Treatment efficiency of combination therapy with diminazene aceturate and quinapyramine sulfate in a horse with dourine

Batbold Davkharbayar[a,*], Batdorj Davaasuren[a,*], Sandagdorj Narantsatsral[a], Banzragch Battur[a], Myagmarsuren Punsantsogvoo[a], Badgar Battsetseg[a], Daiki Mizushima[b, d], Noboru Inoue[b, c], Keisuke Suganuma[b, e, #]

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

[b] *OIE Reference Laboratory for Surra, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan*

[c] *Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan*

[d] *Division of Medical Zoology, Department of Infection and Immunity, Jichi Medical University, Tochigi, 329-0498, Japan*

[e] *Research Center for Global Agromedicine, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan*

E-mail address

Batbold Davkharbayar: b.dawhraa10@gmail.com

Batdorj Davaasuren: davlag_mgl@yahoo.com

Sandagdorj Narantsatsral: naran69@gmail.com

Banzragch Battur: bat912b@yahoo.com

Myagmarsuren Punsantsogvoo: miigaa82@gmail.com

Badgar Battsetseg: bata07@gmail.com

Daiki Mizushima: dmizushima@jichi.ac.jp

Noboru Inoue: ircpmi@obihiro.ac.jp

Keisuke Suganuma: k.suganuma@obihiro.ac.jp

* These authors contributed equally to this work.

Corresponding author. Assistant Prof. Keisuke Suganuma, D.V.M., Ph.D.

Research Center for Global Agromedicine, National Research Center for Protozoan Diseases, OIE Reference Laboratory for Surra, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan

Tel.: +81-155-49-5697; Fax: +81-155-49-5643

E-mail address: k.suganuma@obihiro.ac.jp (K. Suganuma)

Abstract

Dourine is a lethal protozoan disease of equids, and it is caused by *Trypanosoma equiperdum* infection via coitus. To date, treatment strategies against the dourine are not recommended due to the frequent relapses; therefore, the World Organization for Animal Health recommends the stamping-out policy for the control of dourine. Our previous studies have revealed a number of horses with dourine in Mongolia that is the fifth largest horse-breeding country. It is difficult to apply the stamping-out policy for cases of dourine in Mongolia because of an inadequate livestock guarantee system. Therefore, the development of effective treatment measures is an urgent need. In this study, an 8-year-old stallion was definitely diagnosed with dourine based on clinical signs, molecular analysis, and microscopic examination of trypanosomes. Combination therapy with diminazene aceturate and quinapyramine sulfate was applied. Before the treatment, the characteristic clinical signs of dourine were observed, and trypanosomes were detected in the urogenital tract mucosal swab samples by microscopic examination and PCR. Moreover, positive serological results were obtained. After the treatment, we observed an improvement in the health of the treated horse and no trypanosome infection in its urogenital tract by microscopic examination and PCR. Moreover, serological tests showed seronegative results. The horse has showed no relapse for at least 2.5 years after the treatment, and its reproductive ability has improved. Our result suggests that trypanosomes did not invade cerebrospinal fluid when we started the therapy. In conclusion, the combination therapy has therapeutic potential against dourine at an early phase.

Keywords: combination therapy, diminazene aceturate, dourine, *Trypanosoma equiperdum*, quinapyramine sulfate

1 Introduction

Dourine, which is caused by *T. equiperdum* infection during coitus, is a chronic and/or acute contagious disease of equids. *T. equiperdum* primarily parasitizes the genitalia and then parasitizes tissues such as the skin, mammary glands, and cerebrospinal fluid (CSF). In addition to nonspecific clinical signs such as fever and anemia, dourine causes characteristic clinical signs such as local edema of the genitalia and mammary glands, cutaneous eruptions, incoordination, as well as facial and lip paralysis due to the parasitism in tissues and CSF. Dourine is an OIE-listed disease and is well known as a major obstacle for the international movement of horses [1, 2].

Dourine has been thought to be almost completely eradicated worldwide. However, some cases of dourine were recently reported in Mongolia, Italy, Ethiopia, and Venezuela [3-9]. In Mongolia, dourine has occasionally occurred near the border where horse movement was free in the past (unpublished data). The seropositive cases of dourine were estimated to be 5.5% in Töv Province, Mongolia in 2003 [10]. Recently, we revealed an endemic dourine farm case in Töv Province [5] and isolated *T. equiperdum* from horses with dourine, which were bred in different provinces [3]. Moreover, the seroprevalence of horse trypanosomoses was estimated to be 4.8% by recombinant *T. evansi* GM6 (rTeGM6)-based enzyme-linked immunosorbent assay (ELISA) in whole Mongolia (Mizushima et al., unpublished data). Our reports clearly indicate the nationwide endemic situation of dourine and the necessity of an urgent introduction of its control.

Mongolia is estimated to be the fifth largest country in terms of horse population worldwide, and it has bred >3.9 million horses according to the Food and Agriculture Organization [11]. Horses play particularly important roles in Mongolia not only in terms of livestock production (including transportation, milk production, and meat production) but

also in terms of culture, tradition, and nomadic beliefs. The stamping-out policy has been practiced as the only effective control measure against dourine following the recommendation of the World Organization of Animal Health (OIE) [12]. However, the elimination of all infected horses might cause huge economic loss and social disorder in nomadic countries such as Mongolia. Therefore, the case identification and treatment of dourine are important for its control in nomadic countries.

Previous studies have reported experimental treatment of dourine with naganol (Suramin®) and neoarsphenamine [13] or quinapyramine sulfate (QS) [14]. Dourine are frequently relapsed because *T. equiperdum* invade into and parasitize CSF, and several trypanocidal dugs have inability to pass through blood–brain barrier; therefore, treatment measures are not yet established and recommended for the disease [15, 16]. Recently, several reports have indicated that horses experimentally infected with *T. equiperdum* can be treated with diminazene diaceturate (Diminasan®) [17] or bis (aminoethylthio) 4-melaminophenylarsine dihydrochloride (Cymelarsan®) [18, 19]. In addition, *in vivo* (mice) and *in vitro* drug sensitivity tests against some *T. equiperdum* strains suggested that several trypanocidal compounds equally inhibit the growth of *T. evansi* and *T. brucei* [20-23]. In the present study, we evaluated the treatment efficacy of combination therapy with diminazene aceturate (DA) and QS against a clinical case of dourine.

2 Material and methods

2.1 Study site and the history of dourine-suspected horse

This study was conducted from January 2017 to June 2019 in Bayankhutag Sum, Khentii Province, Mongolia, which is located in the east of the country. The edema around the genitalia of an 8-year-old palomino-collared stallion was detected in July 2016 by the

stallion owner. The stallion was treated by Penstrep-400 (Interchemine, Waalre, Holland) following which the edema temporally disappeared; however, it relapsed in November 2016, and the body condition of the horse kept deteriorating. In January 2017, the stallion owner requested to perform definitive diagnosis on a particular stallion expressing dourine-like signs (Fig. 1, Table 1).

Blood sample was collected from the jugular vein of the stallion in an EDTA tube for DNA extraction and blood analysis as well as in a plane tube for serological analyses. Blood parameters [white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), and hematocrit (HCT)] were analyzed using the pocH-100iV Diff automated hematology analyzer (Sysmex, Hyougo, Japan). In addition, urogenital tract mucosal swab samples were serially collected for parasitological analyses.

All procedures described in the present study were approved by the Committee on the Ethics of Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine (Approval number: 18-57).

2.2 Parasitological examination

Parasitological examination of trypanosomes was performed according to the method reported by Suganuma et al. (2016) [3]. In brief, to isolate trypanosomes from the urogenital tract of the stallion, Hirumi's modified Iscove's medium-9 was injected into its urogenital tract to detach trypanosomes from the urogenital tract mucosa. Subsequently, to observe live trypanosome, samples collected from the urogenital tract mucosa using a cotton swab were placed as droplets on a glass slide and immediately observed using light microscopy. For the observation of trypanosome using Giemsa-stained samples by light microscopy, samples

were smeared on glass slides, fixed with 100% methanol, and stained with Giemsa staining solution (Merck, Darmstadt, Germany).

2.3 Molecular analysis

The total DNA from urogenital tract mucosal swab samples and EDTA-treated blood samples was extracted and purified using phenol/chloroform/isoamyl alcohol solution (Sigma-Aldrich, Japan). Purified DNA was stored at −30°C until use. PCR was performed using the KIN primer sets [forward (Kin2) 5′-CGC CCG AAA GTT CAC C-3′ and reverse (Kin1) 5′-GCG TTC AAA GAT TGG GCA AT-3′] [24].

2.4 Serological examination

Immunochromatographic test (ICT) and ELISA using recombinant *T. evansi* GM6 (rTeGM6) antigen were performed following the methods reported in a previous study [5].

2.5 Treatment strategy

The stallion with dourine was treated with DA (Demin®; Brilliant Bio Pharma Private Limited, Telangana, India) at a dosage of 3.5 mg/kg every 24 h for 3 days by subcutaneous injection in the neck. A week after DA treatment was completed, QS was subcutaneously injected (Triquin™-S; Vetoquinol India Animal Health Pvt. Ltd, Mumbai, India) twice at a weekly dosage of 3 mg/kg.

3 Results

3.1 Clinical signs and results of diagnostic testing of the stallion before the treatment

The characteristic clinical signs of dourine around the genitalia; edema of the testicles, thin sheath surrounding a testicle, and scrotum; depigmentation of the penis; and accumulation of smegma around the penis were observed (Fig. 1-B and C). In addition to these clinical signs, its body condition was moderately thin (Fig. 1-A). WBC (9.1×10^3/μL), RBC (7.1×10^6/μL), and HCT (32.3%) levels were relatively low but within the normal range, whereas the HGB level (9.8 g/dL) was lower than the normal range (Table 1). Slight unilateral paralysis of the nose and lower lip was observed, whereas other characteristic clinical signs such as skin plaques and ataxia were not observed (Fig. 1-D). Active trypanosomes were observed in the urogenital tract mucosal swab samples, and PCR with a 540-bp amplicon showed positive results; however, trypanosome DNA was not detected in blood by PCR (Fig. 3, Supplemental Fig. 1, Supplemental Movie 1). ICT and ELISA results showed that the stallion was seropositive for horse trypanosomosis (Supplemental Fig. 2). In addition to this stallion, 7 mares in the herd were suspected to have dourine by ELISA (Supplemental Fig. 3).

3.2 Improvement of clinical signs, fertility and negative results on diagnostic testing after the treatment

After post-treatment 2 weeks, its clinical signs around the genitalia and paralysis gradually improved (Fig. 2). In addition, WBC, RBC, HGB, and HCT levels increased to 9.4 $\times 10^3$/μL, 8.7 $\times 10^6$/μL, 11.8 g/dL, and 36.9%, respectively (Table 1). The HGB level was still lower than the normal range, and the body condition remained moderately thin; however, anemia improved compared with that before treatment. ICT results remained seropositive for dourine (Supplemental Fig. 2-A).

After post-treatment 2 months, the characteristic clinical signs were not observed (Fig. 2). The levels of blood parameters and body condition improved to normal (Table 1). The antibody titers, which were analyzed by ELISA, decreased from the positive range (OD_{450} = 0.41, pretreatment) to the seronegative range (OD_{450} = 0.12, post-treatment 2 months) and remained in the negative range thereafter (OD_{450} = 0.07, post-treatment 6 months; OD_{450} = 0.04, post-treatment 1 year; and OD_{450} = 0.04, post-treatment 2 years) (supplemental fig. 2). ICT results changed to seronegative from post-treatment 1 month and remained negative for post-treatment 1 year (supplemental fig. 2). Trypanosomes were not detected in the urogenital tract mucosal swab samples upon microscopic examination. Moreover, *Trypanozoon* DNA was not detected in the urogenital tract mucosal swab samples by PCR (supplemental fig. 1). The stallion did not relapse for at least post-treatment 2.5 years.

The reproductive rate of the stallion was observed for 3 years. The reproductive rate was 0% in 2016, 50% in 2017 and 25% in 2018 (Supplemental Table 1).

4 Discussion

Even though curative treatment strategy is available against dourine, OIE does not recommend the treatment due to the frequent relapse. Because no vaccine, OIE recommended the stamping-out policy for infected horses as the only effective control measure [12]. Mongolia is one of the largest horse-breeding countries and still faces situations of dourine outbreak [5, 10]. Although the stamping-out policy is recommended by OIE for the control of dourine, it is difficult to apply this strategy in Mongolia and other nomadic countries due to social and economic reasons. Therefore, the establishment of effective treatment measures against dourine is an urgent need in the nomadic horse industry. In the present study, we

evaluated the treatment efficacy of combination therapy with DA and QS against a clinical case of dourine.

The clinical signs of dourine include fever, anemia, emaciation, local edema of the genitalia and mammary gland, skin lesion, paralysis, and ataxia [25]. Despite no apparent skin lesion, the clinical picture of the stallion in the present study corresponded well with those of reported cases of dourine (Fig. 1, Table 1) [26]. rTeGM6-based ELISA and ICT can help detect the anti-*T. equiperdum* antibody in serum because this antibody showed crossreaction among other trypanosome species [5, 27, 28]. The positive results obtained on serological analyses by ICT and ELISA suggested past or present infection of trypanosomes in the stallion. Trypanosome DNA was detected in the DNA extracted from urogenital tract mucosal swab samples but not in that extracted from whole blood by PCR. The amplicon size of 540 bp suggested that the etiological agent was *Trypanozoon*. From the epidemiological point of view, this result suggests that the stallion was infected with *T. equiperdum* or *T. evansi*. As a result of microscopic examination, active trypanosomes were observed in urogenital tract mucosal swab samples. Unlike *T. evansi*, *T. equiperdum* primarily parasitizes tissues and is rarely observed in blood [25]. Our results strongly suggested that the stallion had dourine. The clinical signs around the genitalia were characteristic of dourine, whereas the neurological signs were relatively weak without ataxia in the stallion. In our previous observations of clinical cases of dourine, high numbers of live *T. equiperdum* were observed in the genital samples collected from infected horses. In such cases, neurological signs were not obvious. However, in progressed cases of dourine, trypanosomes were mainly observed in CSF and infected horses usually presented severe neurological signs [4] (Suganuma et al., unpublished data).

Hébert *et al* [29] established an experimental Welsh pony infection model using the *T. equiperdum* OVI strain. They collected CSF samples by ultrasound-guided cervical centesis

and observed *T. equiperdum* in CSF in the acute phase (11 and 28 days) after the infection via the jugular vein. In the present study, the stallion seemed to be naturally infected with *T. equiperdum* from spring to summer, which is its mating season. Further, its owner had observed a local edema in the stallion's genitalia in July 2017. Therefore, it is thought that the stallion had chronic dourine. When the combination therapy trial was started in the following January, CSF samples could not be examined because of the lack of equipment for CSF collection. Although minor neurological signs were observed in this case, *T. equiperdum* had probably not invaded CSF of the infected stallion when the combination therapy was started. Dourine did not relapse in the treated stallion for at least 2.5 years after the combination therapy, despite the fact that the two drugs (DA and QS) do not cross the blood–brain barrier [30-35]. However, the transferability of these drugs to tissues, particularly genitalia, remains unknown, but the result of the complete elimination of trypanosomes from the urogenital tract mucosal swab samples suggested that these drugs affect trypanosomes that parasitize tissues.

In the present case of dourine, the characteristic skin plaques, so-called "silver dollar plaques," were not observed. In our previous observations, the characteristic skin plaques were also not observed in other dourine-suspected Mongolian local breed horses [4]. However, these plaques were sporadically observed in 62% of Friesian and half-breed horses, which were naturally or experimentally infected with *T. equiperdum* [26]. These differences in clinical signs and trypanosome parasitism suggest that clinical signs and pathogenicity rely on the etiological strain of *T. equiperdum* and/or horse breed. After post-treatment 2 months, the body condition of the stallion recovered well and became moderately fleshy according to the Henneke's horse body condition scoring system (Fig. 2-A) [36]. In addition, the clinical signs of dourine started to disappear within post-treatment 2 months (Fig. 2-B and C). Slightly low levels of RBC, HGB, and HCT indicated anemia with emaciation. The levels of

blood parameters also improved after the treatment, and anemia was cured within post-treatment 2 months (Table 1). The improvement in the levels of these blood parameters clearly indicated the positive effects of the combination therapy. A treatment study using Cymelarsan® alone reported the reversion of blood parameters in treated horses [18, 19]. The infected stallion mated 20 mares in 2016 (before the combination therapy but its owner found edema of the genitalia), but the stallion had no reproductive ability because of none of the offsprings in 2017 (Supplemental Table 1). On the other hand, in 2017 and 2018, the reproductive ability of the stallion slightly improved after the combination therapy (Supplemental Table 1). This result indicates that reproductive ability could be reverted by the combination therapy.

During the combination therapy, the antibody titer for the rTeGM6-4r antigen gradually decreased and turned to be negative after post-treatment 1 and 2 months by ICT and ELISA, respectively. These results indicated that the rTeGM6-4r antigen was useful for monitoring the elimination of *T. equiperdum* from the infected horse and the effectiveness of the treatment. Supportive results have been reported in previous studies [19, 35, 37].

5 Conclusions

Because of this study only based on treatment of single stallion and we did not check the parasite in CSF of the stallion, it possible to happen relapse in other case. In this case, dourine did not relapse for at least 2.5 years after the combination therapy; therefore, it can be concluded that the combination therapy with DA and QS has the potential to treat dourine. In the future, the possibility of dourine relapse in the stallion should be carefully analyzed. In addition, trypanosomes in the semen and reproduction ability should be carefully observed.

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289 **Figure legends**

290 Fig. 1. Clinical signs of the stallion before the combination therapy

291 (A) Overall body condition. (B) Edema of the testicles, thin sheath surrounding a testicle, and
292 scrotum. (C) Depigmentation of the penis and accumulation of smegma around the penis. (D)
293 Slight unilateral paralysis of the nose and lower lip.

294

295 Fig. 2. Improvement in clinical signs of the stallion 2 months after the combination therapy
296 with diminazene aceturate and quinapyramine sulfate

297 (A) Overall body condition. (B) Improvement in edema of the testicles, thin sheath
298 surrounding a testicle, and scrotum. (C) Depigmentation of the penis, whereas smegma
299 cleared out around the penis.

300

301 Fig. 3. Microscopic examination of *Trypanosoma equiperdum*

302 Giemsa-stained *T. equiperdum* detected in urogenital tract mucosal swab samples
303 before the combination therapy with diminazene aceturate and quinapyramine sulfate. Arrow
304 indicates the kinetoplast in trypanosome.

305

306 Supplemental Fig. 1. PCR for trypanosomes

307 PCR was performed using KIN primer sets. M: 100-bp DNA ladder, lane 1: DNA
308 sample extracted from urogenital tract mucosal swab samples before combination therapy,
309 lane 2: DNA sample extracted from blood before the combination therapy, lane 3: DNA
310 sample extracted from urogenital tract mucosal swab samples 2 months after the combination

311 therapy, N: Negative control (distilled water) and P: Positive control (*Trypanosoma*
312 *equiperdum* DNA).

313

314 Supplemental Fig. 2. Serological analyses of the stallion with dourine by
315 immunochromatographic test (ICT) (A) and enzyme-linked immunosorbent assay (ELISA)
316 (B)

317 Serological analyses were performed by ICT and ELISA before and after the
318 combination therapy. The reference serum for positive and negative controls was confirmed
319 by ELISA, ICT, CATT, PCR, and microscopic examination of the host stallion [5]

320 A) Diagnosed by ICT. T: Test line, C: Control line, P: Positive control, N: Negative
321 control, 1: Before the combination therapy, 2: Two weeks after the combination therapy, 3:
322 One month after the combination therapy, 4: Two months after the combination therapy, 5:
323 Three months after the combination therapy, 6: Six months after the combination therapy,
324 and 7: One year after the combination therapy. The ICT detection test showed seropositive
325 results.

326 (B) The change in the antibody titer during the period of combination therapy. Pos:
327 Positive control, Neg: Negative control, 1: Before the combination therapy, 2. Two months
328 after the combination therapy, 3: Six months after the combination therapy, 4: One year after
329 the combination therapy, and 5: Two years after the combination therapy.

330

331 Supplemental Fig. 3.

Serological analysis was performed against all horses in the horse farm by ELISA before the combination therapy. Pos: Positive control, Neg: Negative control, Stallion: The stallion that was treated in the present study, and 1–20: Mares in the horse farm.

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438

439 Table 1. Summary of the case and treatment results

General information	
Horse farm information	Khentii Province, Bayakhutag Sum
Sex	Male
Age	8 years
Color	Palomino
Treatment	Diminazene aceturate: dosage of 3.5 mg/kg every 24 h for 3 days Quinapyramine sulfate: 1 week after the diminazene aceturate treatment at dosage of 3 mg/kg twice a week

Disease condition before treatment	
Clinical signs	Edema of the testicles, thin sheath surrounding a testicle, and scrotum; depigmentation of the penis; and accumulation of smegma around the penis
Body condition	Moderately thin
Blood parameters	WBC: 9.1×10^3/μL, RBC: 7.2×10^6/μL, HGB: 9.8 g/dL, and HCT: 32.3%*

Disease condition after post-treatment 2 weeks	
Clinical signs	Reduced edema of the testicles, sheath, and scrotum; depigmentation of the penis; and accumulation of smegma around the penis
Body condition	Moderately thin

Blood parameters	WBC: 9.4 × 10^3/μL, RBC: 8.7 × 10^6/μL, HGB: 11.8 g/dL, and HCT: 36.9%*	440
Disease condition after post-treatment 2 months		441
Clinical signs	No clear clinical sign	442
Body condition	Moderately fleshy	
Blood parameters	WBC: 9.6 × 10^3/μL, RBC: 10.2 × 10^6/μL, HGB: 13.6 g/dL, and HCT: 43.5%*	443
Diagnostic test results before treatment		444
Microscopic examination	Positive, live, actively moving parasites observed in the urogenital tract mucosal swab (Fig. 3 and Supplemental movie 1)	445
PCR	Positive, in DNA extracted from the urogenital tract mucosal swab (Supplemental Fig. 1, line 1)	446
Immunological diagnosis	Positive, ELISA (OD_{405} value: 0.41 compared with the cut-off value of 0.15) and ICT (Supplemental Fig. 2-B, line 1, and Supplemental Fig. 2-A, line 1, respectively)	447
Diagnostic test results after post-treatment 2 months		448
Microscopic examination	Negative, in the blood and urogenital tract mucosal swab	449
PCR	Negative, in DNA extracted from the urogenital tract mucosal swab (Supplemental Fig. 1, line 3)	
Immunological diagnosis	Negative, ELISA (OD_{405} value: 0.11 compared with the cut-off value of 0.15) and ICT negative (Supplemental Fig. 2-B, line 2, and Supplemental Fig. 2-A, line 4, respectively)	450
		451

452

*Normal range of blood parameters: WBC: 5–12 × 10^3/μL, RBC: 7–13 × 10^6/μL, HGB: 12–17 g/dL, and HCT: 32%–52%

WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; ELISA, enzyme-linked immunosorbent assay; ICT, immunochromatographic test

Fig. 1

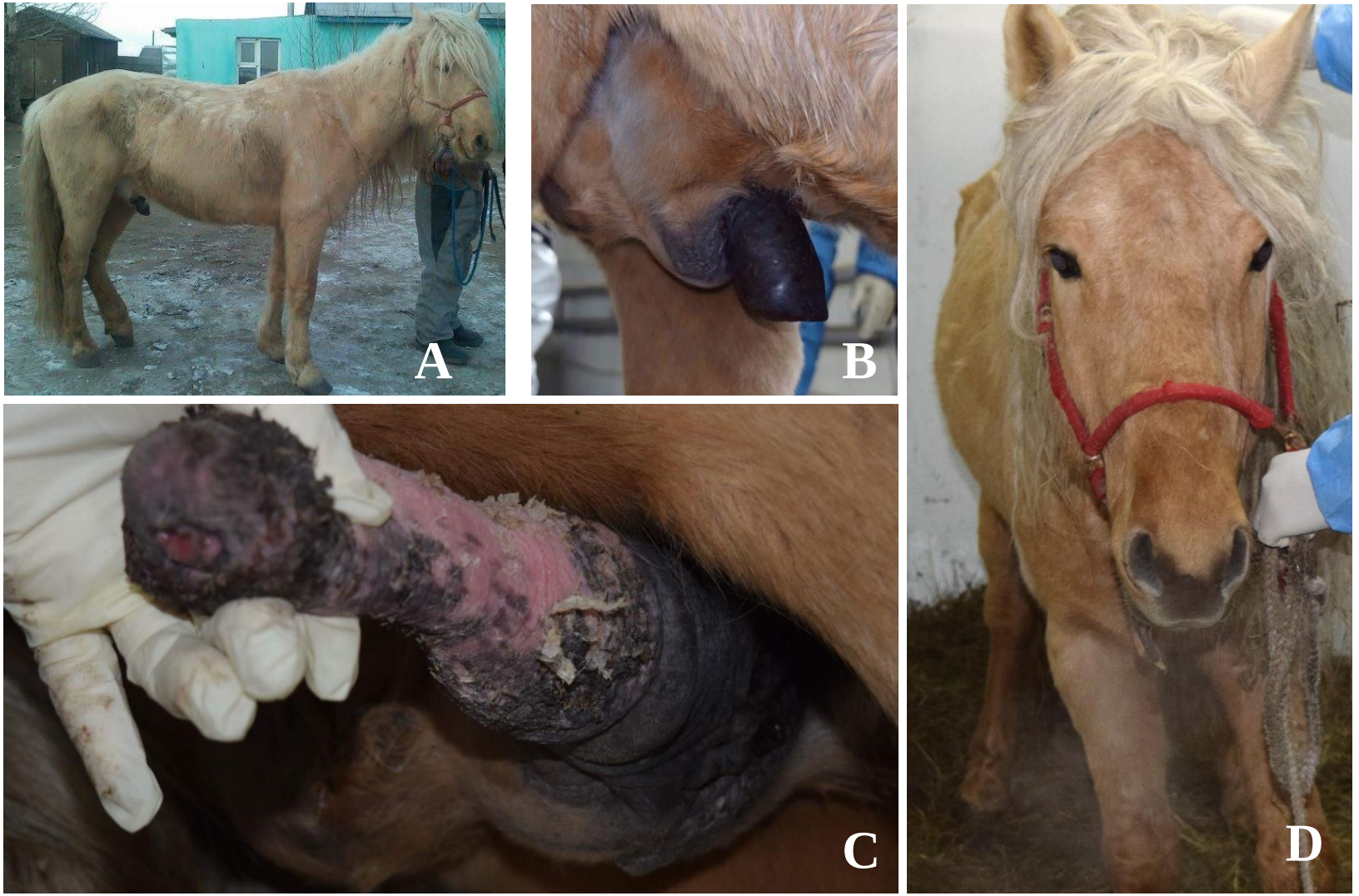

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Fig. 2

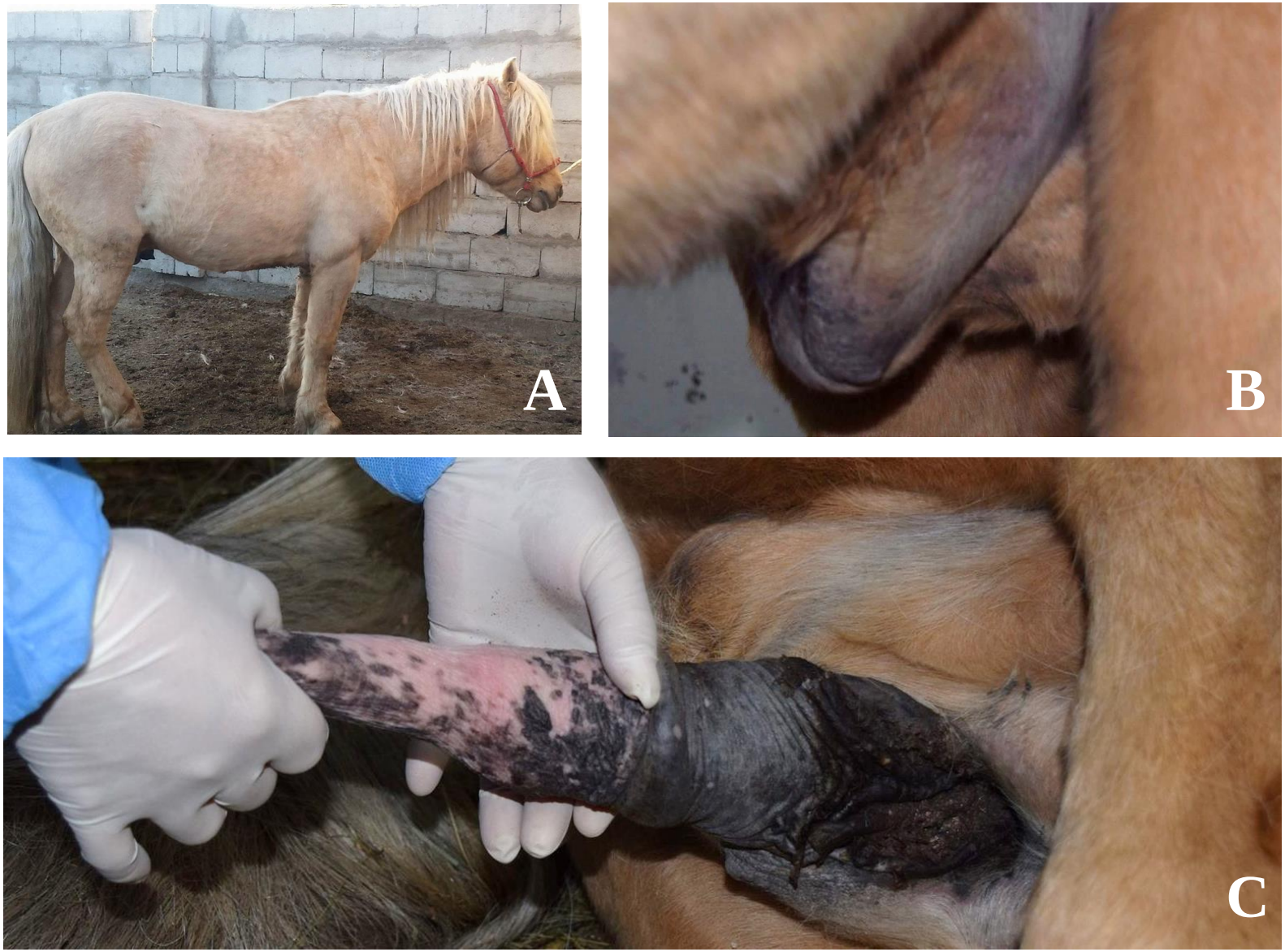

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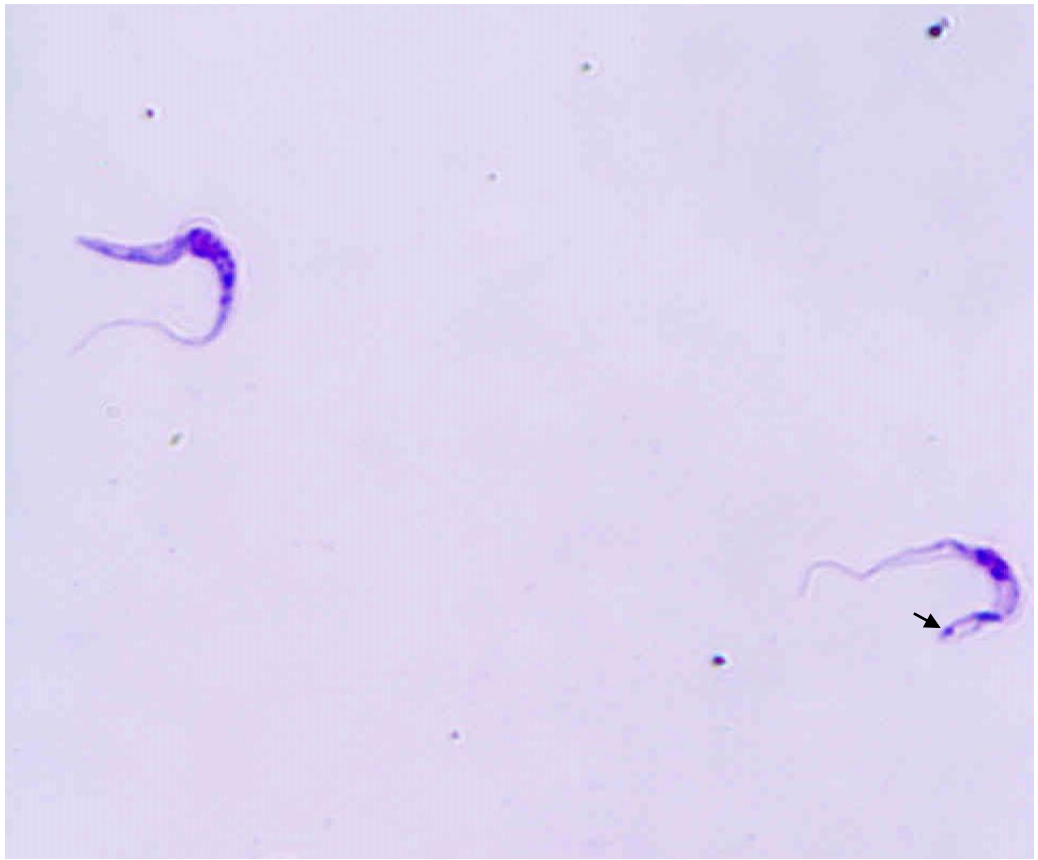
Fig. 3



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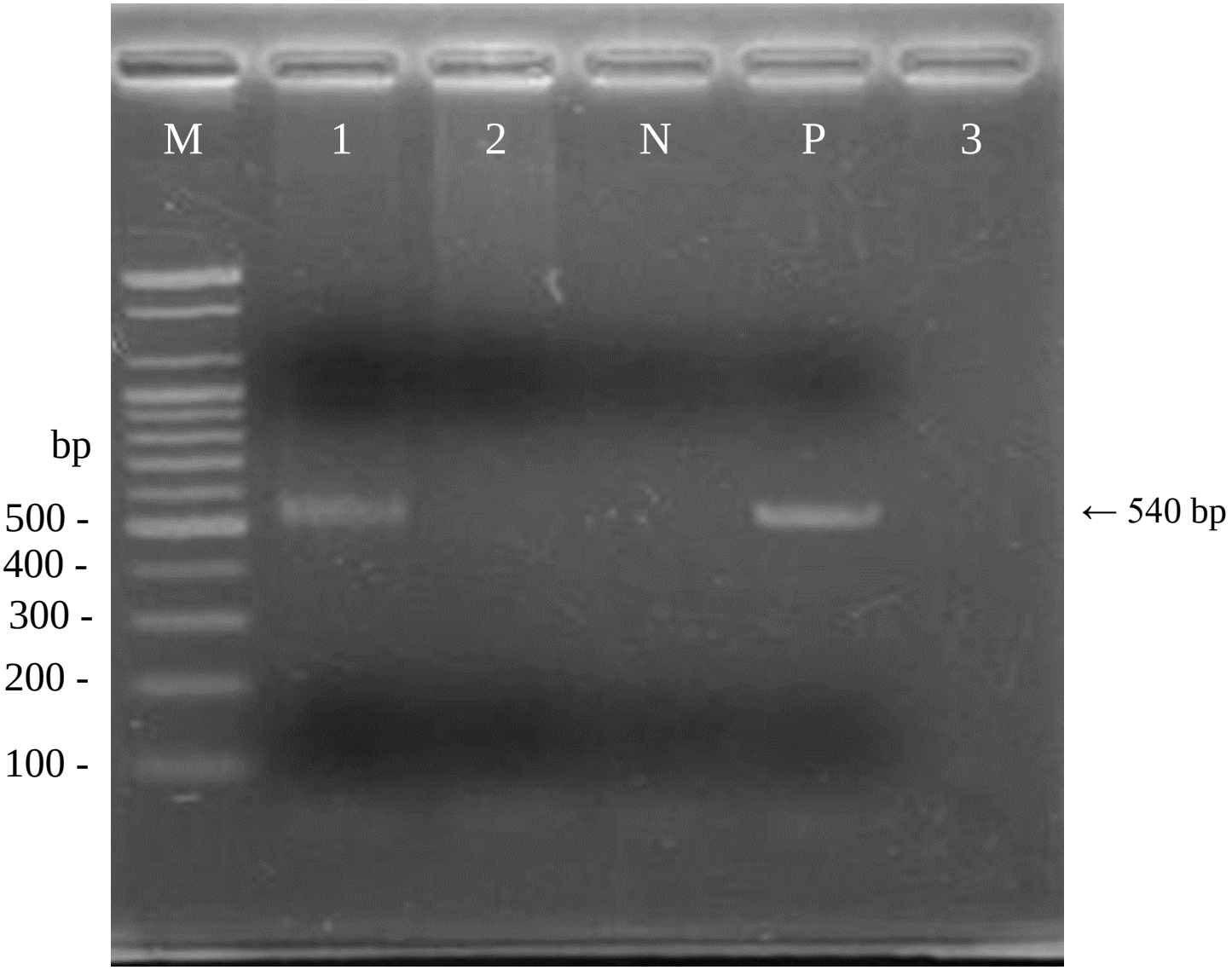
Supplemental Fig. 1



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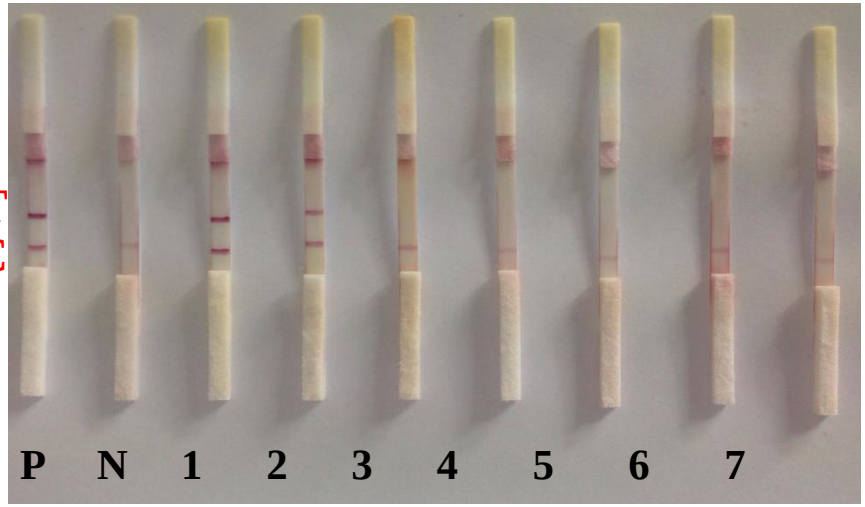
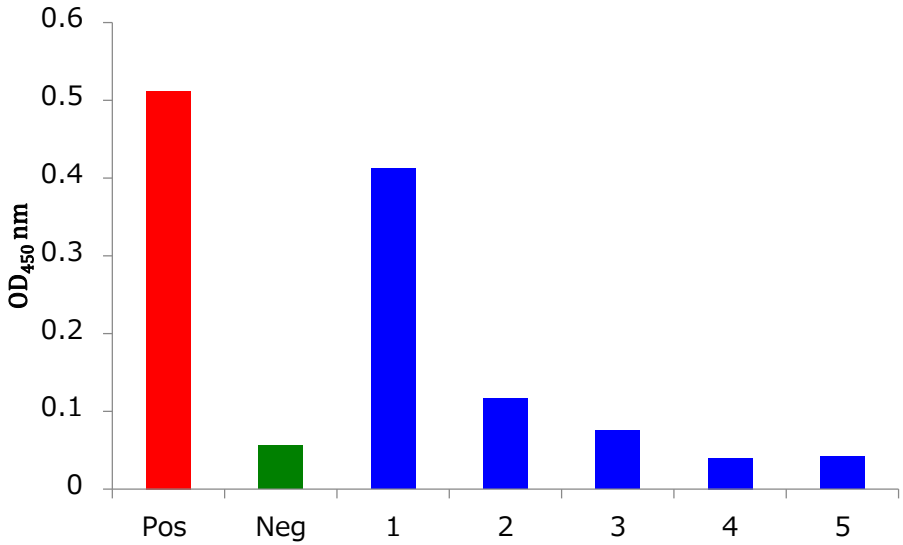
Supplemental Fig. 2.

A.



B.



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Supplemental Fig. 3

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