Veterinary Parasitology 268 (2019) 87–97

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Tissue (re)distribution of *Trypanosoma equiperdum* in venereal infected and blood transfused horses

Ahmed Yasine[a,c,d,*], Merga Daba[b], Hagos Ashenafi[c], Peter Geldhof[d], Leen Van Brantegem[d], Griet Vercauteren[e], Tilaye Demissie[c], Merga Bekana[c], Alemu Tola[c], Ann Van Soom[d], Luc Duchateau[d], Bruno Goddeeris[d,f], Jan Govaere[d]

[a] Wollo University, School of Veterinary Medicine, P.O Box 1145, Dessie, Ethiopia
[b] Alage Agricultural college, P.O Box 77, Ziway Alage, Ethiopia
[c] Addis Ababa University, College of Veterinary Medicine, P. O Box 34, Bishoftu, Ethiopia
[d] Ghent University, Faculty of Veterinary Medicine, 9820, Merelbeke, Belgium
[e] Vet-Path bvba, 9991, Adegem, Belgium
[f] Katholieke Universiteit Leuven, Faculty of Bioscience Engineering, 3001, Heverlee, Belgium

ARTICLE INFO

Keywords:
Trypanosoma equiperdum
Dourine
Horse
Venereal transmission
Pathology
Cymelarsan
Haematology

ABSTRACT

Dourine, caused by *Trypanosoma equiperdum*, is a life-threatening venereal disease in equidae. So far, there is no clear evidence on how and when stallions become infectious, nor which tissues are affected by the parasite in diseased animals. Post-infection, after a transient, temporary phase of parasitaemia, the parasite disperses to different tissues in an unknown distribution pattern. This study describes the distribution of the parasite after infection by artificial insemination (AI) or blood transfusion. Mares (N = 4) were artificially inseminated with *T. equiperdum* spiked semen whereas stallions (N = 4) were infected by blood transfusion. The course of the disease was monitored by parasitological (Woo) and molecular (PCR) tests and clinical signs and haematological parameters were recorded. At 120 days post infection, horses had a full necropsy, histopathology and PCR. A similar pattern of parasitaemia, disease progression and tissue distribution were seen in all horses. Ejaculated semen in the preclinical stage and epididymal semen in the chronic stage of the disease was positive on PCR and caused infection in mice. Cymelarsan® treatment in the chronic stage did not result in a clinico-haematological or histopathological improvement. At necropsy, lesions were observed in the nervous and reproductive system. Histopathological lesions were most severe in the peripheral nerves and associated ganglia, the testicles and genital mucosae with multifocal infiltration of lymphocytes, plasma cells and histocytes. The parasites disseminated to several tissues including the nervous system, testicles and semen. The results indicate that transmission of *T. equiperdum* is possible through semen even from symptomless stallions post-treatment.

1. Introduction

Ethiopia has the largest equine population in Africa with 2.1 million horses (CSA, 2017). Nearly 90% of agricultural operations depend on manual labour and because of the rugged mountainous terrain of the country, equines are extremely important and used to transport both people and agrarian products, cart goods, ride, till and carry water. Despite their huge numbers and significant contribution to the community and national economy, the attention given to the health aspects of horses in Ethiopia is limited (Guyo et al., 2015). Dourine is one of the life-threatening disease affecting the horse population in Ethiopia which, due to its large morbidity, severe symptomatology and mortality, can make horses unusable or even decimate a population in a short time (Alemu et al., 1997; Clausen et al., 1999; Hagos et al., 2010a; Fikru et al., 2010).

Dourine is a disease of equidae directly transmitted from animal to animal during mating (Barrowman, 1976; Brun et al., 1998). An infected mare may also transmit the disease to its foal through milk or through udder lesions (Brun et al., 1998). *Trypanosoma equiperdum*, the causative agent of dourine, can be isolated directly from the urethral tract of an infected stallion (Suganuma et al., 2016). There is no clear evidence-based knowledge on how and when stallions become infectious. It is still unknown whether the transmission of the parasite occurs through the bruises and abrasions caused by intense contact

* Corresponding author at: Wollo University, School of Veterinary Medicine, P.O Box 1145, Dessie, Ethiopia.
E-mail address: yasineahmed11@gmail.com (A. Yasine).

https://doi.org/10.1016/j.vetpar.2019.03.007
Received 31 December 2018; Received in revised form 21 March 2019; Accepted 25 March 2019

during live covering (Blue, 1985) or by the presence of the parasite in the semen and genital secretions. Since the parasite is a tissue parasite it stays only for a brief period of time in circulation. It remains hitherto unknown how the parasite distributes into the different tissues of the host, once it has disappeared from the peripheral blood circulation. Treatment with melarsamine hydrochloride (Cymelarsan® MERIAL, France) at an early stage of the disease has been shown to clear off the parasite from the circulation, improve packed cell volume (PCV) and eliminate clinical signs (Hagos et al., 2010b). However, after treatment, the parasite can still be found in the cerebrospinal fluid (CSF) (Cauchard et al., 2016). An efficient treatment to remove the parasite from the semen and genital secretions of stallion and the effect of treatment in a chronic stage of the disease is currently not available.

This study was conducted (1) to examine the infectiousness of *T. equiperdum* spiked semen in the absence of intense physical contact or genital abrasions by use of artificial insemination, (2) examine the infectiousness of semen and preputial discharge of infected stallions in the preclinical period and after Cymelarsan treatment, (3) describe the symptomatology and haematology post artificial infection in mares and stallions and, finally (4) examine the distribution of the parasite in the horse and the pathological lesions encountered at necropsy in artificially infected horses.

2. Material and methods

2.1. Study animals

Eight horses, four stallions (S01–S04) and four mares (M01–M04) of Selale horses (a local breed) were purchased from Burayu District in the central highlands of Ethiopia where dourine has not reported before and confirmed to be free of trypanosome infection through parasitological (Woo), serological (CATT/*T. evansi*) and PCR (ITS1) tests. They were housed in fly-proof stables supplemented with 2 kg concentrated ration twice daily and grass hay ad libitum.

Swiss albino mice (N = 34), 8 weeks old from the National Veterinary Institute (NVI), Bishoftu, Ethiopia were used for the inoculation experiment. They were kept in plastic cages on wood shavings as bedding. Pelleted feed and water were given ad libitum.

2.2. Ethical statements

All procedures were approved by the Ethical Review Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture (Certificate Ref. No: VM/ERC/004/07/015).

2.3. Experimental infection procedures

The inoculum used in this trial was stabilate of *T. equiperdum* Dodola 943 originally isolated in Dodola, Ethiopia and cryopreserved in liquid nitrogen in Addis Ababa University College of Veterinary Medicine (Hagos et al., 2010b). The isolated *T. equiperdum* stabilate had maxicircle genes (A6, ND4 and ND7) (Domingo et al., 2003; Dean et al., 2013) as has been shown by PCR for Dodola 940, a sister isolate of the current stabilate, by Birhanu et al. (2016) prior to the experimental infection. Since the location of isolation (Dodola) is out of the Tsetse belt, the presence of the tsetse transmitted *T. brucei* also could not be a confusing factor. To investigate the transmission of *T. equiperdum* through artificial insemination, cryostabilates were thawed at room temperature for 3–5 minutes. Concentration was checked using the rapid matching technique (Herbert and Lumsden, 1976). Five ml of a fresh semen sample harvested from a non-infected stallion was spiked with the stabilate containing approximately 36,000 trypanosomes. Mares in natural oestrus with a preovulatory follicle close to ovulation were inseminated with this trypanosome-semen mixture in the uterus just cranial to the cervix with a routine AI. All clinical and haematology parameters of the inseminated mares were checked, and the Woo tests and wet smear examinations were performed on a daily basis until parasitaemia was detected and weekly thereafter until the end of the study.

Stallions (N = 4) were infected by blood transfusion. Each stallion received blood containing approximately 100,000 trypanosomes from donner mares which developed parasitaemia after infection by artificial insemination with *T. equiperdum* spiked semen. In total, 2 ml blood was used with a parasite concentration of 50,000 trypanosomes/ml as assessed by a 'rapid matching technique' (Herbert and Lumsden, 1976). Stallions were monitored daily for clinical signs and weekly for haematology. On top of the clinical follow-up, ejaculated semen was collected every week by use of an artificial vagina. Stallions were allowed to mount a phantom and semen was collected with a Colorado-model artificial vagina (AV) with a disposable plastic inner liner and a collection bottle. When clinical symptoms were apparent, preputial fluid was also collected from the oedematous areas of the prepuce of the diseased stallions.

At post-mortem examination, epididymal semen was collected according to Roels et al. (2014). In short, testicles were collected via a routine castration procedure and a ligature of surgical material was placed proximally on the ductus deferens. After removal, the testicles were rinsed with a sterile saline solution, wrapped in a towel and placed in a box with ice and transported to the laboratory for semen collection. Upon arrival at the laboratory, care was taken to remove the surrounding fascia of the cauda and the ductus deferens to avoid blood contamination of the semen before collection. Semen was collected from the epididymis and vas deferens by retrograde flushing. A syringe connected to a pipette tip was inserted in the proximal part of the ductus deferens (Melo et al., 2008). An incision was made in the junction between the cauda and corpus of the epididymis. Thereafter, semen extender (INRA 96®) was passed through the cauda and ductus deferens under gentle pressure.

The presence of *T. equiperdum* in blood and semen was checked by wet smear examination, PCR and mice inoculation. Intraperitoneal inoculation of mice with the ejaculated semen obtained in the preclinical period of one of the stallions at day 13 post-inoculation was performed (N = 7), as well as inoculation with preputial fluid from all the four stallions at day 63 post-infection (N = 3) and of epididymal semen of three stallions (S02, S03, S04) at day 120 post-infection (N = 5) of each stallion. Confirmation of infectivity of the inoculans (whether ejaculated semen of a stallion in the preclinical phase, preputial fluid at day 63 post-infection or epididymal semen at post-mortem) was based upon a diagnosis of parasitaemia of the mice on wet smear samples.

2.4. Cymelarsan treatment

The effect of Cymelarsan® treatment (bis-aminoethylthio-4-melaminophenylarsine dihydrochloride, MERIAL, France) in a chronic stage of the disease was evaluated. Three of the infected horses (M04, S02, and S03) were treated with Cymelarsan® while four (M01, M02, S01 and S04) were left untreated. Cymelarsan® was administered intramuscularly SID at a dose of 0.25 mg/kg body weight at day 60 post-infection when horses showed clear clinical signs of dourine such as swelling of the genitalia, staggering from the hindquarters in their movement and emaciation. Thereafter, changes in parasitaemia, clinical signs, and haematological values were recorded once per week for 4 consecutive weeks.

2.5. Clinical examination, Parasitological and PCR tests

2.5.1. Clinical examination

Animals were examined daily for clinical signs throughout the study period. Rectal temperature was taken on every other day basis in the morning, using a digital thermometer.

2.5.2. Parasitology

Blood samples were collected from the jugular vein on a daily basis until parasitaemia was detected and weekly thereafter until the end of the study. Parasitological examination was performed by wet smear examination and Haematocrit centrifugation (Woo) test. Wet blood films were examined under the microscope at x400 magnification to look for motile trypanosomes. The Woo test was performed on the negative samples of wet smear examination. Briefly, micro-haematocrit capillary tubes were filled with approximately 50 µl of blood from the vacutainer tube, sealed and centrifuged for 5 min in micro-haematocrit centrifuge at 3000x*g*. The tubes were then mounted in a specially designed viewing chamber and examined under the microscope at x100 magnification for the presence of motile trypanosomes at the interface of the buffy coat and plasma (Woo, 1970).

2.5.3. DNA extraction, conventional PCR and real-time PCR

DNA extraction was performed using a DNA extraction kit for blood and tissues (DNeasy Blood and Tissue Kits, Qiagen, Germany) following the protocol recommended to isolate DNA from animal tissue (Qiagen, 2006). After extraction, DNA was stored at −20 °C until PCR analysis. The DNA concentrations were measured using Nanodrop ND-2000 UV–vis Spectrophotometer (Nanodrop Technologies, USA).

The DNA samples from blood and semen were tested by conventional PCR using ITS1 primer, with forward primer 5'TGTAGGTGAAC CTGCAGCTGGATC3′ and reverse primer 5'CCAAGTCATCCATCGCGAC ACGTT3′ (Fikru et al., 2012). The cycling condition of ITS1 PCR was initial PCR reaction at 95 °C for 5 min, 34 cycles of denaturation at 94 °C for 30 s, annealing temperature of 60 °C for 30 s, and elongation reaction at 72 °C for 30 s and a final extension at 72 °C for 5 min. All PCR amplifications were carried out in 200 µl thin-wall PCR tubes (Thermo Fisher Scientific, USA) in Veriti thermal cycler 96 wells (Applied Biosystems, USA). The reaction mixture was a 25 µl containing 50 ng DNA, 1x Green GoTaq G2 Flexi buffer, 2 mM of MgCl2, 0.2 mM of each dNTPs, 0.5 µM of each primer, 1.25 U GoTaqG2 Flexi DNA polymerase. Ten microliters of the amplified product were used for electrophoresis in 2% agarose gel at 85 V for 35 min and stained with ethidium bromide for visualization under UV light.

Trypanosome DNA testing from tissues collected at necropsy was performed using a Real-time PCR (RT- PCR) to amplify the internal transcribed spacer (ITS1) of the Trypanozoon subgenus and gave a constant product of approximately 450 bp (Njiru et al., 2005; Fikru et al., 2012). The method was carried out on a Step One Plus Real-Time PCR System (Applied Biosystems, USA) and output data were analysed by the Step one ™ version 2.3 software (Applied Biosystems). The reaction mixture (20 µL total volume) contained 1 µL of the two primers (ITS1F and ITS1R) each, 10 µL of Power SYBR®Green PCR Master Mix (Applied Biosystems) and 2 µL of the extracted DNA and 6 µL of nuclease-free water. Pure *T. equiperdum* DNA extracted from mini Anion-Exchange Centrifugation Technique (mAECT) purified trypanosome (*T. equiperdum* 943 Dodola) strain from ITM Antwerp (Belgium) was used as a positive control, while blanks contained nuclease-free water and DNA extracted from tissues of Belgian horses, negative for *T. equiperdum* as negative controls. The application protocol (Fikru et al., 2012) was modified as follows: 10 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 30 s, annealing temperature of 60 °C for 30 s, elongation reaction at 72 °C for 30 s and a final extension at 72 °C for 5 min followed by 1 min at 95 °C and 1 min at 60 °C. After amplification, the samples underwent temperature ramping from 60 °C to 95 °C to calculate the PCR product dissociation curve. Samples were considered positive based on the observed amplification and melting curve in comparison to positive and negative control samples.

2.6. Haematological analysis

About 5 ml of blood from the jugular vein of the horses was taken using ethylene diamine tetra-acetic acid (EDTA) coated vacutainers (Golden Vac™, Zhenjiang Gonggdong medical technology Co. Ltd.). The haematological analysis was performed on a weekly basis starting from one week prior to infection up to week 8 of the experiment to assess the impact of the infection on the haematology parameters. Haematology parameters were followed up in the same way in the treated horses (Cymelarsan treatment) for four consecutive weeks.

The PCV, Haemoglobin concentration (Hgb), RBC count and total and differential white blood cell counts (WBC) were assessed. The PCV was measured by the haematocrit centrifugation technique using a microhaematocrit reader (Hawksley, UK). Total RBC and WBC counts were quantified using the improved Neubauer Haemocytometer. Haemoglobin concentration was determined using an acid haematin method. Thin blood smears were prepared and stained with differential quick stain (Diff-Quick stain) for differential leukocyte counts which were based on 100 cells per slide according to their characteristics, shape of the nucleus, and presence or absence of granules in their cytoplasm (Coles, 1986). The absolute numbers of leukocytes; eosinophils, lymphocytes, neutrophils, basophils and monocytes were obtained by using the differential white cell count percentages and the total leukocytes count.

2.7. Necropsy and histological observations

All horses were subjected to necropsy at the end of the experiment at 120 days post-infection. They were humanely euthanized by an intravenous administration of an over dose of sodium pentobarbital after sedation with xylazine. Necropsy was performed according to standard procedures (Whitwell, 2009). They were examined thoroughly for gross pathological lesions in various organs. Impression smears were taken from different organs after lightly impressing a freshly cut surface of the organs on a slide, allowing it to dry at room temperature and staining it in Diff-Quick stain like a thin blood film (Swierczewski et al., 2013). Tissues samples from different organs were also collected for histopathology and DNA extraction for PCR. Tissue specimens were collected and processed using standard methods (Slaoui and Fiette, 2011) and stained with haematoxylin and eosin.

2.8. Data analysis

Haematological data were entered into an Excel spreadsheet and analysed by R statistical software version 3.5.1 (R Core Team, 2018). Descriptive statistics were used to describe the data. The analysis for the continuous response variables was based on a mixed model including animal as random effect and time as categorical fixed effects factor. Each time point was compared with time 0 using the Dunnett's multiple comparisons technique. The significance level was set at $P < 0.05$.

3. Results

3.1. Venereal infection of mares by artificial insemination

3.1.1. Diagnosis of dourine

Mares inseminated with spiked semen all got dourine based on clinical symptoms visible from day 8 post-insemination/infection and parasites could be observed in Woo test starting from day 6 post-insemination. But later once the parasitaemia was well established in the acute infection, Trypanosomes were appreciated by wet smear as well. Blood collected during the prepatent period for DNA extraction and PCR test showed the presence of the parasite starting from day 5. The clinical signs of dourine such as swelling and depigmentation of the external genitalia, vaginal discharge, depigmentation of the perineal area and ataxia were observed (Table 1).

The level of parasitaemia was fluctuating for about 6–8 weeks of the study period and later, animals became aparasitaemic at chronic stages. In the chronic stage, only a few trypanosomes were exceptionally visible by the Woo test. The body temperature increased after infection

Table 1
Interval between *T. equiperdum* infection and positive test results in the various diagnostic tests and clinical symptoms in mares following artificial insemination with *T. equiperdum* spiked semen.

Horses ID	Positive for *T. equiperdum* (days post infection)		Symptoms of dourine noticed (days post infection)				
	Woo test	PCR	Oedema of the vulva	Oedema of mammary gland	Oedema of limbs	Depigmentation	Ataxia
M01	6	6	15	–	32	21	37
M02	15	12	9	–	24	37	75
M03	14	14	9	15	–	–	18
M04	8	5	8	–	–	56	–
Average	10.75	9.25	10.25	15	28	38	43.3

(–) indicates clinical signs absent.

and thereafter oscillated usually in line with the waves of parasitaemia from an average of 36.5 °C at day 0 to a maximum of 40 °C at day 28 and then to 36.8 °C at day 56. When parasitaemia was not apparent anymore, the rectal temperature remained around physiological values (37–38 °C). Although the appetite remained unchanged, a gradual decline in body weight and condition leading to progressive emaciation and weakness, was obvious. Dull hair coat, muscular atrophy and loss of skin elasticity were observed throughout the study period. Depigmentation was observed on the skin around the vulval areas. Swelling and mucopurulent vaginal discharge were seen. Oedema of lower hind limb below the stifle joint was observed in two of the four mares. Lameness and paralysis of the hindquarters were observed in all infected horses leading to partial dragging or stiffness of the hind legs, a staggering gait, posterior ataxia and an inability to stand upright after prolonged recumbency. In a standing position, the asymmetrical posture and tendency to shift weight from one leg to another was also observed. As the disease progressed horses were unable to stand up and remained in a recumbent position. Inflammation of the conjunctiva and lacrimal discharge of the eyes was noticed in 1 of the 4 infected mares that eventually resulted in corneal opacity and led to permanent blindness.

3.1.2. Haematology

Three of the mares were included for haematological analysis since M03 was euthanized for animal welfare issue due to paralysis before the completion of the experiment. The weekly mean haematological changes during the study period are summarized in Fig. 1A and B. A fluctuation in the haematological values throughout the study period was observed with a significant ($p < 0.05$) decrease in mean RBC count and PCV starting from week 2. No significant variation ($P > 0.05$) was observed in the in the Hgb concentration throughout the study period (Fig. 1A). There was a significant ($p < 0.05$) decrease in the total WBC count and neutrophils count at week 2 and significantly increased by week 4 (Fig. 1B).

3.1.3. Tissue distribution of the parasite and pathological lesions on necropsy

The parasites were found in a number of organs such as the brain, uterus, ovary, heart, kidney and liver as demonstrated from stained impression smears. The presence of the parasite in these organs was further confirmed by RT-PCR (Table 2). Trypanosomes were demonstrated on impression smears on slides made at necropsy and stained by Diff-Quick stain from different organs (Fig. 2). At necropsy, 120 days post-infection in the chronic stage of the disease, no clear visible gross lesions were observed in most organs except for the nervous system and the reproductive tract. Common gross lesions were yellowish fluid accumulations in the peritoneal and thoracic cavities, yellowish discoloration of the synovial fluid, cerebrospinal fluid (sometimes), reduction of perirenal and pericardial adipose tissue with yellowish discoloration, oedema and yellowish discoloration and fluid accumulation at the base of the gluteal muscle following the caudal nerves (obturator and sciatic nerves). Congestion of the brain capillaries,

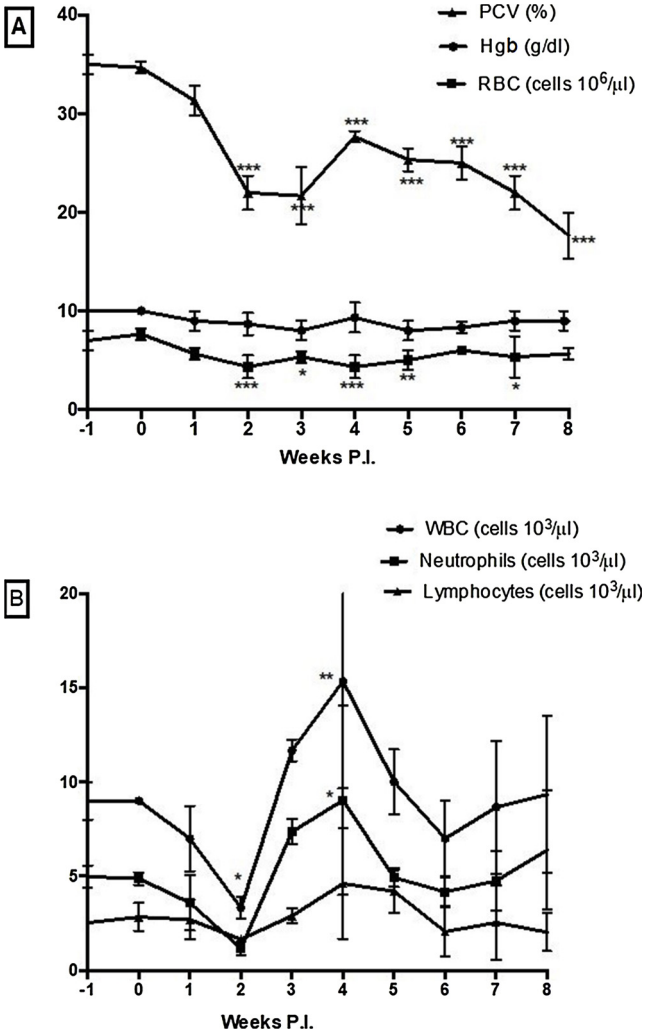

Fig. 1. Mean haematological values in mares experimentally infected with *T. equiperdum* by artificial insemination (A) RBC count, PCV and Hgb concentration (B) total WBC, lymphocytes and neutrophils counts. Significant (*** p = 0.001, ** P = 0.01, * p = 0.05).

oedema of the sacrococcygeal spinal cord and haemorrhagic inflammation at the caudal nerves were observed in the nervous system. In the reproductive tract, diffuse haemorrhagic endometritis was observed especially in the uterine horn, together with low-grade slight vaginitis, depigmentation of the vulva and perineal skin, haemorrhagic and inflamed cervix (in some animals).

On histological examination of the nervous system, histopathological lesions were most severe and consistent in the peripheral nerves (and associated ganglia). The majority of the peripheral nerves showed multifocal infiltration of lymphocytes, plasma cells and fewer

Table 2
Results of impression smear and RT PCR analysis on tissue samples collected from mares at 120 days after an artificial *T. equiperdum* infection.

Tissue sample	M01		M02		M03*	$M04_{Tx}$	
	PCR	Smear	PCR	Smear	PCR	PCR	Smear
Nervous system							
Cerebrum	+	+	+	+	ND	+	+
Cerebellum	+	ND	+	+	+	+	+
Brain stem	+	ND	+	–	+	+	ND
Lumbar spinal cord	+	ND	+	ND	+	+	ND
Sacral spinal cord	+	ND	+	ND	+	+	ND
Obturator nerve	+	ND	+	ND	ND	+	ND
Reproductive system							
Uterus	+	–	+	+	+	+	+
Ovary	ND	–	+	+	+	+	–
Vagina	+	ND	+	ND	+	+	ND
Mammary gland	+	ND	+	ND	+	+	ND
Others							
Lung	+	–	–	–	+	–	–
Liver	–	–	+	+	+	+	–
Kidney	+	+	+	+	+	–	–
Heart	+	+	+	+	+	–	–
Pancreas	+	–	–	ND	–	–	–
Adrenal gland	+	–	ND	–	–	+	–
Spleen	–	–	–	–	ND	+	–

(+) = PCR positive for trypanosomes, (–) = PCR negative, ND = not done, Tx = Cymelarsan treated, *Euthanized for the welfare of the animal since hind quarter paralysis occurred at day 18 post-infection and no impression smear was done.

macrophages between the axons of almost all nerve fascicles sometimes associated with variable axonal swelling and fragmentation (Fig. 3A, B). In the spinal cord, histopathological lesions were confined to the white matter and consisted of chronic axonal degeneration (as of few numbers of empty myelin sheaths with infiltration of histiocytes and phagocytosis of debris, seldom with spheroids). In the lumbar spinal cord, mild meningeal perivascular infiltration of lymphocytes and histiocytes were found (Fig. 3C). In the cerebrum, cerebellum and brain stem, lesions were mostly absent. Only in two animals, mild perivascular infiltration of mononuclear cells in the cerebellar meninges (mild non-suppurative meningitis) were present (Fig. 3D, E). Cerebral meningitis was confined to only one animal. In another animal similar but moderate infiltrates were present in the meninges of the brain stem.

In the reproductive tract, most severe lesions were found in the genital mucosa of the vestibule, vagina and cervix where they consisted of nodular infiltrations of lymphocytes, plasma cells and a few histiocytes sometimes with erosions of the epithelium (Fig. 3F, G). In the uterus, only minimal signs of inflammation were seen. Ovaries and mammary glands had no histological abnormalities.

Kidneys in some animals showed multifocal, intraluminal eosinophilic, amorphous protein-rich material (protein-rich urinary filtration fluid) and cellular debris. There were some areas with small infiltrates of lymphocytes and plasma cells in the interstitium of the cortex. Occasionally, some tubules contained intraluminal degenerated epithelial cells and neutrophils. There was also basophilic amorphous material of intraluminal calcification. There was a mild portal infiltration of lymphocytes, plasma cells and a moderate infiltration of eosinophils in the liver. The bile canaliculi were distended and filled with brown pigment (bile stasis). Many hepatocytes had intracytoplasmic brown granules and within the sinusoids, there were many Kupfer cells with brown granules, which stained blue with Berlin blue staining (hemosiderin). In the lamina propria and in a lesser amount in the submucosa of the intestines, a mild to moderate infiltration of mainly lymphocytes and plasma cells and some eosinophils was observed.

In the spleen, there were many macrophages with intracytoplasmic brown granules (hemosiderin). Some giant cells and many Russell bodies were observed. The white pulp was enlarged (reactive). In the red pulp, there was a mild infiltrate of eosinophils. Different lymph nodes such as the prescapular, prefemoral, inguinal, popliteal, hepatic and renal lymph nodes showed similar microscopic features. The white pulp was enlarged and paler (reactive). In the medullary and intermolecular sinuses, there were some giant cells and a higher amount of macrophages. In the interalveolar septa of the lung, some macrophages with intracellular brown pigment were observed, as well as in the peribronchial areas moderate infiltrations of mainly lymphocytes and some macrophages containing brown granules. The pigment was blue on Berlin blue staining indicating the presence of iron (hemosiderin).

No specific microscopic changes were detected in the urinary bladder, stomach, heart, pancreas and adrenal glands of all horses. The

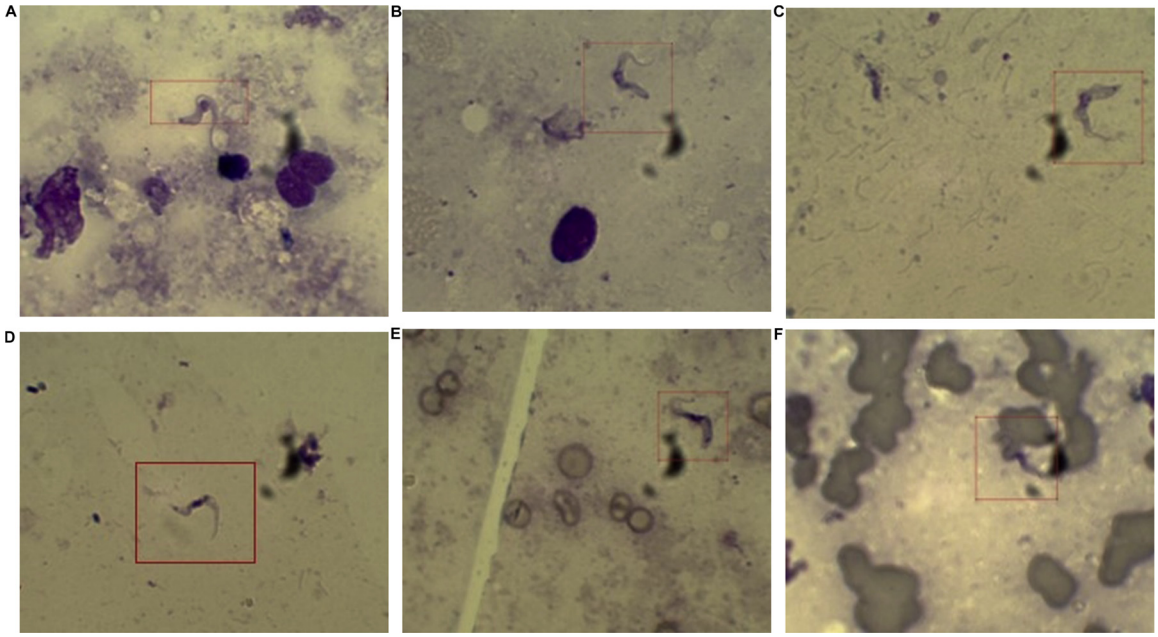

Fig. 2. *T. equiperdum* from impression smears of the organs at necropsy (Diff-Quick stain) (A) cerebrum, (B) cerebellum (C) uterus, (D) ovary, (E) kidney and (F) testicle.

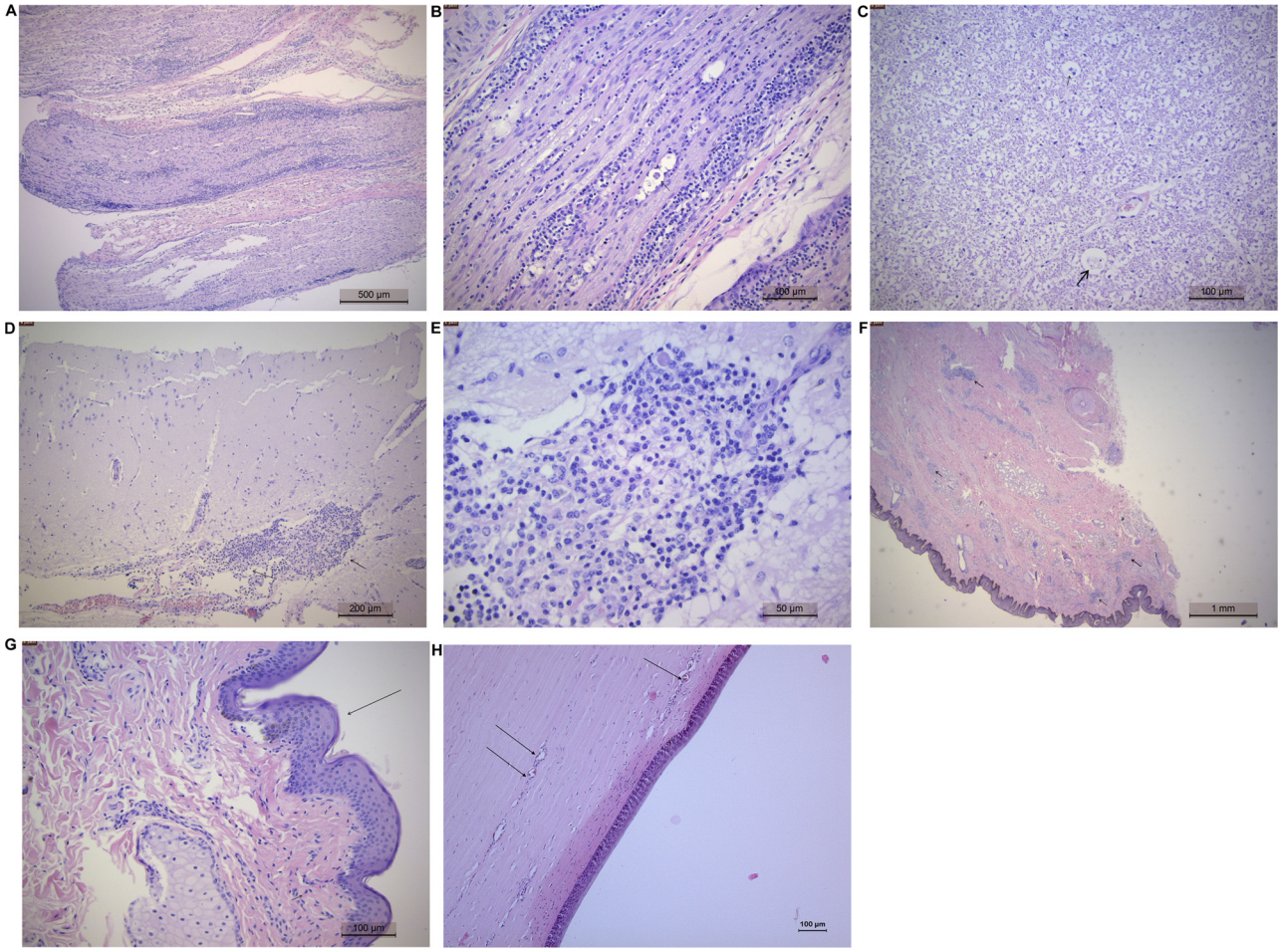

Fig. 3. Photomicrograph of the histopathological lesions of some tissues of dourine diseased mares (Haematoxylin-eosin staining) (A) peripheral nerve: diffuse non-suppurative neuritis (B) peripheral nerve: intraneural infiltration of lymphocytes, plasma cells and histiocytes with axonal swelling and fragmentation (arrow) (C) spinal cord- empty myelin sheaths with phagocytosis of debris (arrows) (D) cerebrum: meningeal infiltration of lymphocytes, plasma cells and histiocytes (E) detail of cerebrum meningeal infiltrate (F) vaginal mucosa: nodular infiltrations of lymphocytes, plasma cells and histiocytes (arrows) (G) depigmented skin of the vulva with multifocal areas of epidermal depigmentation, melanocytes loss (large arrow at junction of pigmented and non-pigmented, small arrow at minimal dermal lymphocyte-infiltration) (H) cornea: small vessels with plump endothelial cells (neovascularization) (arrows).

eyes with corneal opacity contained microscopic small vessels with plump endothelial cells (neovascularization) in the superficial stroma of the cornea (Fig. 3H).

3.2. Infection by blood transfusion in stallions

3.2.1. Diagnosis of Dourine and infectiousness of semen and preputial discharge

Stallions, inoculated by blood transfusion, showed the clinical symptoms of dourine such as swelling of the prepuce from day 10 post-infection (S01), day 19 (S04) and day 21 (S02 and S03), with intact libido but unable to mount due to incoordination and ataxia from day 13 (S01, S02, S03) and cachexia at later stages. Trypanosomes were observed in the blood from day 5 post-inoculation onwards by the Woo test (Table 3). Semen could be collected successfully on day 7 post-inoculation from 3 of the four stallions (S02, S03, and S04) to check for the presence of the parasite by PCR. However, the result of the PCR on these semen samples for the parasite DNA was negative. Due to the difficulties to mount caused by the disease, no semen could be obtained anymore in 3 out of 4 stallions (S01, S02 and S03) later on. Chemical ejaculation, using Imipramine hydrochloride and xylazine and ground collection were not successful. Only one of the four stallions (S04) was able to mount the phantom at day 13 post-infection. This ejaculated semen was used to inoculate mice (N = 7) and DNA was extracted for PCR. Parasite DNA was detected by PCR test and mice inoculated by this ejaculated semen became parasitaemic (7/7).

Since the route of infection in stallions was directly into the bloodstream and the infection-dose was higher, the detection of trypanosomes by Woo test was earlier compared to that of the mares. Preputial swelling was manifested in all infected stallions and later extended to the scrotal area. Depigmentation was observed on the skin around the anal region in one of the stallions. Oedema of lower hind limbs below the stifle joint was observed in another stallion. Signs of dourine such as back pain, lameness and paralysis of the hindquarters leading to partial dragging or stiffness of the hind legs, a staggering gait, posterior ataxia and an inability to stand upright after prolonged recumbency were similar to what was observed in the mares. Stallions still exhibited libido but were unable to mount the mares nor the breeding phantom due to facing difficulties to jump. Inflammation of the conjunctiva and lacrimal discharge of the eyes was noticed in 2 of the 4 infected stallions that eventually resulted in corneal opacity and led to permanent blindness.

3.2.2. Haematology

In stallions after infection, a significant decrease in mean PCV, Hgb concentration, RBC and WBC counts compared to the values prior to infection was observed. There was a significant ($p < 0.05$) decrease in the PCV, Hgb concentration starting from week 2 and RBC count from

Table 3
Interval between *T. equiperdum* infection and positive test results in the various diagnostic tests and clinical symptoms in stallions following artificial infection with *Trypanosoma equiperdum* through blood transfusion.

Horses ID	Positive for *T. equiperdum* (days post infection)	Symptoms of dourine noticed (days post infection)			
	Woo test	Oedema of the prepuce	Oedema of limbs	Depigmentation	Ataxia
S01	5	10	26	–	70
S02	5	21	–	43	102
S03	5	21	–	–	92
S04	5	19	–	–	55
Average	5	17.75	26	43	79

(-) indicates clinical signs absent.

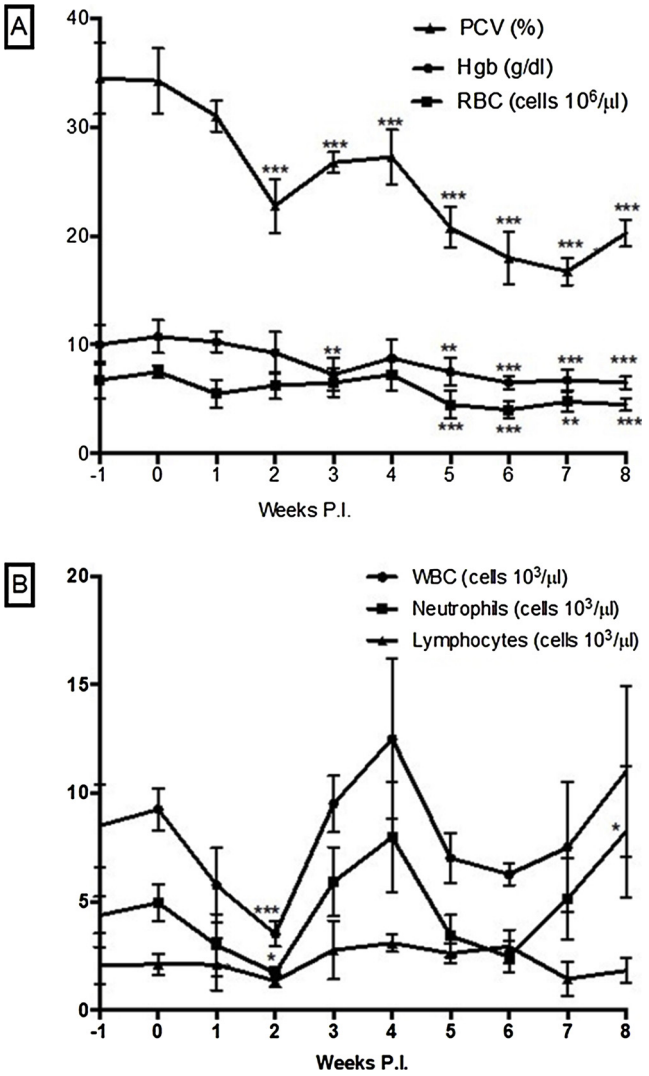

Fig. 4. Mean haematological values in stallions experimentally infected with *T. equiperdum* by blood transfusion, (A) RBC count, PCV and Hgb concentration (B) total WBC, lymphocytes and neutrophils counts. Significant (*** p = 0.001, ** P = 0.01, * p = 0.05).

week 5 post-infection compared with the date of infection (week 0) (Fig. 4A). The mean of total WBC count was significantly ($p < 0.05$) reduced by week 2 post-infection. However, it quickly increased to the level above the pre-infection values and remained fluctuating during the study period. There was a slight increase in lymphocytes and neutrophils (Fig. 4B).

3.2.3. Tissue distribution of the parasite and pathological lesions

At necropsy, 120 days post-infection in the chronic stage of the disease, all gross and histopathological lesions of all organs of the nervous system, other vital organs such as the liver and spleen and the eye with corneal opacity were very similar to that of the mares. The impression smear from the brain, kidney and other vital organs showed the presence of the trypanosomes similar to the mares. Trypanosomes also found in the testicles (Fig. 2F). The presence of the parasite in the tissue of many more organs was also further confirmed by RT-PCR (Table 4). In the reproductive system, clear fluid accumulation was observed at the scrotum and preputial swellings. On histological examination of the male reproductive tract, most severe lesions were found in the testicles (Fig. 5A, B). Testes showed diffuse orchitis characterized by moderate to severe interstitial infiltration of lymphocytes, plasma cells and few macrophages as well as a mild to moderate increase in interstitial fibroblasts (early fibrosis). Seminiferous tubules were severely degenerated and spermatogenesis was mostly absent. The epididymis was normal. The genital mucosae showed superficial lymphoplasmacytic infiltration of varying intensity. In the accessory glands, no histological changes could be noticed.

Table 4
Results of impression smear and RT PCR analysis on tissue samples collected from stallions 120 days after an artificial *T. equiperdum* infection.

Tissue sample	S01	$S02_{Tx}$		$S03_{Tx}$		S04	
	PCR	PCR	Smear	PCR	Smear	PCR	Smear
Nervous system							
Cerebrum	+	+	+	+	+	+	+
Cerebellum	+	+	–	+	–	+	+
Brain stem	ND	+	–	+	–	+	ND
Lumbar spinal cord	+	+	ND	+	ND	+	ND
Sacral spinal cord	+	+	ND	+	ND	+	ND
Obturator nerve	ND	+	ND	+	ND	+	ND
Reproductive system							
Epididymis	+	+	ND	+	ND	+	ND
Testicle	+	+	+	+	+	+	+
Prepuce	+	+	ND	ND	ND	+	ND
Vesicular gland	+	+	ND	+	ND	+	ND
Ampulla	+	–	ND	+	ND	+	ND
Prostate	+	+	–	+	–	–	ND
Others							
Lung	–	+	+	+	–	–	–
Liver	+	+	+	+	+	+	+
Kidney	–	–	–	+	+	+	+
Heart	–	+	+	+	–	+	–
Pancreas	–	–	–	+	–	+	–
Adrenal gland	+	+	–	+	–	–	–
Spleen	+	–	–	+	–	–	–

(+) = PCR positive for trypanosomes, (–) = PCR negative, ND = not done
Tx = Cymelarsan treated.

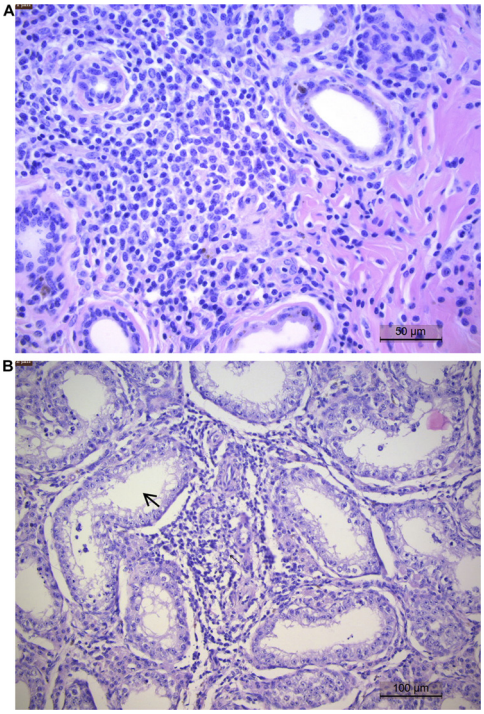

Fig. 5. Microscopic features of testicle of dourine diseased stallions (Haematoxylin-eosin staining) (A) nodular infiltrations of lymphocytes, plasma cells and histiocytes (B) infiltration of lymphocytes and plasma cells (small arrow) and severe degeneration of the tubuli (large arrow).

3.3. Cymelarsan® treatment

Cymelarsan® treatment (at day 60 post-*T. equiperdum* infection) failed to cure chronically infected horses. There was no improvement in clinical signs except a slight, transient improvement in PCV for a few weeks. The presence of the parasite was demonstrated by impression smears at necropsy and further confirmed by RT-PCR from treated horses similar to the non-treated ones. Mice inoculated with preputial fluid of Cymelarsan treated stallions at day 63 post-infection (n = 2) were found to be aparasitaemic (0/3) for each stallion whereas those inoculated with preputial fluid from non-treated stallions (n = 2) developed parasitaemia (3/3) each. Mice inoculated with epididymal semen after day 120 post-infection of the non-treated stallion all developed parasitaemia (5/5). However, when epididymal semen of cymelarsan treated stallions was used, parasitaemia developed only in mice inoculated with epididymal semen from one stallion (5/5) but not from the other one (0/5). All the preputial fluids and epididymal semen samples of both treated and non-treated stallions were found to be positive on PCR.

4. Discussion

Mares infected via artificial insemination (36,000 trypanosomes) developed parasitaemia starting from day 5 post-infection similar to horses infected by blood transfusion, regardless of the higher dose used (100,000 trypanosomes) in the transfusion infection. Dissemination of trypanosomes, even in testicular areas which are supposed to be delineated by a blood-testis barrier, was reported by Pascucci et al. (2013) where the intra-scrotal inoculation of rabbits with udder secretions containing 1000 trypanosomes, resulted in invasion of all tissues.

In the current study, it is shown that ejaculated semen of infected stallions is already infectious to mice in the preclinical period and epididymal semen from cymelarsan treated stallions was still infectious. Moreover, it was shown that *T. equiperdum*-spiked semen was able to infect mares when used in artificial insemination. This clearly indicates that transmission of *T. equiperdum* is possible through semen itself and secondly, that semen from symptomless stallions post-treatment can transmit dourine. Although the presence of the parasite in the semen was previously suggested by its isolation from the urethral tract of diseased horses (Suganuma et al., 2016) the current findings show the infectivity of the semen itself and confirm our previous hypothesis that the spread of dourine to various parts of the world might be enabled through the transportation of infected stallions, mares, donkeys and semen (Ahmed et al., 2018).

All infected animals exhibited a rise in temperature which coincides with the level of blood parasitaemia. The presence of fever is in accordance with previous findings (Brun et al., 1998; Claes et al., 2005) although others did not observe a temperature rise post *T. equiperdum* infection (Barrowman, 1976; Vulpiani et al., 2013). In other types of trypanosomosis (e.g. *T. vivax*) a rise in rectal temperature and temperature fluctuating with the level of parasitaemia is also a common finding (Delespaux, 2004; Adeiza et al., 2008; Dagnachew et al., 2015).

Weight loss, without a loss of appetite, was observed in all animals leading to a considerable degree of cachexia in accordance with previous reports (Barrowman, 1976; Scacchia et al., 2011; Vulpiani et al., 2013). The decline in body condition is a sequel of the parasitaemia resulting in an accumulation of peroxides and free radicals in the body of infected animals causing severe cell damage (Saleh et al., 2009). The uptake of glucose by the parasite necessary for its metabolic activity induces insufficiencies in the host (Hunt, 2010). Similar symptoms have been reported for *T. evansi* in camels (Enwezor and Sackey, 2005). Since, *T. evansi* and *T. equiperdum* exhibit many similarities in biological, biochemical and molecular characteristics (Brun et al., 1998; Ahmed et al., 2018), body weight loss in a *T. equiperdum* infected animal might be attributed to this same mechanism.

Depigmentation of skin around the perineal region observed in the present study has been reported previously (Claes et al., 2003; Hagos et al., 2010a; Pascucci et al., 2013). The probable cause of depigmentation around the vulval skin of infected mares could be due to severe necrosis of the epidermis (which also affects the melanocytes). Gizaw et al. (2017) reported that the depigmented areas were microscopically characterized by severe necrosis of cells, excess free melanin and formation of cystic structures in the epidermis. According to Myers and McGavin (2006), after damage to cells which contain melanin (e.g. melanocytes and basal cells of the skin), the free melanin will be phagocytosed by macrophages which causes loss of melanin pigment in the epidermis resulting in depigmentation.

Preputial swelling in stallions gradually extending to the scrotal area and vulval oedema and mucopurulent vaginal discharge in mares, were consistent findings in previous studies (Alemu et al., 1997; Hagos et al., 2010a; Lelli et al., 2012; Pascucci et al., 2013; Vulpiani et al., 2013). However, oedematous skin plaques, historically regarded as important or even pathognomonic symptoms in cases of dourine (Barrowman, 1976; Claes et al., 2005; Pascucci et al., 2013 and Vulpiani et al., 2013), were not observed in mares or stallions throughout the study period. Differences in *T. equiperdum* strain or breed, nutritional, stress factors and host immune response of horses involved in the experiment are possibly causing these differences in symptomatology (OIE, 2013; Ricketts et al., 2011). The presence of ventral oedema in the experimental horses was restricted to the limbs (in only 3 out of 6 animals), prepuce and scrotum. However, previous reports describe ventral oedema extending to the belly region up to the sternum (Hagos et al., 2010a; Lelli et al., 2012; Pascucci et al., 2013; Vulpiani et al., 2013).

Nervous signs of dourine manifested themselves on average after 64 days in all infected horses and were characterized by hind quarter lameness, incoordination and ataxia. In most studies, nervous signs have been reported to occur only at later stages although not specified in days of occurrence (Scacchia et al., 2011; Vulpiani et al., 2013). In the present study, we noticed that stallions tried to mount mares or the breeding phantom thus exhibiting a normal libido but failed to do so

due to hind quarter lameness or ataxia at early stages of the disease i.e. 7–13 days post infection. In the clinical follow up and attempts to collect semen, stallions were teased to mount on a phantom and/or a mare by keeping an oestrous mare in front of the stallion (to tease the stallion to mount the phantom), stallions had an intact libido, they tried to mount several times, but were unable to jump and mount neither the phantom nor the mare. All stallions were used to this procedure since before the infection trial they all were trained to this way of semen collection. Moreover, dourine as disease is known by these typical signs of hind leg ataxia and is reflected in the local name of the disease as 'Lapessa' or 'Kuta' meaning a 'back bone breaker' (Affan Oromo) (Hagos et al., 2010a). Conversely, these nerves signs were only observed very late in mares (after day 44). However a possible explanation might be that stallions are teased to mount and in this way, early symptoms become easy to notice whereas mares are left in peace at that time. The presence of neurological signs confirms the tropism of *T. equiperdum* for the peripheral nervous system and the lack of involvement of the central nervous system, in contrast with other trypanosomes.

Corneal opacity was found in two stallions and one mare and was consistent with previous reports (Alemu et al., 1997; Vulpiani et al., 2013). Aqueous intraocular fluid collected at necropsy from the eye suffering this corneal opacity was also positive for the parasite DNA in the RT-PCR. That way low concentrations of parasite DNA from conjunctival swabs were already detected in similar cases (Theis and Bolton, 1980; Pascucci et al., 2013).

Using a gentle pressure of a glass slide on a freshly cut surface of the tissues at necropsy and stained with Diff-quick was enough to demonstrate the presence of the parasite in different organs, more specifically in the nervous and reproductive tracts. This rather simple "impression smear" technique, as used to detect Leishmania and Toxoplasma (Swierczewski et al., 2013), has shown its value for quick screening of postmortem tissues for the presence of trypanosomes. In small mammals such as *Crocidura russula* and *Mus spretus* trypamastigote forms of trypanosomes (*Trypanosoma musculi* and *Trypanosoma microti)* from tissues such as heart, liver and kidney had also been previously identified by the use of this technique (Santos-Gomes et al., 1993). Based on the PCR results, trypanosome DNA could be extracted from most tissues of the infected animals (Table 4) and in several organs, the presence of the trypanosome itself could be demonstrated and made visible by use of impression smears, although blood contamination can't be excluded using this technique.

The literature on pathological lesions caused by *T. equiperdum* in horses is scarce. The gross pathological lesions in the reproductive tract, such as congestion of vaginal and uterine mucosa with widespread haemorrhage, are in agreement with the report of Pascucci et al. (2013).

Gross lesions in non-reproductive organs were not common except for abdominal fluid accumulation. This is in line with the report of Pascucci et al. (2013) who stated that no lesions are observed in the parenchymatous organs except for some congestion of the spleen. The presence of an increased amount of fluid in the abdominal cavity is a sequel of the hypoproteinaemia caused by the low albumin level. Orhue et al. (2005) indicated that serum albumin levels decreased in trypanosomosis. The more general oedema during the chronic stage of trypanosomosis might be due to a significant decrease in the albumin levels (Enwezor and Sackey, 2005).

Previous attempts to use molecular techniques to investigate the presence of *T. equiperdum* involved analysis of blood samples (Clausen et al., 1999, 2003; Fikru et al., 2010). Since *T. equiperdum* is a tissue parasite, blood might not be the most reliable matrix for the diagnosis of dourine, as parasitaemia is not constant (OIE, 2013). Pascucci et al. (2013) reported the detection of parasite DNA by RT- PCR in tissues other than blood such as vaginal swabs, joint fluid, lymph nodes and mammary secretions using a set of primers for detection of a gene specific for the trypanozoon group. In the current study, a number of tissues collected at necropsy were found to be positive for *T. equiperdum* by the use of real-time PCR using ITS1 primer. This might significantly improve the diagnosis of dourine in live animals by collecting biopsies from cerebrospinal fluid and lymph nodes. Also, uterine and vulval biopsies collected in this study during the course of the disease, when parasites were not found in the blood, were positive for PCR (data not shown).

The common finding of a plasma cell infiltration, seen in a number of organs, seems to indicate that a diffuse immune-mediated inflammatory reaction could be the basis of the pathogenesis of the disease. Infiltration of tissues with mononuclear inflammatory cells, especially lymphocytes and plasma cells, is a hallmark of chronic inflammation (Jones et al., 1997). The microscopic findings of the present study, show an infiltration of mononuclear cells, especially lymphocytes, in several tissues (e.g. testicle, uterus, spinal cord) and indicate the presence of a chronic inflammatory process in accordance to previous reports (Morrison et al., 1981; Rodrigues et al., 2009; Gizaw et al., 2017).

Mammary glands had no histological abnormalities in the current study. However, trypanosome can be isolated from mammary gland secretions (Vulpiani et al., 2013). PCR test from mammary gland secretions of dourine diseased horses from natural infections in another study were also found positive. In the current study trypanosomes were detected from many more tissues without histological lesions. The same is true for the lesions in the mammary glands. The presence of the trypanosome in the mammary secretion may not end up with lesions, so the absence of tissue lesions might not exclude infectivity of udder secretions.

Starting from the first week post-infection anaemia with a decrease in mean PCV and RBC compared to pre-infection values was observed. The haemolysis of RBC induced by the trypanosomes might be responsible for the anaemia. Anaemia was moderate during early infection and became more severe as the disease progressed. Suganuma et al. (2016) described a slight decrease in PCV and RBC count in dourine infected stallions in Mongolia. Vulpiani et al. (2013) also reported that anaemia progressed from a moderate to a more severe state in dourine diseased horses with chronic oedematous lesions or nervous signs. A haemolytic reaction due to acute RBC destruction could be indicated by the reduction in the RBC count and PCV values. This is a normocytic normochromic type of anaemia (Weatherall, 2003)

The WBC counts of infected stallions decreased immediately one week post infection and increased rapidly to the level above the pre-infection values starting two weeks later and remained fluctuating during the study. The fluctuating WBC is associated with increased numbers of lymphocytes and neutrophils. The increased activity of the mononuclear phagocytic system during trypanosome parasitaemia is an attempt to tackle the trypanosome distribution. In the first two weeks, this results in a decline in WBC numbers due to the massive use of circulating white blood cells more rapidly than they can be produced. The neutrocytopenia observed during the course of infection is thought to increase the susceptibility of infected animals to concurrent infections (Stephen,1986; John et al., 2006; Suganuma et al., 2016).

The mean lymphocyte counts post infection was elevated for some time as compared to values before infection and reduced to normal values after the parasitaemia and as such accompanying the numbers of trypanosomes in the circulation and when the challenge by the parasitaemia decreased, the immune response (WBC count) also diminished. Anosa et al. (1992) ascribed that lymphocytes react to trypanosome antigenic challenge by an increased proliferation of immuno-competent cells into more specific antibody and/or lymphokine producing cells. The fluctuation in the neutrophil counts might be due to the phagocytosis of trypanosomes by the circulating neutrophil cells and their destruction in the spleen and lymph nodes (as been reported in mice infected with *T. brucei*, (Anosa and Kaneko, 1983) and an enhanced activation of neutrophil precursor cells in the bone marrow.

Although cymelarsan® treatment in the acute phase of the disease resulted in a significant improvement in mean PCV values and a cure

rate of 100% (Hagos et al., 2010b), it was found to be ineffective in chronic cases of *T. equiperdum* diseased horses and no improvement of clinical signs was observed. On post-mortem examination, in both treated and non-treated animals, parasites could be found by impression smears and on RT-PCR.

The differences in cure rate between the current study and the treatment in the acute phase (Hagos et al., 2010b) might find its explanation in the fact that once the parasites disseminate out of the peripheral circulation (chronic stage) they escape the treatment. This seems to be confirmed by Cauchard et al. (2016) and Hébert et al. (2018) who indicated a failure of Cymelarsan treatment to clear of *T. equiperdum* OVI from the cerebrospinal fluid. Considering the high tissue distribution capacities of cymelarsan diffusing easily into the brain, uterus, ovaries, testicles and many vital organs as shown in camels (Musa et al., 1994; Youssif et al., 2008), alternative pathways of therapy failure might as well cause the difference between the reported studies. *T. equiperdum* OVI has been reported to be less sensitive *in vitro* to Cymelarsan® than a panel of 19 other *T. equiperdum* and *T. evansi* strains, even though the *T. equiperdum* Dodola strain used by Hagos et al. (2010b) was not included in that study (Gillingwater et al., 2007). Randomly amplified polymorphic DNA (RAPD) cluster analysis (Hagos, 2010) and genome-wide single nucleotide polymorphism (SNP) analysis (Cuypers et al., 2017) of many strains in the trypanozoon subgenus assigned *T. equiperdum* Dodola 943 in to *T. equiperdum* OVI group.

After venereal transmission, *T. equiperdum* reaches the peripheral circulation and vice versa, as seen in stallions, semen contains trypanosomes early in the infection along with the development of clinical signs. However, in the chronic stage, parasites leave the circulation into tissues and cause severe tissue damages responsible for the signs of dourine. In conclusion, we were able to infect mares by AI with *T. equiperdum* spiked semen, resulting in all diagnostic clinical symptoms and changes in blood parameters. The parasites disseminated to blood and several tissues including the nervous system and reproductive organs. In stallions infected intravenously, parasites could be traced back in several organs, including the nervous system, reproductive organs and especially in semen. This study demonstrates and clears doubts to the venereal transmission and dangers for spread of *T. equiperdum* by AI.

Authors contribution

AY, HA, LV, LD, BG, MB, AT, AVS, PG and JG were responsible for the study design, Necropsy and laboratory findings and manuscript preparation. AY and PG analysed the PCR, AY, GV and LV did the histopathology and captured photographs. AY, MD and TD were responsible for the gross pathological examination at necropsy and the haematology analysis. AY, PG and LD were responsible for the data analysis. All authors reviewed the manuscript.

Conflict of interest

The authors declare that they have no competing of interests.

Animals were fed grass hay and supplemented with concentrates and minerals. Water was freely available. The handling of animals during the experiment was based on international guiding principles for biomedical research involving animals, as proposed by the Council for International Organizations of Medical Sciences (2012). Horses were euthanized humanely by intravenous administration of over dose sodium pentobarbital after sedated with xylazine The research was authorized by the Animal Research Ethics Review Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University (Permit No. VM/ERC/004/07/015).

Acknowledgements

The authors would like to acknowledge the financial support of the Flemish Inter-University Council University Development Cooperation (VLIR-UOS). Funding was received from the VLIR (TEAM ZEIN2013PR393). We would like to thank Prof. Philippe Büscher and Dr Nick Van Reet (ITM, Antwerp) for sharing the DNA of *T. equiperdum* 943 (Dodola) for positive control in the PCR. We also would like to thank Dr Birhanu Hadush (Mekelle University, Ethiopia) who gave us Cymelarsan.

References

Adeiza, A.A., Maikai, V.A., Lawal, A.I., 2008. Comparative haematological changes in experimentally infected Savannah brown goats with *T. brucei* and *T. vivax*. Afr. J. Biotechnol. 7, 2295–2298.

Ahmed, Y., Hagos, A., Merga, B., Van Soom, A., Duchateau, L., Goddeeris, B.M., Govaere, J., 2018. *Trypanosoma equiperdum* in the horse – a neglected threat? Vlaams. Diergen. Tijds. 87, 66–75.

Alemu, T., Luckins, A.G., Phipps, L.P., Reid, S.W.J., Holmes, P.H., 1997. The use of enzyme linked immunosorbent assays to investigate the prevalence of *Trypanosoma equiperdum* in Ethiopian horses. Vet. Parasitol. 71, 239–250.

Anosa, V.O., Kaneko, J.J., 1983. Pathogenesis of *Trypanosoma brucei* infection in deer mice (*Peromyscus maniculatus*): haematologic, erythrocyte biochemical, and iron metabolic aspects. Am. J. Vet. Res. 44, 639–644.

Anosa, V.O., Logan-Henfrey, L.L., Shaw, M.K., 1992. Light and electron microscopic study of changes in blood and bone marrow in acute haemorrhagic *T. vivax* infection in calves. Vet. Path. 29, 33–35.

Barrowman, P.R., 1976. Observations on the Transmission, Immunology, Clinical Signs and Chemotherapy of dourine (*Trypanosoma equiperdum* infection) in horses, with special reference to Cerebro-Spinal Fluid. Onderstepoort J. Vet. Res. 43, 55–66.

Birhanu, H., Gebrehiwot, T., Goddeeris, B.M., Büscher, P., Van Reet, N., 2016. New *Trypanosoma evansi* type B isolates from Ethiopian dromedary camels. PLoS Negl. Trop. Dis. 10, e0004556.

Blue, M.G., 1985. Genital injuries from mating in the mare. Equine Vet. J. 17, 297–299.

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

Cauchard, J., Carnicer, D., Madeline, A., Guitton, E., Giraudet, A., Büscher, P., Hebert, L., Laugier, C., 2016. Evaluation of Melarsamine hydrochloride (Cymelarsan®) efficacy for the treatment of dourine nervous form on experimentally infected ponies. J. Equine Vet. Sci. 39, S45eS55.

Claes, F., Agbo, E.C., Radwanska, M., Tepas, M.F., Baltz, T., De Waal, D.T., Goddeeris, B.M., Claassen, E., Buscher, P., 2003. How does *T. equiperdum* fit into the Trypanozoon genus? A cluster analysis and multiplex genotyping approach. Parasitol. 126, 425–431.

Claes, F., Büscher, P., Touratier, L., Goddeeris, B.M., 2005. *Trypanosoma equiperdum*: master of disguise or historical mistake? Trends Parasitol. 21, 316–321.

Clausen, P.H., Gebreselassie, G., Abditcho, S., Mehlitz, D., Staak, C., 1999. Detection of Trypanosoma DNA in serological positive but aparasitemic horses suspected of dourine in Ethiopia. Tokai J. Exp. Clin. Med. 23, 303–308.

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

Coles, E.H., 1986. Veterinary Clinical Pathology, 4th ed. WB Saunders company, Philadelphia. London, pp. 136–170.

CSA, 2017. Agricultural sample survey report on livestock and livestock characteristics, volume II, Statistical Bulletin 585, Addis Ababa. Ethiopia 16–20.

Cuypers, B., Van den Broeck, F., Van Reet, N., Meehan, C.J., Cauchard, J., Wilkes, J.M., Claes, F., Goddeeris, B., Birhanu, H., Dujardin, J.C., Laukens, K., 2017. Genome-wide SNP analysis reveals distinct origins of *Trypanosoma evansi* and *Trypanosoma equiperdum*. Genome Biol. Evol. 9 (8), 1990–1997.

Dagnachew, S., Bezie, M., Terefe, G., Abebe, G., Barry, J.D., Goddeeris, B.M., 2015. Comparative clinico-haematological analysis in young Zebu cattle experimentally infected with *Trypanosoma vivax* isolates from tsetse infested and non-tsetse infested areas of Northwest Ethiopia. Acta Vet. Scand. 57, 24.

Dean, S., Gould, M.K., Dewar, C.E., Schnaufer, A.C., 2013. Single point mutations in ATP synthase compensate for mitochondrial genome loss in trypanosomes. Proc. Natl. Acad. Sci. 2013, 05404.

Delespaux, V., 2004. African animal trypanosomoses. Adapted from: R.J Connor and P. Van Den Bossche In: In: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious Diseases of Livestock, vol. 12. Oxford University Press, Cape Town, pp. 251–295.

Domingo, G.J., Palazzo, S.S., Wang, B., Pannicucci, B., Salavati, R., Stuart, K.D., 2003. Dyskinetoplastic *Trypanosoma brucei* contains functional editing complexes. Eukaryot. Cell 2, 569–577.

Enwezor, F.N.C., Sackey, A.K.B., 2005. Camel trypanosomosis, a review. Vet. Arhiv. 75, 439–452.

Fikru, R.G., Hagos, A., Alemu, T., Goddeeris, B.M., Claes, F., 2010. Comparative diagnosis of parasitological, serological, and molecular tests in dourine-suspected horses. Trop. Anim. Health Prod. 42, 1649–1654.

Fikru, R., Goddeeris, B.M., Delespaux, V., Moti, Y., Tadesse, A., Bekana, M., Claes, F., De Deken, R., Büscher, P., 2012. Widespread occurrence of *Trypanosoma vivax* in bovines of tsetse- as well as non-tsetse-infested regions of Ethiopia: a reason for concern? Vet. Parasitol. 190, 355–361.

Gillingwater, K., Büscher, P., Brun, R., 2007. Establishment of a panel of reference *Trypanosoma evansi* and *Trypanosoma equiperdum* strains for drug screening. Vet.

Parasitol. 148, 114–121.
Gizaw, Y., Megersa, M., Fayera, T., 2017. Dourine: a neglected disease of equids. Trop. Anim. Health Prod. 49, 887–897.
Guyo, S., Legesse, S., Tonamo, A., 2015. A review on welfare and management practices of working equines. Glob. J. Anim. Sci. Livest. Prod. Anim. Breed. 3, 203–209.
Hagos, A., 2010. Isolates of Trypanosomes From Ethiopian Horses. Katholic University of Leuven, Belgium PhD. Thesis.
Hagos, A., Getachew, A., Büscher, P., Goddeeris, B.M., Claes, F., 2010a. Serological and parasitological survey of dourine in the Arsi–Bale highlands of Ethiopia. Trop. Anim. Health Prod. 42, 769–776.
Hagos, A., Goddeeris, B.M., Yilkal, K., Alemu, T., Fikru, R., Yacob, H.T., Feseha, G., Claes, F., 2010b. Efficacy of Cymelarsan® and Diminasan® against Trypanosoma equiperdum infections in mice and horses. Vet. Parasitol. 171, 200–206.
Hébert, L., Guitton, E., Madeline, A., Géraud, T., Zientara, S., Laugier, C., Hans, A., Büscher, P., Cauchard, J., Petry, S., 2018. Melarsomine hydrochloride (Cymelarsan®) fails to cure horses with Trypanosoma equiperdum OVI parasites in their cerebrospinal fluid. Vet. Parasitol. 264, 47–51.
Herbert, W.J., Lumsden, W.H.R., 1976. *Trypanosoma brucei*: a rapid "matching" method for estimating the host's parasitemia. Exp. Parasitol. 40, 427–431.
Hunt, R., 2010. Microbiology and Immunology online: Parasitology. The University of South Carolina Accessed 19/12/18. http://www.microbiologybook.org/parasitology/blood-proto.htm.
John, M.K., John, K.T., Maina, N., Raymond, M., David, M.M., Joseph, M.N., 2006. Haematology of experimental Trypanosoma *brucei rhodesiense* infection in vervet monkeys. Afr. J. Health Sci. 13, 59–65.
Jones, T.C., Hunt, R.D., King, N.W., 1997. Veterinary pathology. Baltmore (Md.), 6th ed. Williams and Wilkins, London, Philadelphia, pp. 177–196.
Lelli, R., Calistri, P., Giovannini, A., Caporale, V., 2012. Evidence of *T. equiperdum* infection in the Italian dourine outbreaks. J. Eq. Vet. Sci. 32, 3–95.
Melo, C.M., Papa, F.O., Fioratti, E.G., Villaverde, A.I.S.B., Avanzi, B.R., Monteiro, G., Dell'aqua Junior, J.A., Pasquini, D.F., Alvarenga, M.A., 2008. Comparison of three different extenders for freezing epididymal stallion sperm. Anim. Reprod. Sci. 107, 331.
Morrison, W.I., Murray, M., Sayer, P.D., Preston, J.M., 1981. The pathogenesis of experimentally induced *Trypanosoma brucei* infection in the dog. I. Tissue and organ damage. Am. J. Pathol. 102, 168.
Musa, M.M., Abdoon, A.M.O., Nasir, B.T., Salim, Y.I., Abdel-Rahman, A.Y., Shommein, A.M., 1994. Efficacy of Cymelarsan® in the treatment of natural chronic *Trypanosoma evansi* infection in camels in the Sudan. Rev. élev. méd. vét. pays trop. 47 (4), 397–400.
Myers, R.K., McGavin, M.D., 2006. Cellular and tissue responses to injury. In: McGavin, M.D., Zachary, J.F. (Eds.), Pathologic Basis of Veterinary Disease, 4th ed. Elsevier, pp. 3–59.
Njiru, Z.K., Constantine, C.C., Guya, S., Crowther, J., Kiragu, J.M., Thompson, R.C., Dávila, A.M., 2005. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol. Res. 95 (3), 186–192.
OIE, 2013. Dourine, chapter 2.5.3. OIE Terrestrial Manual Version Adopted by the World Assembly of Delegates of the OIE in May 2013. pp. 1–6 Paris.
Orhue, N.E.J., Nwanze, E.A.C., Okafor, A., 2005. Serum total protein, albumin and globulin levels in *T. brucei*–infected rabbits: Effect of orally administered scoparia dulcis. Afr. J. Biotech 4, 1152–1155.
Pascucci, I., Di Provvido, A., Cammà, 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.
Qiagen, 2006. DNeasy Blood & Tissue Hand Book 07/2006. QIAGEN GmbH, QIAGEN Strasse 1. 40724. Hilden, Germany.
R Core Team, 2018. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org.
Ricketts, S., McGladdery, A., Crowhurst, J., Newton, R., 2011. Dourine, an emerging venereal threat to European horses. Equine Quarterly Disease Surveillance report 6 7 (2), 15–18.
Rodrigues, A., Fighera, R.A., Souza, T.M., Schild, A.L., Barros, C.S.L., 2009. Neuropathology of naturally occurring *Trypanosoma evansi* infection of horses. Vet. Pathol. 46, 251–258.
Roels, K., Leemans, B., Ververs, C., Govaere, J., Hoogewijs, M., Van Soom, A., 2014. Collection and freezing of equine epididymal spermatozoa. Vlaams. Diergen. Tijds. 83, 321–325.
Saleh, M.A., Al-Salhy, B.M., Sanousi, S.A., 2009. Oxidative stress in blood of camels naturally infected with Trypanosoma evansi. Vet. Parasit. 162, 192–199.
Santos-Gomes, G.M., Abranches, P., Maraghi, S., Dirie, M.F., Silva-Pereira, M.C., Valverde, D., Molyneux, D.H., 1993. Laboratory and field studies on Herpetosoma trypanosomes from Portugal. Ann. Parasitolo. Hum. Com. 68, 163–168.
Scacchia, M., Cammà, C., Di Francesco, G., Di Provvido, A., Giunta, R., Luciani, M., Marino, A.M.F., Pascucci, I., Caporale, V., 2011. A clinical case of dourine in an outbreak in Italy. Vet. Ital. 47, 473–475.
Slaoui, M., Fiette, L., 2011. Histopathology procedures: from tissue sampling to histopathological evaluation. Methods Mol. Biol. 691, 69–82.
Stephen, L., 1986. *Trypanosomosis*. A Veterinary Perspective. Pergamon Press, New York, pp. 351–420.
Suganuma, K., Narantsatsral, S., Battur, B., Yamasaki, S., Otgonsuren, D., Musinguzi, P.S., Davaasuren, B., Battsetseg, B., Inoue, N., 2016. Isolation, cultivation and molecular characterization of a new *Trypanosoma equiperdum* strain in Mongolia. J. Parasitol. Vector Biol. 9, 481.
Swierczewski, B.E., John, C.H., 2013. Examination of blood, other body fluids, tissues, and sputum. In: Magill, Alan J., Hill, David R., Solomon, Tom., Ryan, Edward T. (Eds.), Hunter's Tropical Medicine and Emerging Infectious Disease, 9th ed. W.B. Saunders Elsevier, London, pp. 1082–1083.
Theis, J.H., Bolton, V., 1980. *Trypanosoma equiperdum*: movement from the dermis. Exp. Parasite. 50, 317–330.
Vulpiani, M.P., Carvelli, A., Giansante, D., Iannino, F., Paganico, D., Ferri, N., 2013. Re-emergence of dourine in Italy: clinical cases in some positive horses. J. Equine Vet. Sci. 33, 468–474.
Weatherall, D.J., 2003. Normochromic, normocytic anaemia. In: Warrell, David A., Cox, Timothy M., Firth, John D. (Eds.), Oxford Textbook of Medicine, 4th ed. Oxford press, UK.
Whitwell, K., 2009. Post-mortem examination of horses. In Pract. 31, 104–113.
Woo, P.T.K., 1970. The haematocrit centrifuge technique for the diagnosis of African Trypanosomosis. Acta Trop. 27, 384–386.
Youssif, F.M., Hassan, T., Mohammed, O.S.A., 2008. Residues of CymelarsanR in camels (*Camelus dromedaries*) and Nubian goats infected with *T. Evansi* in Sudan. Afr. J. Food Sci. Technol. 2 (8), 92–97.