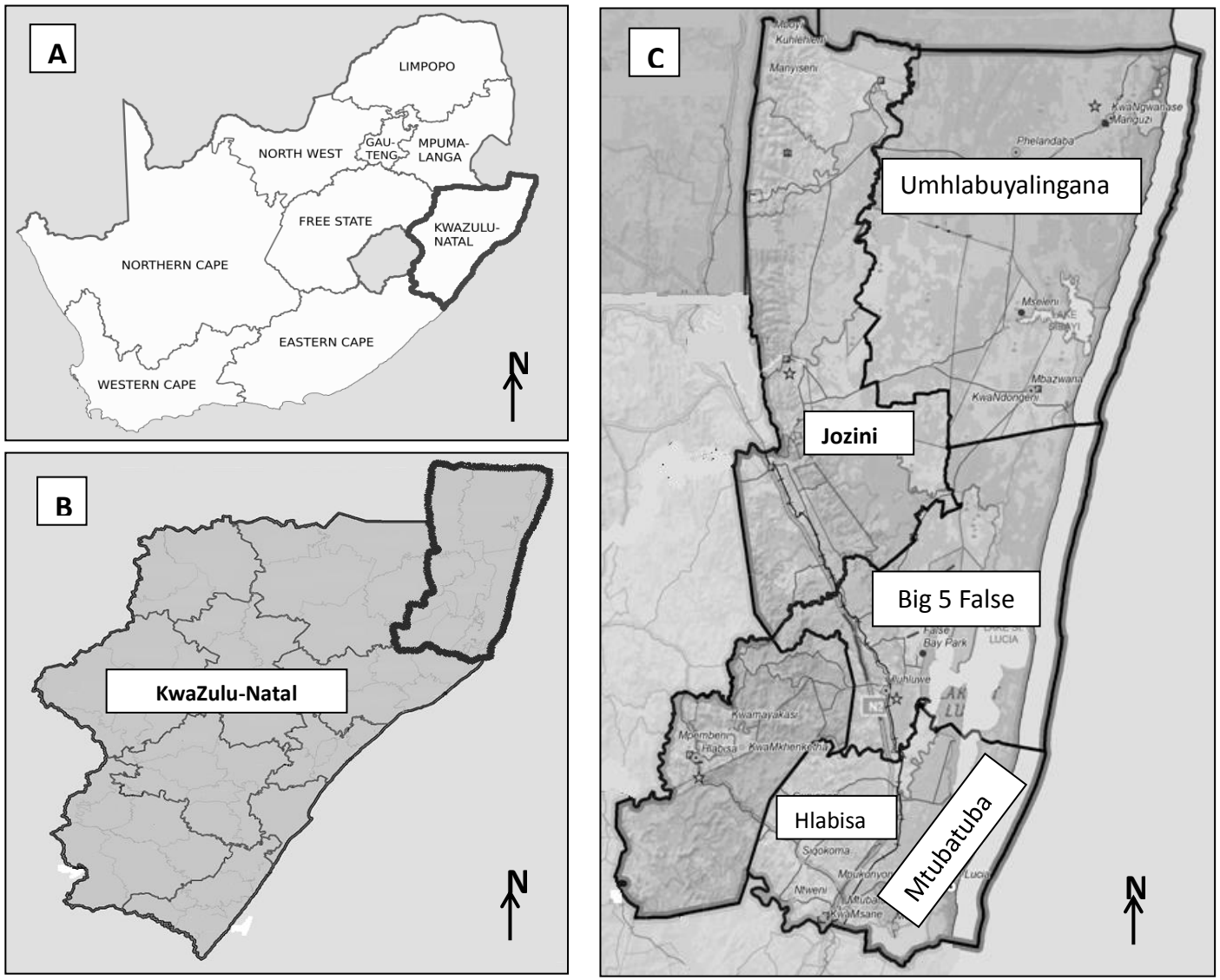

Figure 9: Maps showing the sampled area. (A) South Africa with its nine provinces. (B) KwaZulu-Natal Province. (C) Umkhanyakude district with its five local municipalities. Samples were collected in Hlabisa, Mtubatuba and The Big 5 False Bay local municipalities.

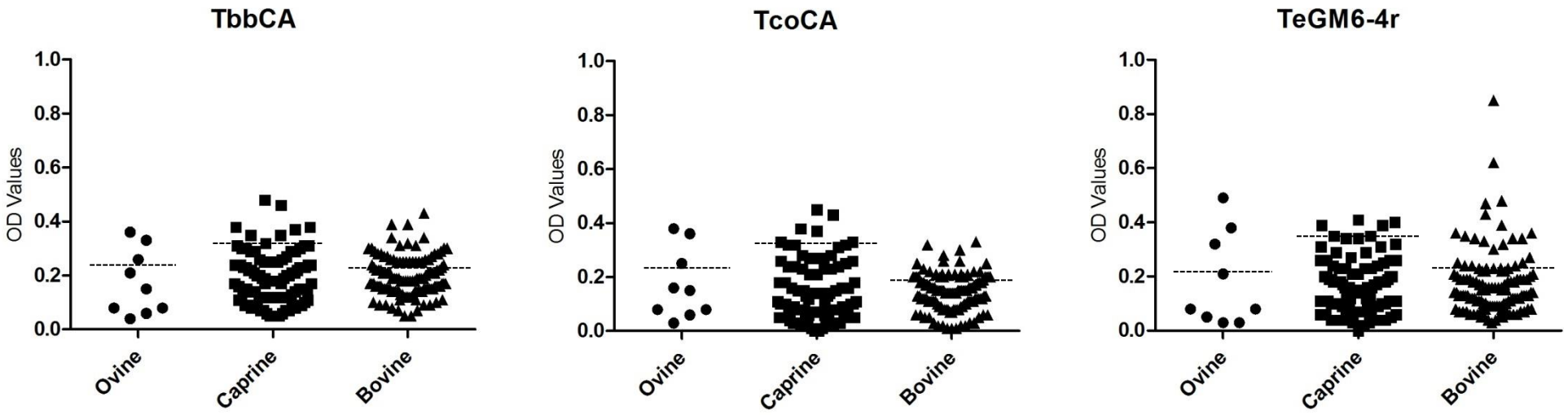

Figure 10: ELISA results of serum samples (n=231) collected from sheep, goats and cattle in KwaZulu-Natal Province of South Africa. The number of sheep, goats and cattle were n = 9, n = 99 and n = 123 respectively. Sera were tested using 3 different antigens: *T. b. brucei* crude antigen (TbbCA), *T. congolense* crude antigen (TcoCA) and recombinant protein TeGM6-4r. Broken lines indicate the cutoff values: TbbCA/ELISA for sheep, goats and cattle were 0.25, 0.34 and 0.22; TcoCA/ELISA for sheep, goats and cattle were 0.25, 0.34 and 0.22 and TeGM6-4r/ELISA for sheep, goats and cattle were 0.22, 0.34 and 0.22, respectively.

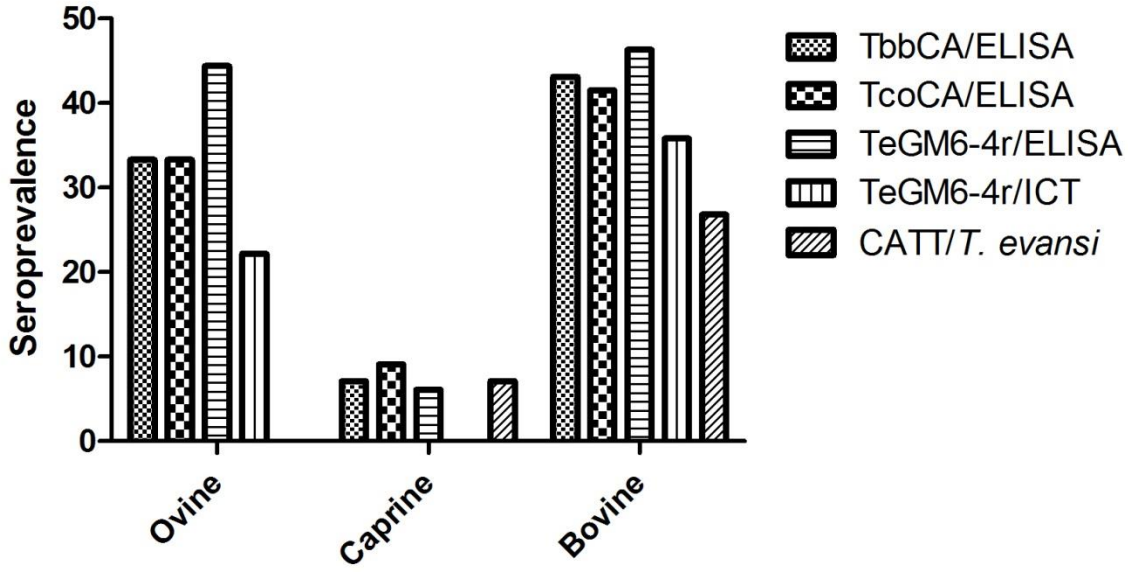

Figure 11: Seroprevalence of animal trypanosomosis in sheep, goats and cattle detected by ELISAs, ICT and CATT. Seroprevalence was variably estimated by different serodiagnostic tests. The prevalence in sheep, goats and cattle was 22.2-44.4%, 0-9.1% and 35.8–46.3%, respectively. No trypanosome-positive caprine samples were detected by ICT.

Table 3. Number of positive samples detected by different serological tests: ELISAs, ICT and CATT/*T. evansi*

Animal species	Sample number	TbbCA/ELISA	TcoCA/ELISA	TeGM6-4r/ELISA	TeGM6-4r/ICT	CATT/*T. evansi*
Sheep	9	3	3	4	2	0
Goats	99	7	9	6	0	7
Cattle	123	53	51	57	44	30
Total	231	63	63	67	46	37
Percentage (%)	-	27.3	27.3	29.0	19.9	16.0

General discussion

Since the first discovery of trypanosomosis by Hoare (1972), enormous efforts have been made to control and prevent the disease (Miles, 2004). Immense knowledge and data relating to diagnosis has become available. At the present, trypanosomosis detection basically depends on parasitological method as a gold standard test. However, low sensitivity of this method has led to introduction of various supplemental and alternative tools in which serological examination is one of the useful technique. In serological diagnosis, recombinant antigen is considered as an important factor in the development of accurate, sensitive, and practical diagnostics (Lejon *et al.*, 2005; Hernández *et al.*, 2010; Tran *et al.*, 2009).

This study proposed recombinant TR protein TeGM6 a novel diagnostic antigen for animal trypanosomosis. The antigen GM6 had been identified from both immunological screening and trypanosome genome informatics, demonstrated high antigenicity to animal sera experimentally infected with trypanosomes (Muller *et al.*, 1992; Thuy *et al.*, 2012). In order to improve the immunoreactivity and binding capacity of the antigen TeGM6 on NC membrane, TeGM6-4r was expressed. As I expected, the new antigen TeGM6-4r showed higher antigenicity than the previously characterized TbbGM6-2r, and clearly distinguished positive samples from negatives in ELISA. Another advantage is that since amino acid sequence of GM6 is highly conserved among the salivarian trypanosome, this universal antigen can detect the animals infected with several *Trypanosoma* spp. including *T. b. brucei, T. evansi, T. congolense* and *T. vivax.*

Evaluation of the TR antigen TeGM6-4r has been conducted using ELISA. The results demonstrated that TeGM6-4r had good characteristics of a universal antigen. The antigen had 100% specificity to *Theileria* and *Babesia* spp., and 81.4% to *T. theileri*. TeGM6-4r based ELISA demonstrated 100% specificity and 80% sensitivity in detection of *T. evansi* infection in experimentally infected WBs. For the field-derived samples, TeGM6-4r/ELISA could effectively determine seroprevalence of surra among WBs in Northern Vietnam. In comparison with CATT/*T. evansi*, TeGM6/ELISA detected higher number of positive sera. This might be attributed to cross-reaction with *T. theileri*; however, existence of *T. theileri* among WBs in Vietnam still needs to be confirmed. With sensitivity of 86.3% and specificity of 58.3% comparing to CATT/*T. evansi*, TeGM6-4r-based ELISA might be an effective and useful tool for assessing seroprevalence of surra in WBs.

Following on the evaluation of antigenicity, specificity and sensitivity of the recombinant antigen TeGM6-4r, the on-site diagnostic test TeGM6-4r/ICT was constructed subsequently. The test demonstrated remarkable performance. In detection of *T. evansi* infection from experimentally infected WBs, TeGM6-4r/ICT was comparable to TeGM6-4r/ELISA which could clearly distinguish negative and positive controls. The test was able to detect *T. brucei*, *T. congolense* and *T. vivax* in field derived samples obtained from cattle in Uganda and Tanzania. The result was in agreement with parasitological test and showed substantial agreement with *T. b. brucei* and *T. congolense* lysate antigen/ELISAs and TeGM6-4r/ELISA (kappa value 0.64, 0.72 and 0.78 respectively). ICT could detect both IgG and IgM in the serum samples while conventional ELISA detects only IgG.

Toward the validation and application of TeGM6-4r based ELISA and ICT for diagnosis of animal trypanosomosis, the tests have been conducted in field-derived serum samples obtained from sheep, goats and cattle in Kwazulu-Natal province, South Africa. This was the first time a variety of serodiagnostic tests has been evaluated for diagnosis of trypanosome infections in South Africa. The results of the study showed that both ELISA assays utilizing crude and recombinant (TeGM6-4r) trypanosome antigens were highly sensitive and efficient. Since production of recombinant antigen is more advantageous, TeGM6-4r ELISA can be a useful and reliable supplementary diagnostic technique to microscopy and PCR for animal trypanosomiasis in South Africa. The TeGM6-4r ICT was less sensitive than ELISA however is relatively specific, simple and rapid. Remarkably, the ICT results were highly in agreement with the results obtained from PCR method, which means ICT was able to detect the truly active infections. With further improvement on sensitivity the trypanosome ICT has the potential for use in both research and on-site diagnosis in trypanosome endemic countries.

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Abstract

Trypanosome infection is the worldwide distributed disease. Various species of animal including water buffaloes, horses and cattle have been affected leading to huge economic losses dues to reduction of the animal products (milk, meat, fur, etc.). Moreover, endemic areas of the disease are often located in the countryside where diagnostic laboratories are costly or inaccessible. Therefore, effective and accurate field test is of great interest to the scientists and so as to the farmers. My study aimed to develop and validate an immunochromatographic test (ICT), a rapid, sensitive and simple method for detection of animal trypanosomosis. For that purpose, identification of a novel antigen was an important and foremost step.

Chapter 1 describes the production of tandem repeat (TR) antigen TeGM6-4r as a novel diagnostic antigen for animal trypanosomosis. The newly expressed TeGM6-4r demonstrated higher immune-reactivity to water buffaloes (WBs) sera, which had been experimentally infected with *T. evansi,* compared to the previously characterized TbbGM6-2r, and clearly distinguished positive samples from negatives in ELISA. Another advantage of TeGM6-4r was that since amino acid sequence of GM6 is highly conserved among the salivarian trypanosome, using a single universal antigen could be able to detect the animls infected with several *Trypanosoma* spp. including *T. b. brucei, T. evansi, T. congolense* and *T. vivax.*

Chapter 2 describes the evaluation of the TR antigen TeGM6-4r using ELISA. The antigen had 100% specificity to *Theileria* and *Babesia* spp., and 81.4% to *T. theileri*.

TeGM6-4r based ELISA demonstrated 100% specificity and 80% sensitivity in detection of *T. evansi* infection in experimentally infected WBs. In the field condition, TeGM6-4r/ELISA had a sensitivity of 86.3% and specificity of 58.3% comparing to CATT/*T. evansi*. The test detected higher number of positive sera than CATT/*T. evansi*. This might be attributed to cross-reaction with *T. theileri*; however, existence of *T. theileri* among WBs in Vietnam still needs to be confirmed. This result indicated that TeGM6-4r-based ELISA might be an effective and useful tool for epidemiological survey of surra in WBs.

In Chapter 3, the on-site diagnostic test TeGM6-4r/ICT was constructed subsequently. The test demonstrated remarkable performance. In detection of *T. evansi* infection from experimentally infected WBs, TeGM6-4r/ICT was comparable to TeGM6-4r/ELISA which could clearly distinguish negative and positive controls. The test was able to detect *T. brucei*, *T. congolense* and *T. vivax* in field derived samples obtained from cattle in Uganda and Tanzania. The result was in agreement with parasitological test and showed substantial agreement with *T. b. brucei* and *T. congolense* lysate antigen/ELISAs and TeGM6-4r/ELISA (kappa value 0.64, 0.72 and 0.78 respectively). Moreover, ICT could detect both IgG and IgM in the serum samples while conventional ELISA detects only IgG.

In Chapter 4, the TeGM6-4r based ELISA and ICT was validated for diagnosis of animal trypanosomosis among sheep, goats and cattle in Kwazulu-Natal province, South Africa. This was the first time a variety of serodiagnostic tests has been evaluated for diagnosis of trypanosome infections in South Africa. Both ELISA assays utilizing crude and recombinant (TeGM6-4r) trypanosome antigens were highly sensitive and efficient. The TeGM6-4r ICT was less sensitive than ELISA however is relatively specific, simple

and rapid. Remarkably, the ICT results were highly in agreement with the results obtained from PCR method, which means ICT was able to detect the truly active infections.

In conclusion, an ICT utilizing a novel antigen TeGM6-4r were successfully developed. The antigen demonstrated high sensitivity (86.3%) and specificity (58.3-100%) in detection of *T. evansi* infection among WBs in Northern Vietnam. The TeGM6-4r/ICT was able to detect both *T. congolense* and *T. vivax* infections in cattle, sheep and goats in Uganda, Tanzania and South Africa. The test was comparable to parasitological test, PCR and TeGM6-4r/ELISA. With further improvement on sensitivity the trypanosome ICT has the potential for use in both research and on-site diagnosis in trypanosome endemic countries. Beside animal trypanosomosis detection the test may also be a good candidate for detection of human African trypanosomosis.

要旨

トリパノソーマ病は地球規模で分布しており、スイギュウ、ウマ、ウシなど様々な動物種に感染し、家畜の生産性を低下させることで莫大な経済的損失をもたらしている。加えて、流行地が農村地帯であることから高度な診断法が利用できないことが多い。よってトリパノソーマ病に対する効果的で正確な野外診断法を開発する事は研究者のみならず農民においても大きな関心事である。本学位論文で私はイムノクロマトグラフィー法（ICT）を応用して簡便、迅速かつ高感度な家畜トリパノソーマ病診断法を開発し、評価した。

第一章では家畜のトリパノソーマ病に対する新規診断用抗原であるタンデムリピート（TR）抗原 TeGM6-4r の組換え体蛋白質調整と評価について、研究を実施した。TeGM6-4r は *Trypanosoma evansi* 実験感染スイギュウ血清に対して以前の研究で評価した TbbGM6-2r よりも強い免疫反応性を示し、同抗原を用いた ELISA 法では陽性と陰性の検体を明確に区別することができた。TbbGM6-2r と TeGM6-4r は全く同じアミノ酸配列であるが、リピートユニットの反復回数だけが異なっている（前者は 2 回、後者は 4 回反復）。一般に TR 抗原を用いた酵素抗体法（ELISA）では、反復回数が増えるほど OD 値が高くなることが知られている。この現象は反復回数を増やすことで TR 抗原中のエピトープが増加し、より多くの抗体分子が結合できるようになるためであると説明されている。トリパノソーマ病診断用抗原として TeGM6-4r を用いるもう一つの利点として、同抗原が病原性トリパノソーマの種間で広く保存されていることがあげられる。これによって本研究

の対象である *T. evansi* に加えて、*T. congolense* や *T. vivax* など、動物アフリカトリパノソーマ病診断へ応用することも期待できる。

第二章では ELISA 法を用いて TeGM6-4r 抗原の実用性を評価した。TeGM6-4r はタイレリアおよびバベシア感染ウシ血清に対しては全く交叉反応を示さず、ウシの非病原性トリパノソーマ *T. theileri* 感染ウシ血清に対しては若干の交差反応性を示し、特異性は 81.4%となった。加えて TeGM6-4r 抗原 ELISA は *T. evansi* 実験感染スイギュウ血清に対して 100%の特異性と 80%の感度を示した。疫学調査で得たスイギュウ血清サンプルを用いた評価では標準血清診断法として採用した市販の血清診断キット CATT/*T. evansi* に対して、58.3%の特異性と 86.3%の感度を示した。TeGM6-4r 抗原 ELISA は CATT/*T. evansi* よりも多くの陽性例を検出したが、この中には *T. theileri* 感染スイギュウに対する交叉反応も含まれている可能性がある。現在ベトナムのスイギュウに *T. theileri* 感染がどの程度存在しているかについては不明であるため、今後の調査で事実を明らかにしていく必要がある。最後に、TeGM6-4r 抗原 ELISA を用いてベトナム北部から採取したスイギュウ血清の *T. evansi* 血清抗体陽性調査を実施した。その結果、同地域のスイギュウ群には *T. evansi* 感染が蔓延していることが明らかとなり、血清抗体陽性率は CATT/*T. evansi* では 27%、TeGM6-4r 抗原 ELISA では 53%であった。以上の結果から TeGM6-4r 抗原 ELISA はスイギュウのスーラ病（*T. evansi* 感染症）血清診断法として効果的かつ有用であることが示唆された。

第三章では開発途上国の獣医臨床現場で実用可能な診断法開発を目的として、TeGM6-4r 抗原を用いた ICT 法の開発を行った。*T. evansi* 実験感染スイギュウ血清を用いた評価において TeGM-4r 抗原 ICT は TeGM6-4r 抗原 ELISA と同等の特異性と感度を示し、陽性と陰性を完全に区別することができた。加えて、TeGM6-4r 抗原 ICT は過去にウガンダおよびタンザニアで採集したウシ由来血清サンプルから *T. brucei*, *T. congolense* ならびに *T. vivax* の動物アフリカトリパノソーマ症における主要病原体感染を検出することもできた。TeGM6-4r 抗原 ICT の結果は顕微鏡検査法、原虫細胞可溶化抗原を用いた OIE 標準 ELISA 法ならびに TeGM6-4r 抗原 ELISA と有意に一致しており、一致性の指標であるカッパ値はそれぞれ 0.64、0.72 ならびに 0.78 であった。以上の結果は動物のトリパノソーマ病血清診断に ICT 法が有用であることを示した初めての報告である。TeGM6-4r 抗原 ICT は同抗原 ELISA よりもやや低い感度を示したが、ELISA と異なり ICT では抗原特異的 IgM と IgG が同時に検出できるという利点もある。さらに現時点で唯一の市販診断キットである CATT/*T. evansi* は *T. evansi* 感染診断専用であるが、TeGM6-4r 抗原 ICT は *T. evansi* 感染に加えて他のアフリカトリパノソーマ感染診断にも利用できる点が優れている。

第四章では南アフリカで採集したヤギ、ヒツジおよびウシ血清サンプルを用いて TeGM6-4r 抗原 ICT による感染調査を実施した。原虫細胞可溶化抗原を用いた OIE 標準 ELISA 法ならびに TeGM6-4r 抗原 ELISA は極めて高感度であったが、TeGM6-4r 抗原 ICT はそれらより感度においては劣っていた。しかし ICT は簡便性

において ELISA に勝っており、ELISA よりもトリパノソーマ検出 PCR との間に高い結果の一致が認められた。この結果は ICT が治療が必要な感染動物を正確に検出できることを示唆している。上述の様々な診断法を用いた感染調査の結果、ヒツジ、ヤギ、ウシの有病率はそれぞれ 0～44.4%、0～9.1%、19.9～29.0%であった。CATT/*T. evansi* ではヒツジ全頭が陰性で、TeGM6-4r 抗原 ICT ではヤギ全頭が陰性を示した。過去に南アフリカで実施された疫学調査においてもウシの有病率が最も高くなっていることから、今回得られた結果は妥当であったと考えられる。加えて今回の感染調査の結果から、ヤギはトリパノソーマ感染に対して他の家畜よりも感受性が低いか、またはベクターであるアブの嗜好性が低いことが示唆された。一方で、ヤギのように感受性の低い宿主は待機宿主となる可能性もあるため、疫学調査対象家畜としての重要性は高いと考えられる。

本学位論文を総括すると、TeGM6-4r を用いた ICT は 86.3%の感度と、58.3～100%の特異性を示し、ベトナム北部のスイギュウ群を用いた評価において *T. evansi* の実用的診断法として優れていると結論付けられた。加えて TeGM6-4r 抗原 ICT はウシ、ヒツジならびにヤギのアフリカトリパノソーマ感染も診断することが可能であり、血清診断法として汎用性が高いことも示された。今後は同 ICT の検出感度をさらに向上させる研究を続けていくことで、実用的簡易迅速診断法が開発できると考えられる。さらに同 ICT 法がヒトのアフリカトリパノソーマ病診断法へ応用可能か否かについても研究を開始する必要がある。