Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2025.262

RESEARCH ARTICLE

Male Fertility Impairment Associated with *Babesia Gibsoni* Infection in Dogs

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ARTICLE HISTORY (25-491)

Received: May 06, 2025
Revised: August 26, 2025
Accepted: August 27, 2025
Published online: October 07, 2025

Key words:
Acquired sterility
Babesia gibsoni
Babesiosis
Dog
Sperm

ABSTRACT

Recent findings indicate a link between *Babesia canis* infection and acquired infertility in male dogs, prompting further investigation into how *Babesia gibsoni* affects male reproductive health. Blood samples of 100 male dogs suspected of having babesiosis were collected for PCR, hematological, biochemical, and serological analyses to verify *B. gibsoni* infection and assess possible co-infections with *Anaplasma* spp., *Ehrlichia canis, Leishmania infantum*, and *Mycoplasma* spp. Out of the 37 *B. gibsoni*-positive dogs, semen samples were collected and analyzed before treatment and six months later from six American Staffordshire Terriers (ASTs), and compared to eight sexually mature, reproductively healthy dogs. At initial examination, *Babesia* spp. was detected in the semen of one dog, while two others tested positive six months posttreatment. Sperm analysis revealed no changes in sperm concentration between initial presentation and six months posttreatment. However, significantly lower percentages of progressively motile spermatozoa (PMOT, median range 26.5%) were observed alongside decreased velocity (VCL – curvilinear velocity, median range 51µm/s; VSL – straight-line velocity, median range 23µm/s; VAP – average path velocity, median range 27µm/s) and viability parameters (Dead, median range, 53.5%; Total defects median range, 37%) compared to healthy dogs (PMOT median range 80.5%; VCL median range 131µm/s; VSL median range 67µm/s; VAP median range 78µm/s; Dead, median range, 6.5%; Total defects, median range, 19.5%). The presence of *Babesia* spp. in semen may adversely affect sperm quality, causing acquired male sterility even six months posttreatment. Further research is essential to improve prevention efforts, resolve legal concerns about parasite transmission through semen, and enhance treatment strategies and reproductive results.

To Cite This Article: Strahinja M, Jelena FA, Slobodanka V, Svetlana N, Milica KF, Miloš D, Dimitrije G and Vladimir M 2025. Male fertility impairment associated with *Babesia gibsoni* infection in dogs. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2025.262

INTRODUCTION

Male infertility in canines remains a relatively underexplored topic, particularly the cases of acquired infertility, which reflects trends observed in human populations. The etiology of acquired infertility in humans remains unidentified in approximately 30% to 50% of cases (Agarwal *et al.,* 2021). Numerous factors can contribute to acquired infertility in male dogs, including 1) anatomical abnormalities such as spermatogenous or spermatocele obstruction, scrotal hernia, and strictures following surgery or injury; 2) orthopedic issues, such as those affecting the hips; 3) prostate problems; 4) epididymal or testicular issues; 5) urinary disorders; 6) retrograde ejaculation; 7) hormonal imbalances; 8) infectious diseases; 9) abnormal sexual behavior; and 10) drugs (Memon, 2007; Fontbonne, 2011).

Among all the causes, infectious diseases are likely the most prevalent cause of acquired infertility in male dogs. Domoslawska and Zdunczyk (2020a; 2020b), for example, recently emphasized a potential link between infection with *Babesia canis* in male dogs and acquired infertility. Damage to several organs during babesiosis sets off a cytokine storm that results in oxidative stress. Spermatogenesis is impacted by intricate interactions between the immune system and germ cells. Histopathological analysis shows tissue destruction,

including necrosis, inflammation, and degeneration of the seminiferous tubules, along with the presence of multinucleated cells within the lumens (Domoslawska and Zdunczyk 2020a). Haemolytic anaemia may cause oxidative stress, decrease blood flow, and affect microcirculation, albeit the precise process is still unknown (Domoslawska and Zdunczyk 2020a). In addition, Karasova *et al*. (2024) showed that in puppies infected with *Babesia gibsoni,* its DNA can be isolated from a variety of tissues, including the spleen, liver, kidneys, heart, brain, and lungs. The significance of looking into *B. gibsoni* infections in male dogs, especially its putative presence in the testicles and potential mode of transmission through semen, was emphasized by these early studies.

This study intends to investigate the reproductive health of male dogs infected with *B. gibsoni,* given that Serbia is endemic for *B. canis* infection and has seen a rise in asymptomatic *B. gibsoni* infections over the last ten years (Davitkov *et al*., 2015; Gabrielli *et al*., 2015), as well as occasional clinical cases in the last five years (Strobl *et al.,* 2021; Milošević *et al.,* 2024).

MATERIALS AND METHODS

Study population: This study, approved by the Ethics Committee of the University of Belgrade - Faculty of Veterinary Medicine and the Ministry of Agriculture, Forestry and Water Management of Serbia (Approval Nos. 323-07-11564/2022-05/1 and 001327728 2024 14841 002 000 323 022), was done from October 2022 to April 2025. It included male dogs that exhibited two or more clinical signs potentially linked with babesiosis (lethargy, depression, weakness, weight loss, anorexia, fever, pale mucosa, hemoglobinuria, and splenomegaly). A total of 100 dogs met this criterion. Detailed medical histories were obtained from the owners, including vaccination status, treatments received within the previous six months, possible tick presence, history of vector-borne diseases (VBD), chronic illnesses, as well as the history of possible dog bites and mating attempts. Following the general clinical examination, a breeding soundness examination was conducted, which included libido assessment, visual scrotum inspection, testes palpation, examination of the penis and prepuce for the presence of discharge, and an ultrasound examination of the urogenital system.

Blood samples were collected for hematological and biochemical analyses, and the conventional PCR test for *Babesia* spp. The blood samples originating from dogs with confirmed *Babesia* infection were sent for PCR sequencing and tested for coinfections with *Anaplasma* spp*., Ehrlichia canis, Mycoplasma haemocanis,* and *Mycoplasma haematoparvum.* An indirect immunofluorescence antibody test (IFAT) was performed for *E. canis*, *A. phagocytophilum, and Leishmania infantum*. Informed consent was given by six infected and eight healthy dogs'owners. Semen was collected for analyses from dogs diagnosed with *Babesia* spp. and re-sampled six months later. Eight sexually mature, reproductively healthy dogs served as controls. Based on the clinicopathological findings, the dogs were treated against babesiosis.

Hematological, biochemical, and urine analysis: Blood was collected in EDTA and serum tubes. Complete blood count (CBC) was analyzed using the automated ProCyte Dx hematology analyzer (Idexx, United States), and routine biochemical parameters were measured on a Mindray BS-240 biochemistry analyzer (China). Blood smears were prepared immediately after sampling to examine the presence of *Babesia* spp. merozoites. Urine was collected through cystocentesis to assess its appearance, and chemical (including the protein-creatinine ratio) and sediment analysis.

DNA extraction, conventional PCR detection, and sequencing: Total DNA was isolated from EDTA blood and semen samples using the Thermo Scientific™ GeneJET kit, following the protocol given by Milošević *et al.* (2024). *Babesia* spp. was detected via PCR with PIRO-A (AATACCCAATCCTGACACAGGG) and PIRO-B (TTAAATACGAATGCCCCCAAC) primers targeting a 410bp 18S rRNA fragment. Anaplasmataceae (including *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Neoehrlichia*, and *Wolbachia*) were identified using 16S rDNA primers EHR16SD and EHR16SR. *M. haemocanis* was detected with a commercial kit (BIORON GmbH), and *Candidatus M. haematoparvum* via qPCR using CMhempar-F (5'-ACGAAAGTCTGATGGAGCAATAC-3') and CMhempar-R primers (5'-TATCTACGCATTCCACCGCTAC-3') (Beus et al., 2024). The positive samples were purified with the GeneJET PCR Purification Kit and sequenced by Macrogen Europe. Only sequences with quality scores >30 were used. Primer sequences were trimmed, and consensus sequences (75% threshold) were uploaded to GenBank. The sequences were aligned with the reference sequences from 49 *Babesia* species, including *B. gibsoni* and *B. canis canis*, using the MAFFT algorithm.

Indirect immunofluorescence antibody test (IFAT): The MegaFLUO® kit was used to detect specific IgG antibodies in serum against *E. canis, A. phagocytophilum,* and *L. infantum*. The fluorescence patterns, including their form and density, of the negative and positive controls were used as references. All reactivity patterns that differed from these controls were considered non-specific and indicated a negative result. Samples were considered positive for *E. canis* and *A. phagocytophilum* when bright yellow-green fluorescence appeared in the inclusion bodies (morulae) at a dilution cut-off of 1:40. *L. infantum* positivity was indicated by light yellow-green fluorescence in promastigotes at a dilution cut-off of 1:100.

Semen collection and analysis: The ejaculates were obtained in the presence of a bitch in estrus, via manual manipulation, into pre-warmed glass tubes (36–38°C) and evaluated immediately after collection. The motility parameters and sperm concentrations were analyzed on the computer-assisted sperm analysis (CASA) system (Minitube, AndroVision, Germany). The sperm morphology was evaluated for abnormalities on 200 eosin-nigrosin-stained spermatozoa per slide.

Statistical analyses: The data were analyzed with SPSS using the Kruskal-Wallis test to assess group differences ($P<0.05$) and the Chi-square test for nominal data, with significance set at $P<0.05$.

RESULTS

Out of 100 dogs, thirty-seven tested positive for *B. gibsoni*. Six of these, all one-to-four-year-old American Staffordshire Terriers (ASTs), were included in the study, along with eight medium-sized reproductively healthy dogs, aged two to six years. All dogs had a BCS (body condition score) of 3/5. They were last vaccinated six to eight months before the initial assessment. None of the dogs had a history of VBD or chronic illnesses, and received any therapy in the previous six months. Only two AST owners reported a history of tick exposure. Two of the six ASTs had fever and pale mucous membranes. All exhibited apathy and a slightly reduced appetite. The clinically healthy reproductive control dogs had no previous history of tick infestation. All of the fourteen dogs had a history of bites: five clinically healthy dogs and two ASTs were bitten by other dogs, while the remaining three clinically healthy dogs and four ASTs bit other dogs. None of the ASTs attempted to mate, which left their owners unsure about possible fertility issues; however, the owners observed no problems with libido. Clinical and ultrasound examinations did not reveal any abnormality of the reproductive system in any dog. Urinalysis indicated no pathological findings in the urinary tract, including the absence of infection. The tests of the prepuce swabs yielded negative results for both bacteria and fungi.

The sequencing method confirmed that all ASTs tested positive for *B. gibsoni* and negative for *Anaplasma* spp.*, E. canis,* and *M. haematoparvum* in the blood. Furthermore, one AST was co-infected with *M. canis* (Table 1). The results of serological tests for ASTs indicated that 5 out of 6 dogs tested positive for *A. phagocytophilum*, 4 out of 6 for *L. infantum*, and 2 out of 6 for *E. canis* (Table 1). All clinically healthy reproductive dogs tested negative for the selected VB pathogens by PCR and serology. Two of the six ASTs exhibited regenerative normocytic normochromic anemia, while four displayed thrombocytopenia. Only one dog had both anemia and thrombocytopenia. All of the ASTs had total leukocyte counts (WBC) within reference ranges but one had neutrophilia and monocytosis. In addition, four out of the six dogs exhibited hyperproteinemia, and three had elevated ALT activity (Table 2). The clinically healthy reproductive dogs showed no changes in CBC and biochemical analyses (data not shown). The ASTs responded positively to therapy, and six months after treatment, they were PCR-negative for *Babesia* spp. with no changes observed in the repeated CBC (results not shown).

All evaluated AST samples' sperm quality parameters at the first and second presentations were below both those of clinically healthy dogs as well as those previously documented for fertile dogs (Domoslawska and Zdunczyk, 2020a) (see Table 3). The percentages of progressively motile sperm notably decreased as did their velocity and viability even though their concentrations stayed constant (Tables 3 and 4). At the first presentation, *Babesia* spp. was only isolated from the semen of Dog no. 4 (Table 1). Follow-up semen analyses showed persistent spermatogenic impairment six months post-infection (Table 4), with only Dog no. 4 exhibiting improved sperm quality (refer to Table 3). Six months after therapy, the PCR results for semen from Dog no. 4 were negative. However, *Babesia* spp. were isolated from the semen of Dog no. 2 and Dog no. 3 during the second presentation. Furthermore, *M. haemocanis* was isolated solely from the prepuce swab of Dog no. 2 at this time. The only difference in sperm quality between the first and second presentations was lower sperm motility at the latter (Table 4).

Table 1: PCR and serological tests conducted on whole blood and serum, and PCR results obtained on prepuce swabs and semen

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6
PCR analysis from blood samples						
Babesia spp.	Yes	Yes	Yes	Yes	Yes	Yes
	Yes	Yes	Yes	Yes	Yes	Yes
M. canis	No	No	No	No	No	Yes
M. haemocanis	No	No	No	No	No	Yes
E. canis	No	No	No	No	No	No
A. phagocytophilum	No	No	No	No	No	No
Immunofluorescence antibody test (IFAT)						
E. canis	Yes	No	No	No	No	Yes
A. phagocytophilum	Yes	No	Yes	Yes	Yes	Yes
L. infantum	Yes	No	Yes	No	Yes	Yes
PCR analysis of preputial swabs						
M. canis	Ist P	2nd P	Ist P	2nd P	Ist P	2nd P	Ist P	2ndP	3rdP	Ist P	2nd P	Ist P	2nd P
	ND	ND	ND	Yes	No	No	No	No	No	ND	ND	ND	ND
PCR analysis of semen samples													
Babesia spp	Ist P	2nd P	Ist P	2nd P	Ist P	2nd P	Ist P	2ndP	3rdP	Ist P	2nd P	Ist P	2nd P
	No	No	No	Yes	No	Yes	Yes	No	No	No	No	No	No

Ist P – first presentation, 2nd P – second presentation, 3rdP – third. presentation, ND – not done.

Table 2: Hematological and biochemical blood analyses.

	RI	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6
Complete blood count(unit)							
RBC (x10^{12}/L)	5.65-8.87	8.34	4.36	7.56	8.27	3.86	6.51
HCT (%)	37.3-61.7	52.9	29.7	51.7	57.3	28.6	45.4
HGB (g/dL)	13.1-20.5	17.9	9.8	17	19.3	9.3	14.7
MCV (fL)	61.6-73.5	63.4	68.1	68.4	69.3	74.1	69.7
MCH (pg)	21.2-25.9	21.5	22.5	22.5	23.3	24.1	22.6
MCHC (g/dL)	32-37.9	33.8	33	32.9	33.7	32.5	32.4
RDW (%)	13.6-21.7	21.5	16.4	20.3	19.3	20.4	18.6
RETIC (%)		1.1	2.7	3.6	1.8	8.6	1.3
RETIC (K/µL)	10-110	87.6	116.8	268.4	148.9	333.1	83.3
RETIC-HGB (pg)	22.3-29.6	20.8	20.7	21.3	23	22.1	21.8
WBC (x10^{9}/L)	5.05-16.76	8.94	15.38	19.97	13.56	11.49	9.55
NEU (x10^{9}/L)	2.95-11.64	5.13	11.85	10.83	9.74	6.44	5.04
LYM (x10^{9}/L)	1.05-5.10	2.86	1.28	4.91	2.83	3.7	3.15
MONO (x10^{9}/L)	0.16-1.12	0.38	1.47	1.24	0.99	0.79	0.82
EOS (x10^{9}/L)	0.06-1.12	0.52	0.7	2.89	0	0.55	0.4
BASO (x10^{9}/L)	0.00-0.10	0.05	0.08	0.1	0	0.01	0.14
PLT (K/µL)	148-484	100	371	37	187	29	137
MPV (fL)	8.7-13.2	13.3	12.7	18.5	12.7	17.5	12.5
PDW (fL)	9.1-19.4	0	17	0	0	0	19.6
PCT (%)	0.14-0.46	0.13	0.47	0.07	0.24	0.05	0.17
Presence of *Babesia spp.* merozoites on blood smear		No	No	No	No	Yes	No
Blood biochemistry parameter (unit)							
Ca (mmol/L)	2.20-3.00	2.17	2.5	2.49	2.24	2.31	2.23
P (mmol/L)	0.80-2.00	1.08	1.29	1.3	1.29	0.73	1.42
Glu (mmol/L)	3.00-6.70	5.1	4.01	6.03	6.36	5.93	5.68
T-bil (µmol/L)	1.00-9.00	0.95	1.74	2.3	1.44	3	0.04
ALT (U/L)	3.0-50.0	44.8	42.3	80.4	76.3	41.8	115.4
AST (U/L)	1.0-46.0	31.4	30.7	56.6	38.8	36.2	38.5
ALP (U/L)	0.0-190.0	37.3	46	60.1	79.5	36.1	48
γ-GT (U/L)	0.0-7.0	4.3	4.2	4.4	5.8	5	4.8
TP (g/L)	55.0-75.0	78.6	95.4	79.7	73.8	77.3	78.5
TG(mmol/L)	0.30-1.30	0.31	0.34	0.29	0.27	0.33	0.43
CREA (µmol/L)	50.0-169.0	85.7	48.5	83.6	81.4	60	42.7
UREA (mmol/L)	3.30-9.20	4.57	4	6.35	3.13	4.67	7.14
TC (mmol/L)	2.50-6.50	5.11	3.68	4.04	4.41	4.36	4.34
CK (U/L)	40.0-254.0	179	173.4	296.8	183.6	195.6	295.5
ALB (g/L)	25.0-40.0	31.6	31.1	33	35.1	29.3	31.2

RI - reference intervals.

Table 3: Semen parameters in dogs infected with *Babesia gibsoni* at first presentation and six months after treatment (second presentation)

Parameter	Dog 1	Dog 1	Dog 2	Dog 2	Dog 3	Dog 3	Dog 4	Dog 4	Dog 4	Dog 5	Dog 5	Dog 6	Dog 6	
	1st presentation	2nd presentation	1st presentation	2nd presentation	1st presentation	2nd presentation	1st presentation	2nd presentation	3rd presentation	1st presentation	2nd presentation	1st presentation	2nd presentation	RI
Live%	34	44	78	48	92	45	54	15	98	64	75	93	98	90–95
Dead%	66	56	22	52	8	55	46	85	2	34	25	7	2	5-10
Head defects (%)	8	18	4	12	8	5	12	24	4	10	4	4	4	4.0±3.0
Middle piece defects (%)	0	2	0	6	0	0	0	0	0	0	2	0	0	
Tail defects (%)	16	16	4	4	4	2	16	34	2	12	6	10	6	12.3±4.6
Proximal cytoplasmic droplets (%)	0	2	0	22	22	4	0	1	22	16	20	0	20	10.3±12.1
Total defects (%)	24	38	8	44	34	36	28	59	28	38	32	14	32	10–25
Other changes and cell types	none		none	Swollen middle part	none	Macro head 13% Micro head 12%	none	Dag defect	None	RBC		Mo, Ba		
Concentration 10^6/mL	12.12	15.45	160.83	46.41	277.2	6.33	120.1	269.2	276.72	38.61	52.94	120.6	276.72	292.6±208.3
TMOT (%)	17.1	16.08	74.84	40.44	91.93	33.02	42.97	14.64	97.27	63.09	73.01	93.81	97.27	88.3±18.4
PMOT (%)	8.06	10.51	34.5	26.66	77.17	25.62	14.91	9.41	86.63	38.08	53.25	19.25	88.63	60–70
PFMOT (%)	5.81	8.48	19.04	18.49	63.35	20.99	7.94	7.39	69.33	27.49	36.74	63.76	69.33	65.2±21.7
PSMOT (%)	2.26	1.9	15.46	8.17	13.66	4.63	6.97	2.03	17	10.58	16.51	15.4	17	19.7±12.8
PCMOT (%)	0	0.13	0	0	0.17	0	0	0	0.3	0	0	0.1	0.3	
LMOT (%)	9.03	5.57	40.34	13.77	14.76	7.44	28.06	5.23	10.64	25.01	19.76	14.55	10.64	
STATC (%)	82.9	83.92	25.16	59.56	8.07	66.98	57.03	85.36	2.73	36.91	26.99	6.19	2.73	11.8±14.4
VCL (µm/s)	27.67	28.67	66.77	50.67	127.4	50.72	40.91	29.92	145.61	68.15	82.27	118.5	145.61	160.7±19.7
VSL (µm/s)	14.13	11.65	21.96	22.21	50.32	23.52	13.61	19.04	60.95	27.84	25.63	49.35	60.95	113.0±20.2
VAP (µm/s)	16.05	14.1	28.43	26.51	60.41	27.1	18.75	21.11	73.53	34.33	34.26	58.84	73.53	124.3±19.7
DCL (µm)	9.64	11.25	27.64	19.46	42.54	22.5	16.71	7.57	43.94	27.51	31.52	43.63	43.94	
DSL (µm)	2.66	2.84	7.27	6.33	15.48	8.75	3.45	2.03	16.05	9.32	7.64	16.88	16.05	
DAP (µm)	3.66	4.08	10.36	8.39	19.29	10.57	5.98	3.09	20.96	12.3	11.35	20.75	20.96	
ALH (µm)	0.34	0.33	0.73	0.58	1.26	0.62	0.51	0.26	1.35	0.76	0.93	1.21	1.35	5.0±0.7
BCF (Hz)	3.26	2.31	8.88	6.36	15.12	5.93	3.91	0.94	16.3	9.72	9.07	15.49	16.3	26.2±4.4
HAC (rad)	0.07	0.07	0.13	0.11	0.23	0.12	0.08	0.08	0.29	0.13	0.18	0.23	0.29	
WOB (VAP/VCL)	0.58	0.49	0.43	0.52	0.47	0.53	0.46	0.71	0.5	0.5	0.42	0.5	0.5	
LIN (VSL/VCL)	0.51	0.41	0.33	0.44	0.39	0.46	0.33	0.64	0.42	0.41	0.31	0.42	0.42	70.1±7.5
STR (VSL/VAP)	0.88	0.83	0.77	0.84	0.83	0.87	0.73	0.9	0.83	0.81	0.75	0.84	0.83	88.9±3.4

RI - reference intervals (according to Domoslawska and Zdunczyk (2020a). Canine babesiosis - a disease rarely considered in the context of male infertility. Ir Vet J 6;73(1):22), ND – not done, RBC – Red Blood Cells, Mo – monocytes, Ba –basophiles, TMOT – Total motility, PMOT - Progressive motility, PFMOT – Progressive fast motility, PSMOT – progressive slow motility, PCMOT – Progressive circular motility, LMOT – Local motility, VCL - Curvilinear velocity, VSL - Straight-line velocity, VAP - Average path velocity, DCL - Curvilinear distance, DSL - Straight line distance, DAP - Distance of average path, ALH - Amplitude of lateral head displacement, BCF - Beat-cross frequency, HAC - Head activity, WOB – Wobble, LIN - Linearity-index, STR – Straightness.

Table 4: Semen analysis parameters comparison between *B. gibsoni*-infected dogs at first (initial) presentation, six months later (second presentation), and clinically healthy dogs.

Parameter/Group	*B. gibsoni* first presentation (N=6)	*B. gibsoni* second presentation (N=6)	Clinically healthy (N=8)
Live%	71 (34-93)^{a*}	46.5 (15-98)^{b*}	93.5 (77-99)$^{a*, b*}$
Dead%	28 (7-66)^{a*}	53.5 (2-85)^{b*}	6.5 (1-23)$^{a*, b*}$
Head defects (%)	8 (4-12)	8.5 (4-24)	4 (1-8)
Middle part defects (%)	0 (0-0)^{a*}	1 (0-6)	1.5 (0-4)^{a*}
Tail defects (%)	11 (4-16)	5 (2-34)^{a**}	5.5 (2-18)^{a**}
Proximal cytoplasmic droplets (%)	0 (0-22)	12 (1-22)	5 (0-10)
Total defects (%)	26 (8-38)	37 (28-59)	19.5 (9-27)
Concentration 10^6/ml	120.5 (12-277)	49.5 (6-277)	72 (55-107)
TMOT(%)	69 (17-94)^{a*}	36.5 (15-97)^{b*}	93 (77-99)$^{a*, b*}$
PMOT (%)	26.5 (8-77)^{a*}	26.5 (9-89)^{b**}	80.5 (68-95)$^{a*, b**}$
PFMOT (%)	23 (6-64)^{a**}	19.5 (7-69)	69 (52-85)^{a**}
PSMOT (%)	12.5 (2-15)	6.5 (2-17)	11.5 (5-17)
PCMOT (%)	0 (0-0)	0 (0-0)	0 (0-1)
LMOT (%)	20 (9-40)$^{a*, b**}$	9 (5-20)^{a*}	7.5 (0-14)^{b**}
Immotile (%)	31 (6-83)	63.5 (3-85)	7 (1-23)
VCL (µm/s)	67.5 (28-127)	51 (29-146)	131 (7-217)
VSL (µm/s)	25 (14-50)^{a**}	23 (12-61)^{b**}	67.5 (48-74)$^{a**, b**}$
VAP (µm/s)	31 (16-60)^{a**}	27 (14-74)^{b**}	78 (60-87)$^{a**, b**}$
DCL (µm)	28 (10-44)^{a**}	20.5 (8-44)^{b**}	49.5 (40-97)$^{a**, b**}$
DSL (µm)	8 (3-17)^{a**}	7±(2-16)^{b**}	21.5 (13-33)$^{a**, b**}$
DAP (µm)	11 (4-21)^{a**}	9.5 (3-21)^{b**}	27 (18-35)$^{a**, b**}$
ALH (µm)	1 (0-1)	1 (0-1)	1 (1-2)
BCF (Hz)	9.5 (3-15)^{a*}	6 (1-16)	16 (11-19)^{a*}
HAC (rad)	0 (0-0)	0 (0-0)	0 (0-0)
WOB (VAP/VCL)	0 (0-1)	0.5 (0-1)	1 (0-1)
LIN (VSL/VCL)	0 (0-1)	0 (0-1)	0 (0-1)
STR (VSL/VAP)	1 (1-1)	1 (1-1)	1 (1-1)

Results are presented as median values with their associated minimum and maximum ranges. The same letters (a-b) indicate significant differences between groups, with significance defined as *P < 0.05 and **P < 0.01. The following abbreviations are used: TMOT – Total motility; PMOT - Progressive motility; PFMOT – Progressive fast motility; PSMOT – Progressive slow motility; PCMOT – Progressive circular motility; LMOT – Local motility; VCL – Curvilinear velocity; VSL – Straight-line velocity; VAP – Average path velocity; DCL – Curvilinear distance; DSL – Straight line distance; DAP – Distance of average path; ALH – Amplitude of lateral head displacement; BCF – Beat-cross frequency; HAC – Head activity; WOB – Wobble; LIN – Linearity index; STR – Straightness.

Depending on the owners' financial capabilities, two treatment protocols were used for *B. gibsoni* infection:, Dogs nos. 1, 4, and 5 received atovaquone (13.3mg/ kg/day), azithromycin (10mg/kg/day), and artesunate (12.5mg/kg/day) for 10 days (Karasová *et al.,* 2022); Dogs nos. 2, 3, and 6 were treated with metronidazole (15mg/kg bid), clindamycin (25mg/kg bid), and doxycycline (5mg/kg bid) for 30 days.

DISCUSSION

The findings of this study highlight the importance of *B. gibsoni* infection and co-infection with other VB pathogens in Serbia, its possible transmission pathways, and the effects of *B. gibsoni* infection on the reproductive health of male dogs.

In this study, 37 out of 100 dogs with clinical signs related to babesiosis tested positive for *B. gibsoni*, but not for the endemic *B. canis*. Although it was established a decade ago that dogs are asymptomatic carriers of *B. gibsoni* (Gabrielli *et al.*, 2015; Davitkov *et al*., 2015; Kovačević Filipovic *et al*., 2018) in Serbia, reports of clinical cases have only surfaced recently (Strobl *et al*., 2021; Milošević *et al*., 2024). Unlike *B. canis*, *B. gibsoni* often leads to asymptomatic and chronic infections, but severe anemia and thrombocytopenia can occur in acute cases (Karasova *et al*., 2022). Low parasitemia may lead to undetectable changes in CBC and blood smears, as noted in several ASTs. This indicates that *B. gibsoni* is more clinically significant in Serbia than previously recognized, highlighting the need for routine PCR testing in dogs. This study also highlights co-infections of *B. gibsoni* with canine hemotropic mycoplasmas, noting a lack of investigation of their prevalence in dogs in Serbia. In this study, only one dog tested positive for co-infection with *M. haemocanis*, and another tested positive from a prepuce swab. *M. haemocanis,* which is possibly part of the urogenital microbiome, has been found in various tissues of dogs (lungs, prostate, epididymis, bladder wall, nasal and vaginal swabs) and also human tissues following dog bites (Tamiozzo 2022, Suhadolc *et al.,* 2024). Although the connection of *M. haemocanis* to fertility issues remains unclear, its detection in a single dog could only suggest the need for further examination during male fertility evaluations.

All *B. gibsoni*-infected dogs in this study were ASTs, one of the most popular dog breeds in Serbia (Janjić *et al*., 2025). Previous research indicated that *B. gibsoni* infections are prevalent in ASTs, with bites from infected dogs potentially increasing infection risk (Imre *et al*., 2013; Birkenheuer *et al*., 2018; Tuska-Szalay *et al*., 2021). American Staffordshire Terriers are often associated with dog-fighting breeds (Imre *et al*., 2013; Tuska-Szalay *et al*., 2021) and have been implicated in causing severe injuries to humans (Bailey *et al*., 2020); however, the connection between breed and aggressive behavior remains untested. It is uncertain whether healthy dogs can acquire infection through bites or if digestive enzymes neutralize the parasite in ingested blood. Currently, no genetic evidence supports the breed's predisposition to *B. gibsoni*, and studies have not confirmed the presence of the parasite in saliva. More investigation is needed to clarify these aspects regarding ASTs and the *B. gibsoni* infection risk.

The venereal transmission of *Babesia* spp. has been debated, with earlier studies suggesting vertical transmission from infected mothers to fetuses (Fukumoto *et al*., 2005). Thus, based on current findings, it has been suggested that bitches with a history of *B. gibsoni* infection should be excluded from breeding. Even if treated and negative for babesiosis at breeding, they remain asymptomatic carriers, and pregnancy may weaken their immune system, increasing the risk of recrudescence (Karasova *et al*., 2022). This study expands the understanding of the potential routes of *Babesia* spp. transmission, as it is the first to detect *Babesia* spp. in semen, thereby prompting further investigation into possible venereal spread. As previously described in other tissues (Karasova *et al*., 2024), *Babesia* spp. are probably present in the genital tract, similar to *Leishmania* amastigotes found in the reproductive tissues and semen of male dogs (Manna *et al*., 2012; Diniz *et al.*, 2005). Remarkably, in this research, *B. gibsoni*-infected dogs did not exhibit inflammatory cells in their semen, or clinical indications of urogenital system inflammation, nor were merozoites seen in their blood smears. This suggests that the presence of *Babesia* spp. in sperm is not associated with inflammation in the genital organs or parasitemia levels, as was found for *L. infantum* (Diniz *et al*., 2005). *Babesia* spp. likely reach the reproductive organs through capillaries, or via capillary rupture during sperm sampling or mating, which enables their penetration through the delicate blood-testis barrier. Furthermore, a high seroreactivity to VB pathogens may indicate an immunological mechanism that aids the invasion of *B. gibsoni's* into the reproductive organs. Quite similarly, *L. infantum* can penetrate the brain parenchyma, either alone or with other zoonotic pathogens such as *E. canis, B. vogeli*, and *Toxoplasma gondii* (Cardinot *et al*., 2016).

The findings of this study demonstrate that males infected with *B. gibsoni* experience decreased sperm quality that remains such over the following six months. Only one of the six dogs with *Babesia* spp. detected in semen at initial presentation showed improvement in sperm quality six months after therapy. By contrast, *Babesia* spp. was detected in the semen of two other dogs after the treatment, whose sperm quality did not improve. Poor sperm quality may have resulted from the medical treatment, as was previously shown in rats: proguanil, especially when used continually, negatively affected the reproductive system, progressive sperm motility, and its viability (Olumide and Raji, 2011). Moreover, spermatogenesis may be impacted by fever. However, only two out of six dogs in this study had it, indicating that, according to Domoslawska and Zdunczyk's (2020a) earlier theory, hyperthermia is not the main cause. Poor sperm quality six months post-therapy may have resulted from persistent *B. gibsoni* infection, the effects of drugs that negatively affected spermatogenesis, or a combination of both. This raises questions regarding the breeding exclusion of male dogs infected with *B. gibsoni* and their long-term fertility. With no cures and frequent relapses, the risk of permanent infertility could be significant, necessitating further research on fertility in male dogs infected with *Babesia* spp.

The limited number of male canines in this study and the inability to conduct sequencing analyses of *Babesia* spp. in the semen samples are its drawbacks.

Conclusions: *Babesia* spp. may be present in the semen of male dogs infected with *B. gibsoni*, which can negatively impact sperm quality, causing acquired sterility. The possibility of venereal transmission of *B. gibsoni* is also highlighted by these findings, underscoring the significance of VBD in researching acquired male sterility and infection pathways. To limit the transmission of illness through semen to non-endemic areas and to inform legislative decisions about prohibiting affected individuals from breeding, ongoing research is essential.

Funding sources: The study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract No 451-03-136/2025-03/200143).

Authors' contributions: MS and MV performed the clinical examination, collected the samples, and created a database. MS took part in writing the manuscript. FAJ wrote the manuscript and performed statistical analysis. DM and GD performed PCR analysis and sequencing. VS and NS performed semen and microbiological analyses and participated in manuscript writing. KFM reviewed the manuscript. All authors have read and approved the manuscript.

Acknowledgements: The authors are thankful to Dr Nevenka Aleksić, a holder of the Cambridge CPE certificate, for language editing and suggestions.

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