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

2 buffaloes

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27 Abstract

28 *Trypanosoma evansi* infection, or surra, is currently affecting various species of
29 animals, especially water buffaloes. Since diagnosis is an important aspect of surra
30 control, development of novel diagnostic antigens is of interest to implement and
31 improve the currently utilized methods. Our study evaluated the tandem repeat antigen
32 TeGM6-4r in *T. evansi* antibody detection in water buffaloes. TeGM6-4r-based ELISA
33 was performed with 20 positive and 8 negative controls and 484 field samples from
34 water buffaloes in Northern Vietnam. To examine cross-reactivity, sera from Japanese
35 cattle that had been experimentally infected with *Theileria orientalis* (n=10), *Babesia*
36 *bovis* (n=3), *Babesia bigemina* (n=7) and *Trypanosoma theileri* (n=59) were included in
37 the study. The sensitivity of the test was 80%. TeGM6-4r did not react with *Theileria* or
38 *Babesia* infected sera, however it showed cross reactivity with 11/59 *T. theileri* infected
39 samples. The reference test, CATT/*T. evansi* also reacted with 3/59 *T. theileri* infected
40 sera. The lysate antigen-based ELISA reacted with 4/59 *T. theileri*, 9/10 *Theileria* and
41 3/10 *Babesia* infected sera. In contrast, TeGM6-4r-based ELISA was 86.3% sensitive
42 and 58.3% specific in the screening of field samples. The average seroprevalence of *T.*
43 *evansi* infection among water buffaloes in Northern Vietnam was 27.1% by CATT/*T.*
44 *evansi* and 53.7% by TeGM6-4r. Seroprevalence in the five surveyed provinces ranged

from 17.4% to 39.8% in the reference test, and 47.3% to 67.3% in the recombinant antigen based test. The finding indicated that the disease is still widely endemic in the area and that surveillance programs need to be carried out regularly to better control surra. We proposed TeGM6-4r as a useful serodiagnostic antigen for the detection and epidemiological surveillance of *T. evansi* infection among water buffaloes.

Keywords: seroprevalence, tandem repeat antigen, *Trypanosoma evansi*, water buffalo, Vietnam

1. Introduction

Serodiagnosis of *Trypanosoma evansi* infection, or surra, mostly relies on the lysate antigen of the parasite. Although lysate antigen production has been standardized, it is usually difficult for the standard protocol to be accepted and strictly adhered to by laboratory workers (Reid and Copeman, 2002; OIE, 2012). Furthermore, the lysate antigen shows cross-reactivity in diagnostic tests, leading to false-positive results. However, recombinant antigens can be developed to be highly specific and they have the additional advantage that laboratory animal usage 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). We reported that recombinant TbbGM6 from *Trypanosoma 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 our 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 water buffalo surra survey that was conducted in five provinces in Northern Vietnam utilizing TeGM6-4r-based ELISA, proposed the recombinant TR antigen “TeGM6-4r” as a novel diagnostic antigen candidate 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. Materials and methods

2.1. 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 MgCl₂, 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°C for 30 sec (denaturation), 54°C for 30 sec (annealing), and 7 °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/>).

2.2. *Expression of recombinant TeGM6-4r*

The expression procedure of the recombinant tandem repeat protein has been previously described (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 OD₆₀₀ 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 hrs. 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°C.

2.3. *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).

2.4. 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 a 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 that were 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).

2.5. *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 1 illustrates the locations and names of the 5 surveyed provinces in a map of Vietnam. Ha Giang, Cao Bang, Thai Nguyen, and Quang Ninh provinces 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. 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

Trypanidum[®] (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. At the sampling site, animals from all age groups and both sexes were randomly selected for sampling. 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°C. Sample size was calculated using sample size calculation software (<http://epitools.ausvet.com.au/content.php?page=SampleSize>) with an expected prevalence of 20% (Holland et al., 2004). In total, 484 serum samples were collected during the survey.

2.6. *CATT/T. evansi*

CATT/T. evansi was conducted according to the manufacturer's instructions (Institute of Tropical Medicine, Antwerp, Belgium) and the OIE manual for surra diagnosis (OIE, 2012).

2.7. *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 detail (Thuy et al., 2012; OIE, 2012). 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 hrs then blocked overnight with 1% bovine serum albumin (BSA). After washing with phosphate buffered saline containing 0.05% Tween 20 (PBS-T), serum samples diluted 200 times in dilution buffer (PBS-T containing 0.1% BSA) were added to each well and incubated at room temperature for 2 hrs. 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).

2.8. 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 adsorption in ELISA plates were performed. The adsorbed serum samples were collected and used within 1 week.

2.9. *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-absorbed with *E. coli* lysate with those after adsorption. Fisher's exact test was performed to compare seroprevalence of surra in different provinces. $P < 0.05$ indicated statistical significance.

3. Results

3.1. 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. Its molecular mass is estimated to be 32 kDa. The single-repeat domain is 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. 2A. Cutoff values (mean + 3SD) for lysate antigen-based ELISA and TeGM6-4r-based ELISA were calculated from the 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 out of 20 positive controls were found to be negative (false negative) by the recombinant protein. Sensitivity and specificity of the lysate antigen, for which there were no false negatives, were both 100%. Sensitivity and specificity 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. 2B). Another reference test, CATT/*T. evansi*, showed only cross-reaction with 3 out of 59 (5.1%) *T. theileri*-infected samples.

In order 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 $\pm$ 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.

3.2. Seroprevalence of surra among water buffaloes in Northern Vietnam determined by CATT/*T. evansi* and TeGM6-4r-based ELISA

Since CATT/*T. evansi* is standardized, widely evaluated and the OIE's recommended field test, we utilized it as a reference antibody detection test to evaluate TeGM6-4r-based ELISA. In this study, 484 field samples from water buffaloes in Northern Vietnam were screened by CATT/*T. evansi* and TeGM6-4r-based ELISA.

Seroprevalence of surra was determined and is 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 ranged from 17.4-22.0% and did not differ significantly ($P>0.05$). In the case of TeGM6-4r-based ELISA, 260 (53.7%) of the tested serum samples 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 (positive in both CATT/*T. evansi* and TeGM6-4r-based ELISA) and 147 were false positives (negative in CATT/*T. evansi* but positive in TeGM6-4r-based ELISA), giving 86.3% sensitivity and 58.3% specificity.

4. Discussion

The serodiagnostic value of the tandem repeat domain of GM6 for surra was first reported in our 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 the 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, 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 traces of *E. coli* proteins in rTeGM6-4r. 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 our study, lysate antigen had 100% sensitivity but showed strong cross-reactivity with *Theileria* and some *Babesia* infected sera, which differed from the report of Reid and Copeman (2002) in presenting high specificity but low to moderate sensitivity of lysate antigen. This indicates that the antigenicity of lysate antigen might be inconsistent due to differences in laboratory protocols and also differences in the interpretation of data.

In utilizing TeGM6-4r for our water buffalo surra survey in Northern Vietnam, we found that the disease was still widely endemic in the region. A seroprevalence of 27% was 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), while the surveys of Verloo et al. (2000) and Holland et al. (2004) reported seroprevalence of 16-22%. The high seroprevalence illustrates the widespread occurrence of *T. evansi* in Northern Vietnam at the present time. 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

288 previously infected buffalo are likely to develop clinical symptoms of diseases.
289 Consequently, outbreaks of surra and other infectious diseases usually occur during
290 winter. Among the surveyed provinces, Thai Nguyen province had the highest
291 seroprevalence (35.7% by CATT/*T.evansi* and 67.3% by Te-GM6-4r/ELISA). We found
292 this to be consistent with surra disease dynamics associated with the movement of
293 animals from highlands to the plains. It has been reported that buffaloes transported to
294 plain areas suffer from stress and are thus more susceptible to the disease. Although the
295 rate of anti-*T. evansi* antibodies detected in our study was high, it does not necessarily
296 reflect a high infection rate of surra. In these areas, local veterinarians provide drug
297 administration and antibodies remain in the blood circulation of the animals long after
298 successful treatment. Moreover, high seroprevalence, especially when determined by
299 TeGM6-4r, might be due to *T. theileri* infection since the recombinant antigen showed
300 81.4 % specificity to *T. theileri* infected sera. Non-pathogenic infection caused by *T.*
301 *theileri* has been reported in buffaloes (Rodrigues et al., 2003). In Vietnam, Sivakumar
302 et al. (2013) found the parasite in 10% of the cattle. However, the seroprevalence in
303 water buffaloes is still unknown. In the screening of field-derived serum samples,
304 TeGM6-4r demonstrated a sensitivity of 86.3% and specificity of 58.3%, in comparison
305 to the CATT/*T. evansi* reference test. The low specificity is best explained by its

cross-reactivity with *T. theileri*. The prevalence of *T. theileri* infection in the water buffaloes of northern Vietnam 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. We 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.

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396

397 FIGURE LEGENDS

398 Figure 1. Map of Vietnam showing the location of the five provinces surveyed in the
399 study during the period 2012-2013.

400

401 Figure 2. Evaluation of lysate antigen and TeGM6-4r in ELISA using confirmed
402 positive and negative water buffalo sera (A). Cutoff values of the tests were 0.33
403 and 0.35, respectively (indicated by broken lines). Samples from Japanese cattle
404 experimentally infected with each of the parasites *Theileria orientalis*, *Babesia*
405 *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

Provinces	Number of serum samples	CATT/ <i>T.evansi</i>			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	

Serum samples were collected during the period 2012-2013. ¹P value was calculated by using Fisher’s exact test.
*Indicates the surra prevalence in the province is statistically different from average prevalence (P value < 0.05).

Table 1. Nguyen *et al.*

Figure

Figure 1. Nguyen et al.

Figure 2. Nguyen et al.