The evaluation of GM6-based ELISA and ICT as diagnostic methods on a Mongolian farm with an outbreak of non-tsetse transmitted horse trypanosomosis

Batdorj Davaasuren[a,b], Tovuu Amgalanbaatar[b], Simon Peter Musinguzi[a], Keisuke Suganuma[a,c], Davaajav Otgonsuren[b], Ehab Mossaad[a, d], Sandagdorj Narantsatsral[b], Banzragch Battur[b], Badgar Battsetseg[b], Xuenan Xuan[a], Noboru Inoue[e, #]

[a] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido, Japan*

[b] *Institute of Veterinary Medicine, Laboratory of Molecular Genetics, Zaisan 17024, Ulaanbaatar, Mongolia*

[c] *Research Center for Global Agro-medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido, Japan*

[d] *College of Veterinary Medicine, Sudan University of Science and Technology, Khartoum, Sudan*

[e] *Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido, Japan*

[#] Address correspondence to Prof. Noboru Inoue, D.V.M., Ph.D., ircpmi@obihiro.ac.jp, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan

Tel.: +81-155-49-5647; Fax: +81-155-49-5643

Abstract

Trypanosoma equiperdum, which is the etiological agent of dourine, spreads through sexual intercourse in equines. Dourine (*T. equiperdum*) has been reported in Mongolia, where it is considered an economically important disease of horses. *T. evansi* has also been reported in Mongolian domestic animals. The objective of this study was to evaluate the potential application of recombinant *T. evansi* GM6 (rTeGM6-4r)-based diagnostic methods on a farm with an outbreak of non-tsetse transmitted horse trypanosomosis.

Ninety-seven percent homology was found between the amino acid sequences of *T. equiperdum* GM6 and the GM6 of another *Trypanozoon*, which also shared the same cellular localization. This finding suggests the utility of rTeGM6-4r-based serodiagnostic methods for epidemiological studies and the diagnosis of both surra and dourine in Equidae.

Fifty blood samples were examined from a herd of horses. The diagnostic value of an rTeGM6-4r-based ELISA and an rTeGM6-4r-based immunochromatographic test (ICT) were measured in comparison to a *T. evansi*

41 crude antigen-based ELISA, which is a diagnostic method recommended by the
42 OIE. However, this is not a perfect diagnostic method for trypanosomosis.
43 Positive serum samples were detected in 46%, 42% and 28% of the tested
44 horses using an rTeGM6-4r-based ELISA, crude antigen-based ELISA and
45 rTeGM6-4r-based ICT, respectively. The sensitivity of rTeGM6-based ELISA was
46 81%, the specificity was 79%, and the agreement was moderate. We conclude
47 that rTeGM6-4r-based ELISA and ICT represent alternative options for baseline
48 epidemiological studies and the on-site diagnosis of horse trypanosomoses in
49 the field, respectively.

50

51 *Keywords:* Dourine; ELISA; Immunochromatographic test; Mongolia; Outbreak

1. Introduction

In Mongolia, animal husbandry has not only been a tradition but also a main source of nutrition and raw materials for centuries. The horse is important in the present-day lives of many Mongolians. Horses are used not only for transportation but also for the herding of other livestock, entertainment and for the myriad goods they yield (Yazdzik, 2011). The livestock sector contributes 90% of the total agricultural production, which accounts for 11% of Mongolia's total GDP, and horses comprise 5.9% of the total livestock of the country (NRSO, 2015). Annually, horse meat production is worth approximately 48 million USD, which represented 4.1% of the livestock production in 2013 (FAO, 2016). During recent years, however, cases of non-tsetse transmitted horse trypanosomosis in Mongolia have tended to increase due to uncontrolled importation and cross-breeding of horses. The Mongolian agricultural sector has been severely affected by various infectious diseases, and the impact of these diseases on the national economy is currently a pertinent issue. (Altangerel et al., 2012). Thus,

67 the impact of non-tsetse transmitted horse trypanosomosis is an issue that
68 cannot be ignored.

69 Protozoan parasites are a common causative factor of equine diseases
70 throughout the world. Horses can be infected by *Trypanosoma brucei brucei*, *T.*
71 *vivax* and *T. congolense* in Africa, *T. cruzi* and *T. vivax* in South America and *T.*
72 *evansi* and *T. equiperdum* worldwide (Nimpaye et al., 2011). One such
73 protozoan parasite—*T. equiperdum*—causes dourine in equines (Stephen,
74 1986). *T. equiperdum* belongs to the *Trypanozoon* subgenus and is closely
75 related to *T. evansi* and *T. brucei. T. evansi*, the etiological agent of surra, has
76 the widest host range and geographical distribution. Its worldwide distribution is
77 attributed to mechanical transmission by biting insects such as tabanids.
78 Transmission can also be vertical, horizontal, iatrogenic and peroral, with
79 various epidemiological significances depending on the season, location and
80 host species (Desquesnes et al., 2013). *T. evansi* is an obligate blood parasite
81 that spreads through the whole body via the bloodstream, whereas *T.*
82 *equiperdum* has the unique ability to propagate in tissue rather than in the blood

83 circulation. This ability enables the parasite to spread through sexual intercourse
84 independently from vectors (Brun et al., 1998).
85 The clinical diagnosis of dourine is not always possible as clinical signs and
86 gross lesions are not always present or are not specific enough. A direct
87 laboratory diagnosis is also problematic, given that a low number of parasites
88 are normally present in infected tissues and during the mild, short-lasting
89 parasitemia (Pascucci et al., 2013). The complement fixation test (CFT) for
90 dourine is the test prescribed by the International Animal Health Code for the
91 testing of equines before they are moved internationally (OIE, 2016a, b).
92 However, the CFT has low sensitivity and sometimes gives inconclusive results,
93 and experienced technicians are required due to the numerous and
94 cumbersome preparatory steps. For the serological diagnosis of surra, the World
95 Organization for Animal Health (OIE) recommends the use of trypanosome
96 lysate antigen in an enzyme-linked immunosorbent assay (ELISA) and the card
97 agglutination test for *T. evansi* (CATT/*T. evansi*). Estimates of predictive values
98 indicate that ELISA for detecting IgG is more likely to correctly classify

uninfected animals, whereas the CATT is more likely to correctly classify truly infected animals. ELISA would thus be suitable for verifying the disease-free status of animals prior to movement or during quarantine (OIE, 2016a). Moreover, ELISA and CFT require laboratory equipment, and therefore, these methods are inconvenient for field use. Because perfect performance of serological diagnostic methods have not yet been established for animal trypanosomosis, it is important that serological tests be validated and standardised if they are to be suitable for correctly identifying infected animals; cross evaluation in different laboratories is thus required. Cross evaluation in different laboratories is therefore required (Desquesnes, 1997). Many of the new techniques in immunology and molecular biology, which have provided a great deal of fundamental information on the tsetse-transmitted trypanosomes, have yet to be applied to *T. evansi* (Luckins, 1988). Thus, good diagnostic methods and countermeasures are urgently needed.

Some protozoan antigens are composed of repeated amino acid sequences that display immunological dominance. The GM6 antigen, which is located within

115 the flagellum on the microtubular fibers of the membrane skeleton was identified
116 by its ability to immunoreact with bovine serum taken during the early phase of a
117 cyclic trypanosomal infection (Müller et al., 1992). GM6 is highly conserved
118 among the trypanosomes (Thuy et al., 2012). The recombinant GM6 antigen
119 (rTeGM6) derived from *T. evansi*, which consists of four repeats, was named
120 TeGM6-4r. This recombinant tandem repeat protein has already shown good
121 diagnostic value in *T. evansi*-infected water buffalo, cattle, goats and sheep
122 (Nguyen et al., 2015a; Nguyen et al., 2014). We therefore estimate that
123 TeGM6-4r might have good diagnostic value for non-tsetse transmitted horse
124 trypanosomoses.

125 An outbreak of non-tsetse transmitted horse trypanosomosis caused by *T.*
126 *evansi* was reported in the early 1980s in Kazakhstan and Russia near
127 Mongolian borders (Luckins, 1988). In contrast, dourine and *T. equiperdum* were
128 not reported in Mongolian horses until a survey carried out using a lyophilized *T.*
129 *equiperdum* crude antigen-based ELISA estimated the sero-prevalence of *T.*
130 *equiperdum* in Mongolian horses to be 5.5% (Clausen et al., 2003). Recently, a

131 new *T. equiperdum* strain was isolated from the urethral mucosa of a horse in
132 Mongolia that was clinically infected with dourine (Suganuma et al., 2016). Thus,
133 reports clearly show the potential risk of dourine in the Mongolian equine
134 industry.

135 The objective of this study was to evaluate the diagnostic potential of
136 rTeGM6-4r-based diagnostic methods on a Mongolian horse farm with an
137 outbreak of non-tsetse transmitted horse trypanosomosis.

138

139

140 **2. Materials and methods**

141 *2. 1. Study area*

142 This study was conducted in the middle of March 2016 in Ulziit Khoroo,
143 Ulaanbaatar city, which is located in the central part of Mongolia. In regard to an
144 individual stallion with edema of genital organ, the owner requested us to
145 conduct further diagnosis. With the objective to determine the main cause of the
146 observed symptoms. The herd included approximately 50 horses.

147

148 *2.2. Sample information*

149 In the present study, 50 blood samples were collected from a herd of horses
150 that included 9 males and 41 females (Supplemental Table 1). Permission was
151 obtained according to the standards of animal experimentation in Obihiro
152 University of Agriculture and Veterinary Medicine (Approval No. 28-45).
153 Approximately 5 mL of blood was drawn from the jugular vein into vacutainer
154 tubes; one with EDTA-2Na for DNA extraction, and the other for serum
155 separation. In addition to the blood samples, genital organ swabs were collected
156 from three selected horses with clinical signs of dourine. The total DNA of each
157 whole blood and genital organ swab sample was purified by phenol-chloroform
158 isoamilalchohol methods, as described by (Sambrook and Russell, 2001). The
159 serum was separated from the clotted blood. All samples were stored at -30ºC
160 until use.

161

2. 3. Cloning and sequence analysis of GM6 from T. equiperdum

Total DNA was extracted from a culture-adapted *T. equiperdum* IVM-t1 strain (Suganuma et al., 2016). The GM6 gene of *T. equiperdum* was amplified by a PCR with GM6 primer sets (Table 1). The amplicon was cloned into pCR™2.1-TOPO® vector, and the nucleotide sequence of *T. equiperdum* GM6 was determined using an ABI Prism 3100 Genetic Analyzer (Thermo Fisher Scientific, MA, USA). The amino acid sequence of *T. equiperdum* GM6 was compared to the GM6 of *T. evansi* (Thuy et al., 2012), *T. b. brucei* (accession number: XP_828202.1) and *T. b. gambiense* (accession number: Q26755.1) by ClustalW multiple alignment (Bioedit 7.2.5).

2. 4. Indirect immunofluorescence antibody test (IFAT) for T. equiperdum

Smears prepared from swabs of the genital organ from horses with suspected dourine infection were fixed with methanol, followed by blocking with 5% skim milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T) at room

177 temperature for 1 hour. The smears were washed 3 times with TBS-T and were
178 then incubated with mice anti-TeGM6-4r antibodies. The smears were
179 subsequently incubated with anti-mouse IgG-DyeLight 488 (Thermo Fisher
180 Scientific) and Hoechest 33348 (Dojindo, Inc., Kumamoto, Japan) for kinetoplast
181 staining and nucleus staining, respectively. The prepared IFAT slides were
182 examined using a confocal laser scanning microscope (TCS-NT, Leica
183 Microsystems GmbH, Wetzlar, Germany).
184

185 *2. 5. Positive and negative control sera*

186 One positive and 20 negative serum samples were obtained from Mongolian
187 horses. The positive serum sample was collected from a horse that was
188 definitively diagnosed as dourine-positive based on the microscopic observation
189 of active movement of *T. equiperdum* that were obtained from a genital organ
190 swab (Suganuma et al., 2016). Negative sera were selected from Mongolian
191 horses by a KIN-PCR using total DNA extracted from whole blood samples and
192 genital organ swabs. The status of surra of each animal was also confirmed by

193 CATT/*T. evansi* (Institute of Tropical Medicine, Antwerp, Belgium), according to
194 the OIE manual for the diagnosis of surra (OIE, 2016a). After substracting the
195 blank well optical density (OD) value, the standard deviation and average values
196 of the OD values of the negative samples were calculated. The cut-off value for
197 ELISA was determined based on the summation of the mean value of negative
198 samples plus 3 times the standard deviation of the OD values of the negative
199 samples.

200

201 *2. 6. PCR*

202 All of the DNA samples that were extracted from the whole blood (n=50) and
203 swabs of the genital organs of selected horses (IDs 14, 15 and 20) showing the
204 characteristic symptoms of dourine were analyzed by an internal transcribed
205 spacer 1 (ITS1) PCR (Njiru et al., 2005) (Table 1). The PCR cycles were as
206 follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for
207 40 s, 58°C for 40 s, 72°C for 90 s, and final extension at 72°C for 5 min.

208

209 *2. 7. Crude trypanosome antigen and recombinant T. evansi GM6-4r-based*

210 *ELISA*

211 The rTeGM6-4r-based ELISA and the trypanosome cell lysate crude

212 antigen-based ELISA plates were prepared in accordance with a previous study

213 and the OIE manual, respectively (Nguyen et al., 2014; OIE, 2016a) with minor

214 modifications. In brief, Maxisorp 96-well plates (Nalgene-Nunc, NY, USA) were

215 coated with 1 µg/well of the crude antigen or 200 ng/well of rTeGM6-4r diluted in

216 a coating buffer (50 mM carbonate-bicarbonate buffer [pH 9.6]) for 4 h. Then the

217 coated plates were blocked overnight with 3% skim milk in PBS-T. After washing

218 with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), 200 times

219 diluted sera in dilution buffer (PBS-T containing 3% skim milk) was added to

220 each well and incubated at room temperature for 2 h. The plates were then

221 washed with PBS-T before adding 5000 times diluted anti-horse IgG rabbit

222 antibodies conjugated with horseradish peroxidase (Thermo Fisher Scientific).

223 After 2 h of incubation, the plates were washed with PBS-T. Then, 2'-azino-bis

3-ethylbenzothiazoline-6-sulphonic acid (ABTS) solution in 0.05M mixture of citric and phosphoric acid and 0.0075% hydrogen peroxide were applied, and the plates were incubated for 30 min at 37ºC. After incubation, the absorbance of each well at 405 nm was read with an MTP-500 microplate reader (Corona Electric, Ibaraki, Japan).

2. 8. rTeGM6-4r-based immunochromatographic test (ICT)

The rTeGM6-4r-based ICT was prepared in accordance with the methods of a previous study (Nguyen et al., 2015b). To perform the ICT, 10 µL of a serum sample was diluted five times with PBS and loaded on the sample pad. An ICT was determined to be positive if it displayed both the test and control lines and was considered to be negative if it only displayed the control line.

2. 9. Statistical analysis

The degree of agreement between diagnostic methods was quantified by

kappa statistics and 95% confidence intervals using the Microsoft Excel software program (Microsoft, Redmond, WA, USA) according to the methods of Jacob Cohen (Cohen, 1960) and Clopper and Pearson (Clopper and Pearson, 1934), respectively. The distribution of OD values for ELISA data was visualized using the GraphPad Prism 6.0 software program (GraphPad Software, Inc., CA, USA).

3. Results

3.1. Characterization of T. equiperdum *GM6 as a candidate serodiagnostic antigen*

The amino acid sequences of GM6 from different *Trypanozoon* parasites were aligned to examine its homology (Fig. 1). The amino acid sequences of the GM6 genes from *T. equiperdum, T. evansi, T. brucei* and *T. b. gambiense* showed 97–98% homology. Among the 68 amino acid repeat units within *Trypanozoon*, there were only two different amino acids. This indicates that the sequence of tandem repeat units in GM6 is highly conserved among the

255 *Trypanozoon* subgenus. In addition, the cellular localization *of T. equiperdum*
256 GM6 was analyzed by IFAT using anti-*T. evansi* GM6 antibodies. *T. equiperdum*
257 GM6 was localized along the flagellum of the parasite, which was identical to the
258 previously reported cellular localization of GM6 in the *Trypanozoon* subgenus
259 (Hayes et al., 2014; Müller et al., 1992) (Fig. 2). Moreover, anti-*T. evansi* GM6
260 antibodies showed a strong cross-reaction with *T. equiperdum* parasites.
261

262 *3. 2. Field trial of rTeGM6-4r-based serodiagnostic methods for horses*

263 Samples from 50 horses collected from one farm, on which an epidemic of
264 trypanosomoses was clinically suspected, were screened. Of the 50 samples,
265 46%, 42% and 28% were found to be positive using the rTeGM6-4r-based ELISA
266 (Fig. 3-A), crude antigen-based ELISA (Fig. 3-B) and rTeGM6-4r-based ICT,
267 respectively. The diagnostic value of the rTeGM6-4r-based ELISA and the
268 rTeGM6-4r-based ICT was measured in comparison to the crude antigen-based
269 ELISA. The sensitivity and specificity of the rTeGM6-4r-based ELISA were 81%
270 and 79%, respectively (Table 2). The Cohen's kappa value between

271 rTeGM6-4r-based ELISA and crude antigen-based ELISA was 0.60, and the
272 strength of agreement between the rTeGM6-4r-based ELISA and crude
273 antigen-based ELISA was considered 'moderate' (Table 2). The kappa value
274 between the rTeGM6-4r-based ICT and crude antigen-based ELISA was 0.53.
275 Additionally, a 2-year-old horse (ID 2) was seropositive by all three tests, even
276 though this horse had not yet mated with a stallion. The horse (ID2) was born to
277 a mare (ID 31) and stallion (ID29) that were found to be positive by all three tests
278 (Supplemental Table 1).

279

280 *3. 3. Results of the ITS1 PCR-based diagnosis of horses*

281 Genital organ swabs from three selected horses (IDs 14, 15 and 20) that
282 were found to be strongly positive by both the ELISA and ICT (Supplemental
283 Table 1), were clearly positive on the PCR (Supplemental Fig. 1). Moreover, the
284 active movement of trypanosomes was observed from a genital swab sample
285 from a horse (ID14) by microscopy. In contrast, all of the whole blood samples
286 were found to be negative by both Giemsa staining and a PCR (data not shown).

4. Discussion

T. equiperdum and *T. evansi* are cosmopolitan trypanosomes. The former trypanosome causes dourine via sexual transmission in Equidae, whereas the latter causes surra via mechanical transmission through bloodsucking insects such as *Tabanus* spp. Nowadays, Mongolian herdsmen and veterinarians have a strong need for diagnostic and mass screenings that can be applied on-site due to the relatively high prevalence of these diseases (Clausen et al., 2003; Desquesnes et al., 2013).

In this study, the *T. evansi* GM6 antigen was evaluated with two diagnostic tools: an ICT and an ELISA for non-tsetse transmitted horse trypanosomosis, especially dourine. In all previous studies, crude antigen-based ELISA for trypanosomes exhibited high sensitivity and specificity, generally >90%. However, the ELISA is not a true gold standard; rather, it is a recommended serological test in trypanosomes (Kocher et al., 2015). Because the measured

303 sensitivity and specificity are calculated using crude antigen-based ELISA as a
304 reference, they are relative rather than true values.

305 *T. equiperdum* GM6 cross-reacted with anti-*T. evansi* GM6 antibody, with
306 localization detected along the flagella (Fig. 2). The GM6 amino acid sequence
307 was observed to have high similarity among *Trypanozoon,* and the antigens
308 were almost the same (Fig. 1). These results suggested that in addition to surra
309 in Equidae, these rTeGM6-based serodiagnostic methods would be useful for
310 epidemiological studies involving dourine and for its diagnosis. Similarly, (Pillay
311 et al., 2013) reported that the *T. vivax* GM6 is an excellent candidate antigen for
312 the development of a point-of-care test for the diagnosis of animal African
313 trypanosomoses in cattle that are caused by *T. vivax* and *T. congolense*. A
314 previous report noted that the considerable sensitivity of rTeGM6-4r would make
315 it a useful antigen for the diagnosis of surra in future surveillance programs
316 (Nguyen et al., 2014). A similar study of TcoCB1 and TvGm6 antigens, which are
317 homologous proteins of TeGM6, showed high levels of sensitivity and specificity
318 of their rapid tests (Boulange et al., 2017).

In the current study involving horses, the sensitivity and specificity of rTeGM6-4r-based ELISA were moderate at 81% and 79%, respectively (Table 2). This higher sensitivity of the ELISA in comparison to the ICT could be due to the enzyme-substrate reaction, which enhances the detection process when there is a low antibody titer. In our previous study involving ruminants, we also found that the rTeGM6-4r based ICT was less sensitive than the ELISA. However, it was relatively specific, simple and rapid (Nguyen et al., 2015a). The higher specificity of the rTeGM6-4r-based ICT in this study might be explained by lower cross-reactivity among the tested samples. The slightly higher OD values in the negative control wells in the crude antigen-coated plates suggests the possibility of cross-reaction with a non-specific antibody in the sera of the horses (Fig. 3-B). It is not surprising that cross-reactions occur with other trypanosome species and even other parasites in most instances in which crude lysates of the antigen of a given trypanosome strain are cultured *in vivo* or *in vitro* (Magez and Radwanska, 2014). Among the samples that showed a positive reaction in the rTeGM6-4r-based ICT, only one sample was found to be negative by the ELISAs.

335 This sample might represent a newly infected horse in which the infection could
336 not be detected using GM6 and the crude antigen-based ELISA because of the
337 low IgG titer. However, ICT can detect both IgG and IgM, which is the dominant
338 immunoglobulin at the early stage of dourine infection (Nguyen et al., 2015b).
339 The DNA extracted from the genital organs from the three selected horses
340 was found to be positive by a PCR, whereas none of the DNA samples from
341 whole blood were found to be positive (Supplemental Fig. 1). These PCR results
342 strongly suggested that these horses had dourine. This is also supported by the
343 fact that *T. equiperdum* is primarily a tissue parasite; thus, although *T.*
344 *equiperdum* can often be found in smears taken from the genitalia or plaques, it
345 is not usually present in the circulating blood (Gunn and Pitt, 2012).
346 Based on the observation of the active movement of *T. equiperdum* by
347 microscopy and the identification of its DNA in a PCR using genital swab
348 samples, we concluded that the horses were truly infected with dourine. This
349 conclusion is strongly supported by the study of Suganuma et al. (2016). The
350 PCR results in our ongoing surveillance project in Mongolia have also revealed

surra epidemics in other domestic animals (data not shown). Suganuma et al. (Suganuma et al., 2016) recently isolated a new *T. equiperdum* strain from a dourine-infected horse in Mongolia. The recorded prevalence (40%) of the non-tsetse transmitted horse trypanosomosis in the herd indicated that the disease has been spreading for a long time. However, the majority of the horses showed no clinical signs.

During the last decades, importation of horses mainly from foreign countries such as Russia and other European countries to Mongolia has tended to result in crossbreeding. Proper veterinary checkups are often not done on imported horses in animal quarantine. Moreover, stallions without veterinary checkup frequently migrate with their owners within the country and spread dourine in their harems in Mongolia (Hund, 2008). These two domestic and international horse migration events might play a key role in the spread of disease in Mongolia. The main risk factor is the herders' inadequate knowledge in relation to the diagnosis and treatment of dourine. In this emerging market economy, inadequate incentives exist for herders to invest in disease control and animal

367 health (Goodland et al., 2009).

368 The possible infection of a young horse (ID 2), which had not been involved
369 in mating, suggests the possibility of vertical transmission of the disease
370 because the young horse was sired by an infected stallion (ID29) and dammed
371 by an infected mare (ID31), and the serodiagnosis of both animals was clearly
372 positive (Supplemental Table 1). This evidence agreed with a report that
373 suggested that the transmission can be vertical, horizontal, iatrogenic and
374 peroral, each of which has varying degrees of epidemiological significance,
375 depending on the season, location and host species (Desquesnes et al., 2013).

376 The results of the present study revealed that the GM6 gene of *T. evansi*
377 and *T. equiperdum* are almost identical in terms of the tandem repeat units. This
378 finding allows this antigen to be used for diagnosing dourine. In the present
379 study, rTeGM6-4r showed good diagnostic value in testing the sera of *T.*
380 *equiperdum*-infected horses. Similar results were found in domestic animals
381 infected with *T. evansi* (Nguyen et al., 2015a; Nguyen et al., 2014). In conclusion,
382 the rTeGM6-4r-based ELISA and ICT may offer alternative diagnostic methods

for large-scale epidemiological studies and the on-site diagnosis of non-tsetse transmitted horse trypanosomoses in the field. Moreover, the results of this preliminary epidemiological study indicated that there are ongoing epidemics of horse trypanosomoses in Mongolia. However, these methods could not distinguish between surra and dourine; thus, new definitive diagnostic methods need to be developed based on the genome information of the new *T. equiperdum* IVM-t1 strain.

391 **Acknowledgments**

392 We thank Ms. Noriko Endo, Ms. Shoko Hayakawa and Ms. Batuyanga for their
393 project management in Mongolia. We thank all of the Mongolian SATREPS
394 project researchers for their support of this study. We also thank Ms. Yoko
395 Matsushita for her excellent technical assistance. This study was financially
396 supported by the Japan Society for the Promotion of Science (JSPS),
397 Grants-in-Aid for Scientific Research from the Ministry of Education, Culture,
398 Sports, Science and Technology (MEXT), and the AMED/JICA SATREPS.

5. References

Altangerel, K., Sivakumar, T., Battsetseg, B., Battur, B., Ueno, A., Igarashi, I., Yokoyama, N., 2012. Phylogenetic relationships of Mongolian Babesia bovis isolates based on the merozoite surface antigen (MSA)-1, MSA-2b, and MSA-2c genes. Vet Parasitol 184, 309-316.

Boulange, A., Pillay, D., Chevtzoff, C., Biteau, N., Come de Graca, V., Rempeters, L., Theodoridis, D., Baltz, T., 2017. Development of a rapid antibody test for point-of-care diagnosis of animal African trypanosomosis. Vet Parasitol 233, 32-38.

Brun, R., Hecker, H., Lun, Z.R., 1998. Trypanosoma evansi and T. equiperdum: distribution, biology, treatment and phylogenetic relationship (a review). Vet Parasitol 79, 95-107.

Clausen, P.H., Chuluun, S., Sodnomdarjaa, R., Greiner, M., Noeckler, K., Staak, C., Zessin, K.H., Schein, E., 2003. A field study to estimate the prevalence of Trypanosoma equiperdum in Mongolian horses. Vet Parasitol 115, 9-18.

Clopper, C.J., Pearson, E.S., 1934. The use of confidence or fiducial limits illustrated in the case of the binomial Biometrika 26, 404-413.

Cohen, J., 1960. A Coefficient of Agreement for Nominal Scales. Educational and Psychological Measurement 20, 37-46.

Desquesnes, M., 1997. [International and regional standardization of immunoenzyme tests: methods, concerns and limitations]. Rev Sci Tech 16, 809-823.

Desquesnes, M., Dargantes, A., Lai, D.H., Lun, Z.R., Holzmuller, P., Jittapalapong, S., 2013. Trypanosoma evansi and surra: a review and perspectives on transmission, epidemiology and control, impact, and zoonotic aspects. Biomed Res Int 2013, 321237.

FAO 2016. FAOSTAT (FAO).

Goodland, A., Sheehy, D., Shine, T., 2009. Mongolia Livestock Sector Study, Volume I– Synthesis Report. Sustainable Development Department, East Asia and Pacific Region, World Bank, Washington, DC.

Gunn, A., Pitt, S.J., 2012. Parasitology : an integrated approach. John Wiley & Sons, Chichester, West Sussex.

Hayes, P., Varga, V., Olego-Fernandez, S., Sunter, J., Ginger, M.L., Gull, K., 2014. Modulation of a cytoskeletal calpain-like protein induces major transitions in trypanosome morphology. J Cell Biol 206, 377-384.

Hund, A. 2008. The Stallion's Mane: The Next Generation of Horses in Mongolia (SIT Digital Collections Donald B. Watt library, Independent Study Project (ISP) Collection).

Kocher, A., Desquesnes, M., Kamyingkird, K., Yangtara, S., Leboucher, E., Rodtian, P., Dargantes, A., Jittapalapong, S., 2015. Evaluation of an Indirect-ELISA Test for Trypanosoma evansi Infection (Surra) in Buffaloes and Its Application to a Serological Survey in Thailand. Biomed Res Int 2015, 361037.

Luckins, A.G., 1988. Trypanosoma evansi in Asia. Parasitol Today 4, 137-142.

Magez, S., Radwanska, M., 2014. Trypanosomes and trypanosomiasis. Springer, Wien, x, 294 pages pp.

Müller, N., Hemphill, A., Imboden, M., Duvallet, G., Dwinger, R.H., Seebeck, T., 1992. Identification and characterization of two repetitive non-variable antigens from African trypanosomes which are recognized early during infection. Parasitology 104 Pt 1, 111-120.

Nguyen, T.T., Motsiri, M.S., Taioe, M.O., Mtshali, M.S., Goto, Y., Kawazu, S., Thekisoe, O.M., Inoue, N., 2015a. Application of crude and recombinant ELISAs and immunochromatographic test for serodiagnosis of animal trypanosomosis in the

Umkhanyakude district of KwaZulu-Natal province, South Africa. J Vet Med Sci 77, 217-220.
Nguyen, T.T., Ruttayaporn, N., Goto, Y., Kawazu, S., Sakurai, T., Inoue, N., 2015b. A TeGM6-4r antigen-based immunochromatographic test (ICT) for animal trypanosomosis. Parasitol Res 114, 4319-4325.
Nguyen, T.T., Zhou, M., Ruttayaporn, N., Nguyen, Q.D., Nguyen, V.K., Goto, Y., Suzuki, Y., Kawazu, S., Inoue, N., 2014. Diagnostic value of the recombinant tandem repeat antigen TeGM6-4r for surra in water buffaloes. Vet Parasitol 201, 18-23.
Nimpaye, H., Njiokou, F., Njine, T., Njitchouang, G.R., Cuny, G., Herder, S., Asonganyi, T., Simo, G., 2011. Trypanosoma vivax, T. congolense "forest type" and T. simiae: prevalence in domestic animals of sleeping sickness foci of Cameroon. Parasite (Paris, France) 18, 171-179.
Njiru, Z.K., Constantine, C.C., Guya, S., Crowther, J., Kiragu, J.M., Thompson, R.C., Davila, A.M., 2005. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol Res 95, 186-192.
NRSO, M. 2015. Mongolian statistical year book Kh.Bajiikhuu, S.M.R.S.A.D., ed. (http://www.1212.mn/en, National Registration and Statistics Office of Mongolia).
OIE 2016a. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016. In World Organization for Animal Healt (http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/ OIE) - World Organisation for Animal Health.
OIE 2016b. Terrestrial Animal Health Code 2016 (http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_dourine.htm OIE), OIE - World Organisation for Animal Health.
Pascucci, I., Di Provvido, A., Camma, C., Di Francesco, G., Calistri, P., Tittarelli, M., Ferri, N., Scacchia, M., Caporale, V., 2013. Diagnosis of dourine in outbreaks in Italy. Vet Parasitol 193, 30-38.
Pillay, D., Izotte, J., Fikru, R., Buscher, P., Mucache, H., Neves, L., Boulange, A., Seck, M.T., Bouyer, J., Napier, G.B., Chevtzoff, C., Coustou, V., Baltz, T., 2013. Trypanosoma vivax GM6 antigen: a candidate antigen for diagnosis of African animal trypanosomosis in cattle. PLoS One 8, e78565.
Sambrook, J., Russell, D.W., 2001. Molecular cloning : a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
Stephen, L.E., 1986. Trypanosomiasis: a veterinary perspective. Wheaton, London, 1-551.
Suganuma, K., Narantsatsral, S., Battur, B., Yamasaki, S., Otgonsuren, D., Musinguzi, S.P., Davaasuren, B., Battsetseg, B., Inoue, N., 2016. Isolation, cultivation and molecular characterization of a new Trypanosoma equiperdum strain in Mongolia. Parasit Vectors 9, 481.
Thuy, N.T., Goto, Y., Lun, Z.R., Kawazu, S., Inoue, N., 2012. Tandem repeat protein as potential diagnostic antigen for Trypanosoma evansi infection. Parasitol Res 110, 733-739.
Yazdzik, E. 2011. The Mongolian Horse and Horseman (SIT Digital Collections Donald B. Wat Library, Independent Study Project (ISP) Collection).

495 **Figure captions**

496 Fig. 1. Comparison of the amino acids in the tandem repeat unit of GM6 among the
497 *Trypanozoon* subgenus. Ninety-seven percent amino acid sequence homology was
498 observed between the GM6 genes from *T. equiperdum* IVM-t1 strain, *T. evansi*
499 Tansui strain, *T. b. brucei* (Accession no. XP_828202.1) and *T. b. gambiense*
500 (Accession no. Q26755.1). A single unit of tandem repeats consists of 68 amino acids.
501 The red colored amino acids indicate differences.

502

503 Fig. 2. The cellular localization of *T. equiperdum* GM6 antigen. *T. equiperdum* GM6
504 cross-reacted with anti-*T. evansi* GM6 antibody. *T. equiperdum* GM6 was localized
505 along the flagella. Left panel: GM6 antigen localization (green signal). Right panel:
506 Merged image (red: a kinetoplast and the nucleus).

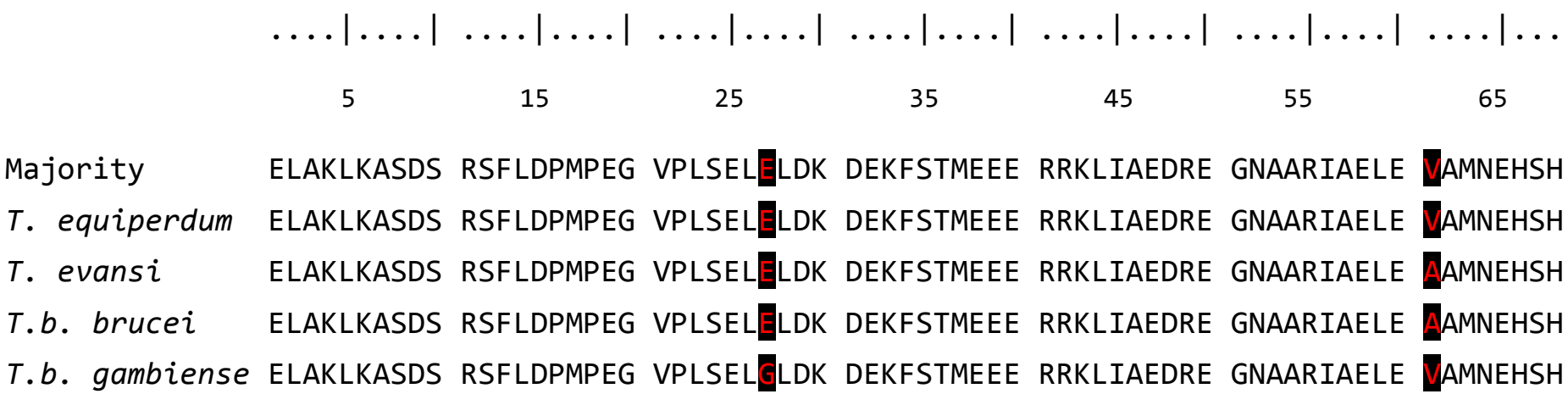
507

508 Fig. 3. Optical density distribution of the two ELISA methods. Optical density
509 distribution of farm samples (N=50) and negative controls (N=20) by
510 rTeGM6-4r-based ELISA (A) and crude antigen-based ELISA (B). Cut-off values of
511 rTeGM6-4r-based ELISA and crude antigen-based ELISA were 0.34 and 0.44,
512 respectively.

513

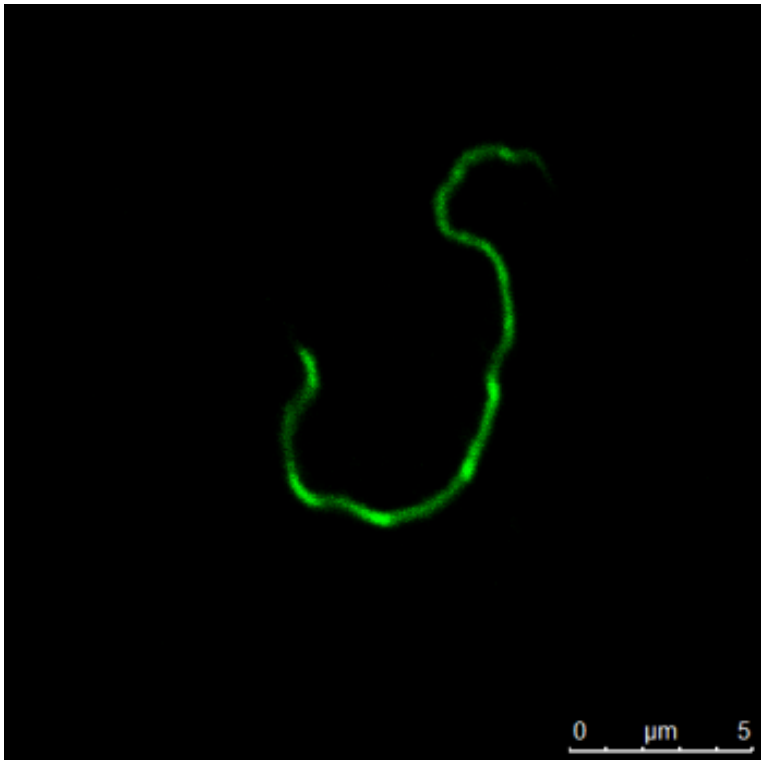
514 Supplemental Fig. 1. ITS1-PCR results from genital organ swabs. The PCR results
515 for selected swab samples. M, a 100-bp DNA marker; 1, Sample ID 14; 2, Sample ID
516 15; 3, Sample ID 20; 4, a negative control (swab sample from a healthy horse); 5, a
517 positive control (total DNA of *T. equiperdum* IVM-t1). Dourine infection was strongly

518 indicated in all three samples based on positive results in three of the serodiagnostic
519 tests and the clinical signs.



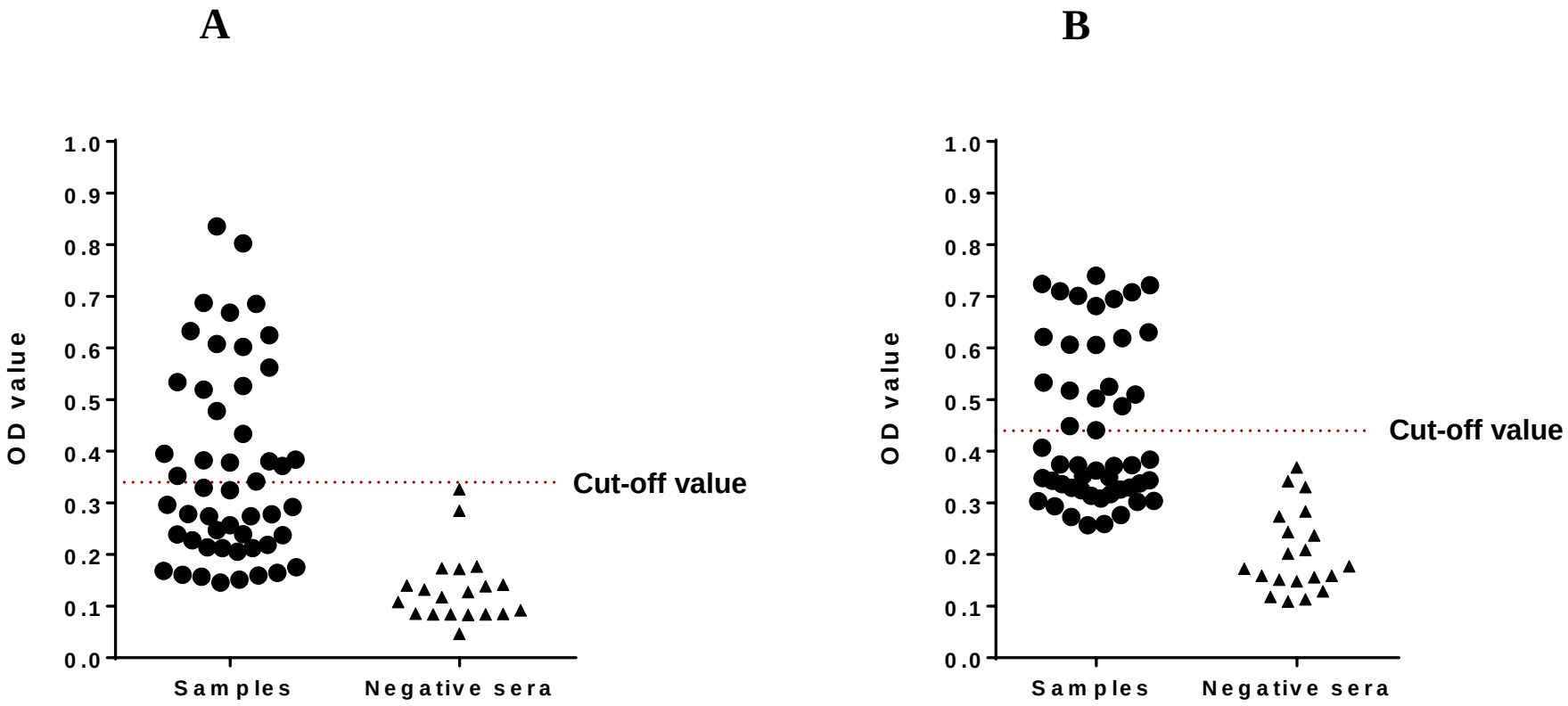
Batdorj *et al.*, Fig. 1

T. equiperdum GM6



Merged

Batdorj *et al.*, Fig. 2



Batdorj *et al.*, Fig. 3

Table 1. The PCR primer sets

Method	Sequence	Target locus	Size (bp)	Reference
ITS1 PCR	5’- CCG GAA GTT CAC CGA TAT TG - 3’ 5’- TTG CTG CGT TCT TCA ACG AA- 3’	ITS1 region	540	Njiru *et al.*
GM6 PCR	5’-GGA TCC ATG GAG CTT GCT AAA-3’ 5’-GAA TTC CTA ATG TGA ATG CTC-3’	GM6 TR units	Variable	Thuy *et al.*

Table 2. Comparison of the results of the different serodiagnostic tests

		Crude antigen ELISA[a]			Sensitivity	95% CI[b]	Specificity	95% CI[b]	Agreement[c]	Kappa[d]
		Positive	Negative	Total						
rTeGM6-4r ELISA	Positive	17	6	50	81%	58%-95%	79%	60%-92%	80%	0.60
	Negative	4	23							
rTeGM6-4r ICT	Positive	12	2	50	57%	32%-78%	93%	77%-99%	78%	0.53
	Negative	9	27							

[a] Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016

[b] Confidence intervals at 95% for sensitivity and specificity

[c] Number of observed agreement

[d] Cohen’s Kappa value