



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

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2 **Mongolian farm with an outbreak of non-tsetse transmitted horse**  
3 **trypanosomosis**

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25 **Abstract**

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

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

38 Fifty blood samples were examined from a herd of horses. The diagnostic  
39 value of an rTeGM6-4r-based ELISA and an rTeGM6-4r-based  
40 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

## 52 **1. Introduction**

53 In Mongolia, animal husbandry has not only been a tradition but also a main  
54 source of nutrition and raw materials for centuries. The horse is important in the  
55 present-day lives of many Mongolians. Horses are used not only for  
56 transportation but also for the herding of other livestock, entertainment and for  
57 the myriad goods they yield (Yazdzik, 2011). The livestock sector contributes  
58 90% of the total agricultural production, which accounts for 11% of Mongolia's  
59 total GDP, and horses comprise 5.9% of the total livestock of the country (NRSO,  
60 2015). Annually, horse meat production is worth approximately 48 million USD,  
61 which represented 4.1% of the livestock production in 2013 (FAO, 2016). During  
62 recent years, however, cases of non-tsetse transmitted horse trypanosomosis in  
63 Mongolia have tended to increase due to uncontrolled importation and  
64 cross-breeding of horses. The Mongolian agricultural sector has been severely  
65 affected by various infectious diseases, and the impact of these diseases on the  
66 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



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

113       Some protozoan antigens are composed of repeated amino acid sequences  
114 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 isoamilalcohol methods, as described by (Sambrook and Russell, 2001). The  
159 serum was separated from the clotted blood. All samples were stored at -30°C  
160 until use.

161

162 2. 3. *Cloning and sequence analysis of GM6 from T. equiperdum*

163 Total DNA was extracted from a culture-adapted *T. equiperdum* IVM-t1  
164 strain (Suganuma et al., 2016). The GM6 gene of *T. equiperdum* was amplified  
165 by a PCR with GM6 primer sets (Table 1). The amplicon was cloned into  
166 pCR™2.1-TOPO® vector, and the nucleotide sequence of *T. equiperdum* GM6  
167 was determined using an ABI Prism 3100 Genetic Analyzer (Thermo Fisher  
168 Scientific, MA, USA). The amino acid sequence of *T. equiperdum* GM6 was  
169 compared to the GM6 of *T. evansi* (Thuy et al., 2012), *T. b. brucei* (accession  
170 number: XP\_828202.1) and *T. b. gambiense* (accession number: Q26755.1) by  
171 ClustalW multiple alignment (Bioedit 7.2.5).

172

173 2. 4. *Indirect immunofluorescence antibody test (IFAT) for T. equiperdum*

174 Smears prepared from swabs of the genital organ from horses with  
175 suspected dourine infection were fixed with methanol, followed by blocking with  
176 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 Hoechst 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 subtracting 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



224 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) solution in 0.05M mixture of  
225 citric and phosphoric acid and 0.0075% hydrogen peroxide were applied, and  
226 the plates were incubated for 30 min at 37°C. After incubation, the absorbance  
227 of each well at 405 nm was read with an MTP-500 microplate reader (Corona  
228 Electric, Ibaraki, Japan).

229

## 230 2. 8. *rTeGM6-4r-based immunochromatographic test (ICT)*

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

236

## 237 2. 9. *Statistical analysis*

238 The degree of agreement between diagnostic methods was quantified by

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

244

245

### 246 **3. Results**

#### 247 *3.1. Characterization of T. equiperdum GM6 as a candidate serodiagnostic* 248 *antigen*

249 The amino acid sequences of GM6 from different *Trypanozoon* parasites  
250 were aligned to examine its homology (Fig. 1). The amino acid sequences of the  
251 GM6 genes from *T. equiperdum*, *T. evansi*, *T. brucei* and *T. b. gambiense*  
252 showed 97–98% homology. Among the 68 amino acid repeat units within  
253 *Trypanozoon*, there were only two different amino acids. This indicates that the  
254 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).

287

288

#### 289 **4. Discussion**

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

297 In this study, the *T. evansi* GM6 antigen was evaluated with two diagnostic  
298 tools: an ICT and an ELISA for non-tsetse transmitted horse trypanosomosis,  
299 especially dourine. In all previous studies, crude antigen-based ELISA for  
300 trypanosomes exhibited high sensitivity and specificity, generally >90%.  
301 However, the ELISA is not a true gold standard; rather, it is a recommended  
302 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 (Boulangue et al., 2017).

319 In the current study involving horses, the sensitivity and specificity of  
320 rTeGM6-4r-based ELISA were moderate at 81% and 79%, respectively (Table 2).  
321 This higher sensitivity of the ELISA in comparison to the ICT could be due to the  
322 enzyme-substrate reaction, which enhances the detection process when there is  
323 a low antibody titer. In our previous study involving ruminants, we also found that  
324 the rTeGM6-4r based ICT was less sensitive than the ELISA. However, it was  
325 relatively specific, simple and rapid (Nguyen et al., 2015a). The higher specificity  
326 of the rTeGM6-4r-based ICT in this study might be explained by lower  
327 cross-reactivity among the tested samples. The slightly higher OD values in the  
328 negative control wells in the crude antigen-coated plates suggests the possibility  
329 of cross-reaction with a non-specific antibody in the sera of the horses (Fig. 3-B).  
330 It is not surprising that cross-reactions occur with other trypanosome species  
331 and even other parasites in most instances in which crude lysates of the antigen  
332 of a given trypanosome strain are cultured *in vivo* or *in vitro* (Magez and  
333 Radwanska, 2014). Among the samples that showed a positive reaction in the  
334 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 Sukanuma et al. (2016). The  
350 PCR results in our ongoing surveillance project in Mongolia have also revealed



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

357       During the last decades, importation of horses mainly from foreign countries  
358 such as Russia and other European countries to Mongolia has tended to result  
359 in crossbreeding. Proper veterinary checkups are often not done on imported  
360 horses in animal quarantine. Moreover, stallions without veterinary checkup  
361 frequently migrate with their owners within the country and spread dourine in  
362 their harems in Mongolia (Hund, 2008). These two domestic and international  
363 horse migration events might play a key role in the spread of disease in  
364 Mongolia. The main risk factor is the herders' inadequate knowledge in relation  
365 to the diagnosis and treatment of dourine. In this emerging market economy,  
366 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

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

390

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494

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

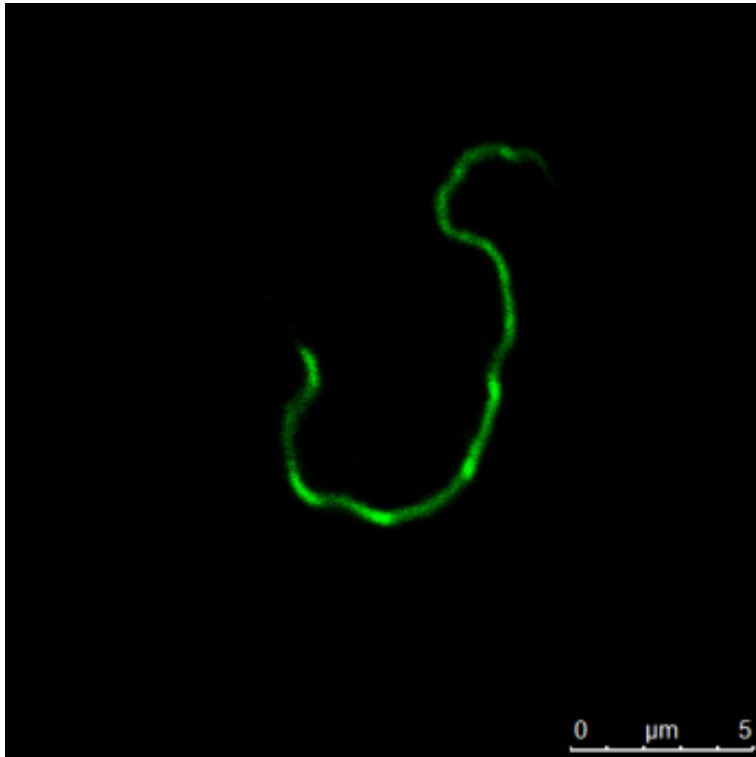
518 indicated in all three samples based on positive results in three of the serodiagnostic  
519 tests and the clinical signs.



.....|.....| .....|.....| .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|.....  
 5                    15                    25                    35                    45                    55                    65

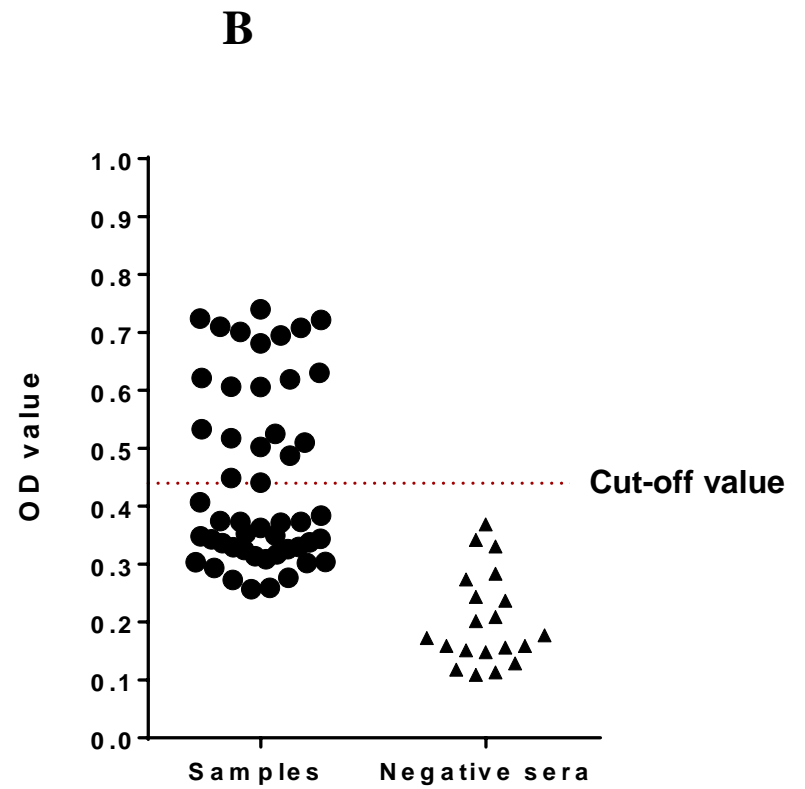
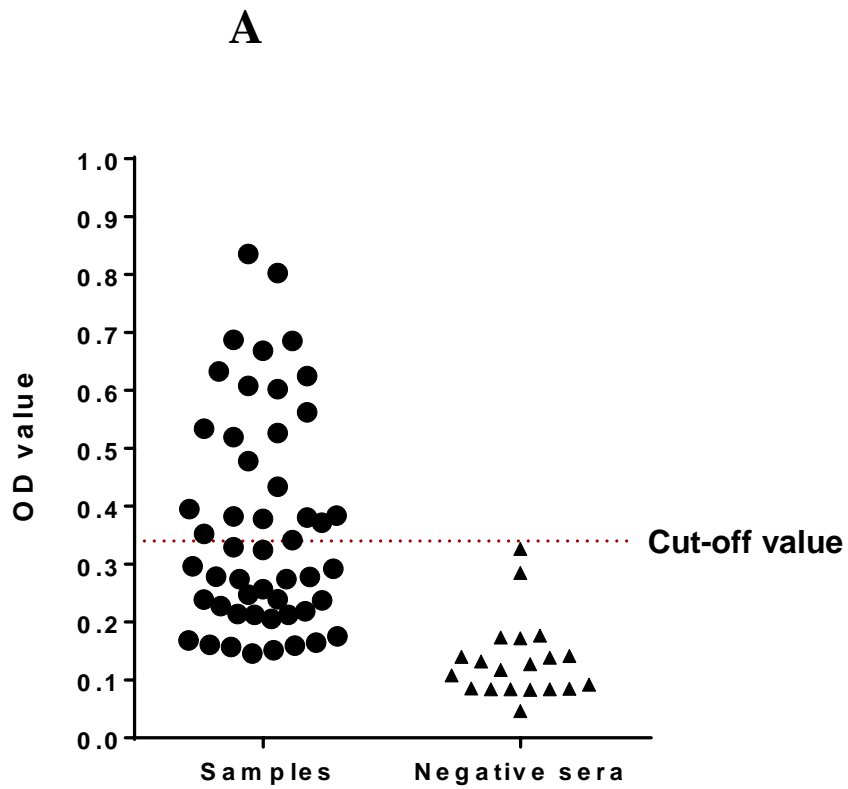
Majority	ELAKLKASDS	RSFLDPMPEG	VPLSEL	<b>E</b> LDK	DEKFSTMEEE	RRKLIAEDRE	GNAARIAELE	<b>V</b> AMNEHSH
<i>T. equiperdum</i>	ELAKLKASDS	RSFLDPMPEG	VPLSEL	<b>E</b> LDK	DEKFSTMEEE	RRKLIAEDRE	GNAARIAELE	<b>V</b> AMNEHSH
<i>T. evansi</i>	ELAKLKASDS	RSFLDPMPEG	VPLSEL	<b>E</b> LDK	DEKFSTMEEE	RRKLIAEDRE	GNAARIAELE	<b>A</b> AMNEHSH
<i>T.b. brucei</i>	ELAKLKASDS	RSFLDPMPEG	VPLSEL	<b>E</b> LDK	DEKFSTMEEE	RRKLIAEDRE	GNAARIAELE	<b>A</b> AMNEHSH
<i>T.b. gambiense</i>	ELAKLKASDS	RSFLDPMPEG	VPLSEL	<b>G</b> LDK	DEKFSTMEEE	RRKLIAEDRE	GNAARIAELE	<b>V</b> AMNEHSH

*T. equiperdum* GM6



Merged





**Table 1. The PCR primer sets**

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

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

		Crude antigen ELISA <sup>a</sup>			Sensitivity	95% CI <sup>b</sup>	Specificity	95% CI <sup>b</sup>	Agreement <sup>c</sup>	Kappa <sup>d</sup>
		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							

<sup>a</sup> Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016

<sup>b</sup> Confidence intervals at 95% for sensitivity and specificity

<sup>c</sup> Number of observed agreement

<sup>d</sup> Cohen's Kappa value