Szkoła Główna Gospodarstwa Wiejskiego
w Warszawie
Instytut Medycyny Weterynaryjnej

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Wpływ różnych gatunków *Mycoplasma* spp., Herpesvirus canis typu 1 i *Chlamydia* spp. na jakość nasienia psa domowego (*Canis familiaris)*

The influence of various species of *Mycoplasma* spp., Herpesvirus canis type 1 and *Chlamydia* spp. on canine semen quality (*Canis familiaris*)

Rozprawa doktorska

Doctoral thesis

Rozprawa doktorska wykonana pod kierunkiem
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Instytut Medycyny Weterynaryjnej SGGW w Warszawie

Warszawa, 2024

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Mojemu Promotorowi Prof. dr hab. Piotrowi Jurce,
za poświęcony czas, nieocenioną pomoc przy powstawaniu niniejszej pracy, a także za motywację do rozwoju naukowego i zawodowego.

Moim Rodzicom,
za możliwość kształcenia, nieocenione wsparcie oraz za to, że zawsze we mnie wierzyli.

Mojemu Chłopakowi Filipowi,
za wiarę we mnie i wsparcie w dążeniu do osiągnięcia zamierzonych celów oraz za niezliczoną ilość poprawek językowych moich artykułów.

Wszystkim, którzy w jakikolwiek sposób przyczynili się do powstania niniejszej pracy.

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1. STRESZCZENIE

Wpływ różnych gatunków *Mycoplasma* spp., Herpesvirus canis typu 1 i *Chlamydia* spp. na jakość nasienia psa domowego (*Canis familiaris)*

Niepłodność stanowi istotny problem w populacji psów hodowlanych. Pomimo dostępu do zaawansowanych technik diagnostycznych, znalezienie przyczyny obniżonej jakości nasienia niejednokrotnie stanowi wyzwanie. W ostatnich latach coraz częściej zwraca się uwagę na rolę czynników mikrobiologicznych w przebiegu niepłodności. Dane dotyczące wpływu *Mycoplasma* spp. na płodność psów są sprzeczne. Sugeruje się, że *Mycoplasma* spp. jest przyczyną obniżonej jakości ich nasienia. Z drugiej jednak strony istnieją płodni nosiciele tej bakterii. Wpływ CHV-1 na układ rozrodczy suk został dobrze poznany i opisany. Zarówno u suk, jak i u psów, podczas infekcji obserwuje się występowanie grudkowo-pęcherzykowych zmian na powierzchni błony śluzowej przedsionka pochwy oraz u podstawy prącia. Wpływ zakażenia CHV-1 na jakość nasienia psów nie został jeszcze wyjaśniony, jednakże obniżona płodność spowodowana zakażeniem herpeswirusem u innych gatunków sugeruje, że podobne zjawisko może wystąpić u psów. Dotychczas nie opisano związku przyczynowego między zakażeniem chlamydiami a chorobami układu moczowo-płciowego u psów, chociaż odnotowano zmniejszoną liczebność miotów u psów-nosicieli *C. psittaci*. Nie wiadomo jednak czy i w jaki sposób *Chlamydia/Chlamydohpila* spp. wpływa na jakość nasienia.

Celem badań była ocena wpływu drobnoustrojów *Mycoplasma* spp. (rożnych gatunków), Herpesvirus canis i *Chlamydia (Chlamydophila)* spp. na jakość nasienia psa domowego oraz oszacowanie częstości występowania tych mikroorganizmów w układzie rozrodczym psów- samców w województwie mazowieckim. Badania przeprowadzono na psach (samcach psa domowego) w wieku 1-8 lat. Każdy pies był poddawany rutynowemu badaniu klinicznemu. Oznaczano u niego stężenia hormonów surowicy krwi, pobierano nasienie i oceniano jego jakość. Ponadto pobierano wymazy z napletka oraz z nasienia w celu przeprowadzenia badania mikrobiologicznego oraz oznaczeń PCR w kierunku *Mycoplasma* spp., Herpesvirus canis oraz *Chlamydia* (*Chlamydophila*) spp. W przypadku uzyskania pozytywnego wyniku w kierunku *Mycoplasma* spp., przeprowadzano identyfikację gatunkową tej bakterii.

Wykazano, że *Mycoplasma* spp. częściej występuje na powierzchni napletka (83,3%) niż w nasieniu (60,3%). Jednocześnie nie wykazano wpływu tej bakterii na jakość psiego nasienia. Zarówno w nasieniu jak i napletku najczęściej wykrywanymi gatunkami mykoplazm były *Mycoplasma* HRC689 oraz *M. canis.* Szczególną uwagę zwraca fakt, że u większości nosicieli *Mycoplasma* spp. (72,3%) wykryto w napletku więcej niż jeden gatunek tej bakterii. Ponadto, u 10 psów- nosicieli *Mycoplasma* spp. użycie standardowych starterów nie pozwoliło na określenie gatunku tej bakterii, co sugeruje, iż psy mogą być nosicielami nieopisanych dotychczas gatunków tej bakterii. Tylko u jednego psa potwierdzono obecność *Chlamydia (Chlamydophila)* spp., a jedynym odchyleniem od normy była zmniejszona ruchliwość postępowa plemników. CHV-1 nie został zdiagnozowany u żadnego z badanych psów.

Mycoplasma spp. występuje powszechnie w różnych częściach układu rozrodczego psów i jej obecność nie jest związana z występowaniem niepłodności. Prawdopodobnie nie wszystkie gatunki *Mycoplasma* spp. zasiedlające psi układ rozrodczy zostały poznane i opisane. Występowanie CHV-1 i *Chlamydia* (*Chlamydophila*) spp. w populacji polskich psów-samców jest sporadyczne.

Słowa klucz: *Mycoplasma*, Herpesvirus Canis, *Chlamydia*, CASA, PCR

2. SUMMARY

The influence of various species of *Mycoplasma* spp., Herpesvirus canis type 1 and *Chlamydia* spp. on canine semen quality (*Canis familiaris*)

Infertility is a significant problem in the breeding dog population. Despite access to advanced diagnostic techniques, finding the cause of reduced semen quality is often a challenge. In recent years, increasing attention has been paid to the role of microbial factors in infertility. Data on the influence of *Mycoplasma* spp. on canine fertility are conflicting. It has been suggested that *Mycoplasma* spp. is the cause of reduced semen quality. However, there are fertile carriers of this bacterium. The effects of CHV-1 on the reproductive system of female dogs have been well understood and described. In both female dogs and males, papulopustular lesions are observed on the surface of the vaginal vestibule mucosa and at the base of the penis during infection. The effect of CHV-1 infection on canine semen quality hasn't been evaluated so far. However, reduced fertility caused by herpesvirus infection in other species suggests that a similar phenomenon may occur in dogs. To date, a correlation between chlamydial infection and urogenital disease in dogs has not been described, although reduced litter size in *C. psittaci* carrier dogs has been reported. However, it is unknown if and how *Chlamydia/Chlamydophila* spp. affect semen quality.

The aim of the study was to evaluate the effect of the microorganisms *Mycoplasma* spp. (various species), Herpesvirus canis, and *Chlamydia* (*Chlamydophila*) spp. on the quality of domestic dog semen and to estimate the prevalence of these microorganisms in the reproductive system of male dogs in the Mazovia voivodeship. The study was conducted on male dogs aged 1-8 years. Each dog underwent a routine clinical examination, and serum hormone concentrations were determined. Semen was collected from each dog, and its quality was assessed. Additionally, swabs were taken from the foreskin and semen for microbiological examination and PCR assays for *Mycoplasma* spp., Herpesvirus canis, and *Chlamydia* (*Chlamydophila*) spp. In case of a positive result for *Mycoplasma* spp., species identification of this bacterium was carried out.

The study results showed that *Mycoplasma* spp. is more commonly found on the surface of the foreskin (83.3%) than in semen (60.3%). At the same time, the negative

effect of this bacterium on the quality of dog semen was not demonstrated. In both semen and foreskin, the most frequently detected mycoplasma species were *Mycoplasma* HRC689 and *M. canis*. In the majority of *Mycoplasma* spp. carriers (72.3%), more than one species of this bacterium was detected in the foreskin. Additionally, in 10 *Mycoplasma*- positive dogs, the use of standard primers failed to identify the species of this bacterium, suggesting that dogs may be carrying previously undescribed species of this bacterium. Only one dog was confirmed to have *Chlamydia* (*Chlamydophila*) spp., and the only abnormality observed was reduced sperm progressive motility. CHV-1 was not diagnosed in any of the dogs tested.

Mycoplasma spp. is commonly found in various parts of the canine reproductive system, and its presence is not associated with infertility. It is likely that not all *Mycoplasma* spp. colonizing the canine reproductive system have been recognized and described. The occurrence of CHV-1 and *Chlamydia* (*Chlamydophila*) spp. in the Polish male dog population is sporadic.

Key words: *Mycoplasma*, Herpesvirus Canis, *Chlamydia*, CASA, PCR

3. WYKAZ SKRÓTÓW

AKC (*American Kennel Club***) -** Amerykański Klub Kynologiczny

BPH (*Benign prostatic hyperplasia*) - Łagodny rozrost prostaty

C. – *Chlamydia*/ *Chlamydophila*

Ca. – *Candidatus*

CASA (*Computer Assisted Sperm Analysis*) - Komputerowo wspomagana analiza nasienia

CHV-1 (*Canine herpesvirus type 1*) - Herpeswirus typu 1 psów

ELISA (*Enzyme-linked immunosorbent assay*) - Test immunoenzymatyczny

E2- estradiol

FCI (*Fédération Cynologique Internationale*) - Międzynarodowa Federacja Kynologiczna

ISDS (*International Sheep Dog Society)* **-** Międzynarodowe Stowarzyszenie Psów Pasterskich

IL-1α - Interleukina 1α

IL-1ß - Interleukina 1ß

LIN (Linearity) – liniowość ruchu plemnika (%)

LPS (*Lipopolysaccharide*) – Lipopolisacharyd

M. – *Mycoplasma*

NGS (*Next Generation Sequencing*) - sekwencjonowanie nowej generacji

PCR (*Polymerase chain reaction*) – Reakcja łańcuchowa polimerazy

spp. (*species*) – gatunki

Real-time PCR (*real-time polymerase chain reaction*) - reakcja łańcuchowa polimerazy w czasie rzeczywistym

STR (*Straightness*) -prostoliniowość ruchu plemnika wyrażona stosunkiem VSL/VAP

T4- tyroksyna

TES – testosterone

TNF-α (*Tumor necrosis factor alpha*) – czynnik martwicy nowotworów α

VAP (*Average path velocity*) – średnia prędkość plemnika po przybliżonej ścieżce ruchu (μm/s)

VCL (*Curvilinear velocity*) -średnia prędkość plemnika po rzeczywistej ścieżce ruchu (μm/s)

VSL (*Straight line velocity*) – średnia prędkość plemników wzdłuż linii prostej

wyznaczonej między początkowym a końcowym położeniem gamety (μm/s)

WHO (*World Health Organization*) - Światowa Organizacja Zdrowia

WOB (*Wobble*) – indeks oscylacji wyrażony stosunkiem VAP/VCL

ZKwP – Związek Kynologiczny w Polsce

4. WYKAZ PUBLIKACJI STANOWIĄCYCH DYSERTACJĘ

Badania zostały szczegółowo przedstawione w spójnym tematycznie zbiorze artykułów opublikowanych w czasopismach naukowych stanowiących integralną część manuskryptu:

1. **Domrazek K,** Kaszak I, Kanafa S, Sacharczuk M, Jurka P. The influence of *Mycoplasma* species on human and canine semen quality: a review. Asian J Androl. 2023;25(1):29-37. DOI: 10.4103/aja 2021124.
   (IF 2023: **2.9**, pkt. MNiSW **70**).
2. **Domrazek K,** Jurka P. Prevalence of Chlamydophila spp. and Canid herpesvirus-1 in Polish dogs. Veterinary World. 2024; 17(1): 226-232. DOI:10.14202/vetworld.2024.226-232
   (IF 2024: **1.6**, pkt. MNiSW **70**).
3. **Domrazek K**, Konieczny P, Majka M, Czopowicz M, Cywińska A, Jurka P. The lack of the influence of various species of Mycoplasma spp. on canine semen quality. Theriogenology. 2024; 219: 86-93. DOI: 10.1016/j.theriogenology.2024.02.018
   (IF 2024: **2.8**, pkt. MNiSW **140**).
4. **Domrazek K**, Konieczny P, Majka M, Czopowicz M, Jurka P. The impact of microorganisms on semen quality. Animals 2024, 14, 1267. DOI: 10.3390/ ani14091267
   (IF2024: **3.0**, pkt. MNiSW **100**).

   Sumaryczny IF wynosi: **10,3**
   Sumaryczna punktacja Ministerstwa Nauki i Szkolnictwa Wyższego wynosi: **380**

Kopie powyższych publikacji wraz z oświadczeniami współautorów znajdują się w załączeniu.

5. Wstęp

Według Światowej Organizacji Zdrowia (WHO) niepłodność jest globalnym problemem zdrowia publicznego (Cerván-Martín i wsp., 2020), który dotyka nie mniej niż 186 milionów ludzi na całym świecie - przy czym niepłodność męska przyczynia się do ponad połowy wszystkich przypadków bezdzietności (Inhorn & Patrizio, 2015). Podobnie jak u ludzi, niepłodność u psów jest powszechnym i narastającym problemem (England i wsp., 2010). Ze względu na rozwój hodowli psów oraz doskonalenie technik wspomaganego rozrodu, zjawisko występowania słabej jakości nasienia od lat jest przedmiotem dyskusji i badań. Niepłodność stanowi istotny problem w populacji psów hodowlanych. Podczas, gdy sukces reprodukcyjny jest niezbędny dla utrzymania zdrowia i różnorodności genetycznej gatunku, występowanie niepłodności stanowi wyzwanie dla programów hodowlanych. Zaobserwowano, że niektóre rasy psów są częściej dotknięte zaburzeniami reprodukcyjnymi, takimi jak obniżona jakość nasienia lub obniżone wskaźniki płodności (Axnér i wsp., 2022; Dahlbom i wsp., 1997). Niepowodzenie krycia można przypisać suce, reproduktorowi lub czynnikom wpływającym na płodność obu osobników. Podczas, gdy przyczyna niepłodności u suki może być trudna do ustalenia, jakość nasienia stanowi biologiczny marker płodności u samca (Hallberg i wsp., 2024).

W przeszłości ocena jakości nasienia opierała się o subiektywne badanie makroskopowe oraz mikroskopowe, a koncentracja plemników w nasieniu była szacowana przy użyciu komór Bürkera-Türka, Thoma oraz Maklera (Christensen i wsp., 2005). Wraz z rozwojem technik wspomaganego rozrodu, zostały opracowane zobiektywizowane metody oceny jakości nasienia takie jak systemy CASA (*Computer Assisted Sperm Analysis*) (Rijsselaere i wsp., 2012), spektrofotometria (Strzeżek i wsp., 2013) czy cytometria przepływowa (Niżański i wsp., 2012). Obecnie ocena parametrów opisujących nasienie przy użyciu systemu CASA jest złotym standardem diagnostyki niepłodności ludzi i procedura ta została poddana walidacji dla nasienia psa. Na podstawie licznych badań ujednolicono metodykę i opracowano normy dla najważniejszych parametrów opisujących jakość nasienia (Núñez-Martínez i wsp., 2006). Podstawowa analiza nasienia w systemie CASA obejmuje ocenę następujących parametrów: koncentracja plemników, całkowita ilość plemników w ejakulacie, ruchliwość całkowita oraz ruchliwość postępowa (Rijsselaere i wsp., 2012). Bardziej

zaawansowane aparaty, dzielą plemniki na subpopulacje o różnych właściwościach ruchowych (Núñez-Martínez i wsp., 2006).

Pomimo dostępu do zaawansowanych technik diagnostycznych, znalezienie przyczyny obniżonej jakości nasienia niejednokrotnie stanowi wyzwanie. Niepłodność psów obejmuje spektrum zaburzeń, od nieprawidłowości anatomicznych i zaburzeń równowagi hormonalnej po zakażenia i predyspozycje genetyczne (Memon, 2007). W ostatnich latach coraz częściej zwraca się uwagę na rolę czynników mikrobiologicznych, w tym infekcji bakteryjnych, wirusowych i grzybiczych, w pogorszeniu zdrowia reprodukcyjnego psów (Graham & Taylor, 2012; Muñoz i wsp., 2020; Siemieniuch i wsp., 2009). Mikroorganizmy te mogą kolonizować układ rozrodczy, prowadząc do stanów zapalnych, odpowiedzi immunologicznej, a ostatecznie do upośledzenia płodności (Tamiozzo, 2022). Jednak pomimo ich potencjalnego znaczenia, dokładny wpływ zakażeń na płodność psów jest słabo poznany.

Dostępne komercyjnie laboratoria, oferują badania profilowe umożliwiające diagnostykę niepłodności i obejmują one testy w kierunku patogenów: *Mycoplasma* spp., *Chlamydia* (*Chlamydophila*) spp. oraz Herpesvirus canis (CHV-1). Niektóre dodatkowo umożliwiają wykrycie *Brucella canis*. Badania te są często zlecane przez lekarzy weterynarii, a interpretacja uzyskanych wyników u psów niewykazujących objawów klinicznych stanowi duży problem.

5.1 *Mycoplasma* spp.

Bakterie z rodzaju *Mycoplasma* spp. są najmniejszymi samoreplikującymi się organizmami należącymi do rodziny *Mycoplasmataceae*. Mogą one być wykrywane u ludzi, zwierząt oraz roślin (Whitman i wsp., 2010). Są to gram-ujemne, pleomorficzne komórki o średnicy 300-800 nm (Whitman i wsp., 2010). Bakterie te nie posiadają ściany komórkowej i są ograniczone jedynie błoną plazmatyczną. Cechują się one również obecnością uniwersalnego kodonu stop UGA dla tryptofanu (Yiwen i wsp., 2021). Kolonie *Mycoplasma* spp. mają zwykle średnicę mniejszą niż 1 mm, a ich wygląd w literaturze jest opisywany jako zbliżony do „smażonego jajka" lub „główki kalafiora" (Rakovskaya i wsp., 2019). Wielkość genomu bakterii *Mycoplasma* spp. waha się od około 580 do 1350 kb (Whitman i wsp., 2010).

Bakterie *Mycoplasma* spp. pierwszy raz zdiagnozowano i opisano u psów w 1934 roku (Shoetensack, 1934). Zgodnie z dostępną literaturą, dotychczas poznano i opisano 15 gatunków tej bakterii występujących u psów: *Mycoplasma arginini, Mycoplasma bovigenitalium, Mycoplasma canis, Mycoplasma cynos, Mycoplasma edwardii, Mycoplasma feliminutum, Mycoplasma felis, Mycoplasma gateae, Mycoplasma haemocanis, Mycoplasma maculosum, Mycoplasma molare, Mycoplasma opalescens, Mycoplasma* sp. HRC689, *Mycoplasma* sp. VJC358 oraz *Mycoplasma spumans* (Chalker, 2005). Niektórzy autorzy opisują także gatunek *M. collis* jako psi (Johansson & Pettersson, 2002), jednakże brak jest danych dotyczących występowania *M. collis* u psów. Doniesienia wskazują przy tym, że gatunek ten został pierwotnie wyizolowany od gryzoni (Hill, 1983), dlatego też sugeruje się, iż *M. collis* mógł zostać błędnie zidentyfikowany jako pochodzący od psów (Chalker & Brownlie, 2004).

Psie mykoplazmy mają zróżnicowane pochodzenie filogenetyczne (Domrazek i wsp., 2022). Większość gatunków została umieszczona w grupie taksonomicznej *Hominis*, z wyjątkiem *M. haemocanis* (grupa *Pneumoniae*) oraz *M. feliminutum (*gr. *Acholeplasma*) (Whitman i wsp., 2010). Różnorodność ta znajduje odzwierciedlenie w zawartości mol G + C każdego gatunku oraz w wielkościach genomów (Chalker, 2005).

Mykoplazmy są zdolne do produkcji szeregu czynników zjadliwości np.: adhezyn powierzchniowych i ich białek pomocniczych, polisacharydów otoczkowych, enzymów inwazyjnych i biofilmu, które umożliwiają ich osiedlanie się, namnażanie i rozprzestrzenianie *in vivo*. Podczas cyklu życiowego bakterie te pochłaniają składniki

odżywcze z komórek gospodarza i uwalniają dużą liczbę metabolitów, takich jak nadtlenek wodoru (H_2O_2), amoniak (NH_3) i siarkowodór (H_2S) czym powodują miejscowe uszkodzenie tkanek (Yiwen i wsp., 2021). Niektóre gatunki *Mycoplasma* spp. mogą również wydzielać egzotoksyny i hemolizyny. Mykoplazmy mogą również produkować patogenne enzymy: peptydazy, fosfatazy, ekto-ATPazy, cytotoksyczne nukleazy i nukleotydazy, które są uważane za czynniki chorobotwórcze (Yiwen i wsp., 2021). Mykoplazmy rozwinęły mechanizmy oporności na reakcje układu immunologicznego swoich gospodarzy: np. mechanizm modulujący wpływ na układ odpornościowy gospodarza dzięki wysoce plastycznemu zestawowi zmiennych białek powierzchniowych oraz zakażanie niefagocytarnych komórek gospodarza (Gautier-Bouchardon, 2018). Mechanizmy te przyczyniają się do utrzymywania się mykoplazm w ich żywicielach i do rozwoju przewlekłych infekcji.

Szacuje się, iż *Mycoplasma* spp. zasiedla górne drogi oddechowe 100% zdrowych psów, będąc tym samym elementem ich mikrobioty (Chalker, 2004). Najczęściej wykrywanymi gatunkami są *M. canis* i *M. edwardii,* które mogą występować samodzielnie lub w połączeniu z innymi gatunkami (Chalker, 2004). *Mycoplasma* spp., może również powodować zapalenie płuc (Chalker, 2004). Badania oceniające mikrobiom zdrowych psów wykazały, że mykoplazmy występowały z malejącą częstością od jamy nosowej do dolnych dróg oddechowych (Ericsson i wsp., 2016).

Mycoplasma haemocanis powoduje niedokrwistość hemolityczną o różnym stopniu nasilenia. Innymi objawami zakażenia tym gatunkiem mykoplazmy mogą być gorączka, apatia, powiększenie węzłów chłonnych, brak koordynacji ruchowej, powiększenie śledziony, brak apetytu, żółtaczka, odwodnienie, utrata masy ciała, brak reakcji na bodźce i nagła śmierć (Beus i wsp., 2024). Opisano również przypadek posocznicy i ostrego septycznego zapalenia wielostawowego spowodowanych bakteriami *Mycoplasma* spp., jako powikłania po przeprowadzonym zabiegu adrenalektomii (Stenske i wsp., 2005). Badania wykazały, że u około 30% psów *Mycoplasma* spp. bytuje w obrębie okrężnicy, gdzie jest ona uważana za część mikrobioty (Bowe i wsp., 1982). Z drugiej jednak strony *Mycoplasma* HRC 689 została opisana jako przyczyna zapalenia jelita grubego u 5 psów rasy Bokser (Bowe i wsp., 1982). *M. canis* może być również czynnikiem etiologicznym zapalenia pęcherza moczowego. Do objawów infekcji tą bakterią zalicza się krwiomocz, bolesne oddawanie moczu, strangurię, polidypsję i poliurię oraz nietrzymanie moczu (Hemmatzadeh i wsp., 2019; L'Abee-Lund i wsp., 2003).

Szacuje się, że nosicielami *Mycoplasma* spp. w układzie rozrodczym może być nawet 88% suk i 85% psów (Doig i wsp., 1981). Dane dotyczące wpływu *Mycoplasma* spp. na płodność samców i samic psów są sprzeczne. Niektórzy autorzy sugerują *M. canis* jako przyczynę niepłodności i śluzowo-ropnego wypływu z pochwy suk (L'Abee-Lund i wsp., 2003). Bakterie *Mycoplasma* spp. są również opisywane jako przyczyna zapaleń błony śluzowej macicy, niepłodności, resorpcji, ronień lub rodzenia słabych i martwych szczeniąt (Alves i wsp., 2023; Chierchia i wsp., 2023). Inni z kolei, izolowali różne gatunki *Mycoplasma* spp. od zdrowych i płodnych suk (Jagódka i wsp., 2023; Janowski i wsp., 2008; Maksimović i wsp., 2018).

Analogiczna sytuacja ma miejsce w odniesieniu do płodności psów samców. W dostępnej literaturze istnieją doniesienia opisujące *M. maculosum* oraz *M. canis* jako przyczynę obniżonej jakości nasienia (Laber & Holzmann, 1977; Tamiozzo, 2022). Badania przeprowadzone *in vitro* wykazały, iż bakterie *Mycoplasma* spp. mogą przyczepiać się do plemników, tym samym upośledzając ich ruchliwość (Svenstrup, 2003). Z drugiej jednak strony wielu autorów nie potwierdziło wpływu tej bakterii na jakość nasienia (Lechner i wsp., 2023; Schäfer-Somi i wsp., 2009). W dostępnej literaturze, opisywane są również przypadki występowania stanu zapalnego żołędzi prącia, napletka, jąder najądrzy, gruczołu krokowego oraz łagodnego rozrostu prostaty (BPH) u psów- nosicieli *Mycoplasma* spp. (Schäfer-Somi i wsp., 2009).

Obecność *Mycoplasma* spp. w badanej próbce może być potwierdzona za pomocą: hodowli komórkowej, badań serologicznych i reakcji łańcuchowej polimerazy (PCR) (Chalker & Brownlie, 2004). Badanie hodowlane nie jest zalecane ze względu na wysokie wymagania tej bakterii i wysokie koszty pożywek hodowlanych (Chalker, 2005). Ponadto, ze względu na podobne właściwości biochemiczne poszczególnych gatunków, ich różnicowanie może dawać fałszywe wyniki (Razin, 1983). Techniki serologiczne obejmują: hamowanie wzrostu, immunofluorescencję, hamowanie metabolizmu i podwójną immunodyfuzję (Chalker, 2005). Ze względu na fakt, iż mogą wystąpić reakcje krzyżowe z niektórymi innymi gatunkami, metoda ta również nie jest zalecana do diagnostyki gatunkowej tej bakterii (Chalker, 2005). Ponadto fizjologiczna powszechność występowania tych bakterii w wielu układach i narządach może skutkować wysokimi mianami przeciwciał przy jednoczesnym braku aktywnej infekcji. PCR jest wysoce użyteczną metodą wykrywania *Mycoplasma* spp. i jest rutynowo ona wykonywana w laboratoriach komercyjnych i niekomercyjnych (Messick, 2003). Metoda ta pozwala ona na precyzyjną identyfikację gatunków *Mycoplasma* spp. oraz

potwierdzenie występowania patogenu w badanym układzie bądź narządzie (Chalker & Brownlie, 2004). Obecnie brak jest możliwości szczegółowej identyfikacji wszystkich gatunków tej bakterii w komercyjnych laboratoriach. Głównym ograniczeniem badania PCR jest wykrywanie zarówno żywych, jak i martwych patogenów, co może skutkować uzyskiwaniem fałszywie dodatnich wyników w przypadku wykonywania kontrolnych badań w trakcie lub po leczeniu (Wolffs i wsp., 2005).

Ze względu na trudności w identyfikacji poszczególnych gatunków mykoplazm u psów, znaczna część badań dotyczy obecności lub braku tej bakterii, niewiele jednak wiadomo na temat zakażeń wywołanych przez konkretne gatunki (Alves i wsp., 2023; Domrazek i wsp., 2022; Schulz i wsp., 2015).

Ze względu na brak ściany komórkowej antybiotyki beta-laktamowe, glikopeptydy i fosfomycyna, nie są skuteczne w leczeniu infekcji bakteriami *Mycoplasma* spp. Biologiczne cechy charakterystyczne dla tych bakterii sprawiają, że wiele innych substancji (sulfonamidy, trimetoprim, ryfampina, polimyksyny, kwas nalidyksowy, linezolid i kilka innych) również nie wykazuje efektu terapeutycznego (Chernova i wsp., 2016). Fluorochinolony wykazują działanie bakteriobójcze dla mykoplazm, podczas gdy antybiotyki z grupy tetracyklin i makrolidów wykazują działanie bakteriostatyczne (Gautier-Bouchardon, 2018; Tamiozzo, 2022). Dlatego antybiotyki te są szeroko stosowane do tłumienia infekcji mykoplazmą *in vivo* oraz skażeń kultur komórkowych (Uphoff & Drexler, 2014).

Podsumowując, niewiele jest informacji na ten temat wpływu bakterii *Mycoplasma* spp. na płodność psów. Dane dostępne w literaturze są sprzeczne. Dotychczasowe badania w dużej mierze skupiały się przede wszystkim na wykrywaniu tego rodzaju bakterii bez jednoczesnej identyfikacji gatunkowej. Ponadto nieznana jest częstość występowania poszczególnych gatunków *Mycoplasma* spp. oraz wpływ każdego z nich na zdolności reprodukcyjne psów. W codziennej praktyce lekarsko-weterynaryjnej rutynowo wykonuje się badania diagnostyczne poprzez pobranie wymazu z napletka, co niekoniecznie musi korelować z obecnością tej bakterii w innych częściach układu rozrodczego psów- samców.

5.2 Herpesvirus canis typ 1 (CHV-1)

Herpesvirus canis to dwuniciowy, otoczkowy wirus DNA opisany po raz pierwszy w 1965 roku (Carmichael i wsp., 1965). Należy on do rodziny *Herpesviridae*, podrodziny *Alphaherpesvirinae* i rodzaju *Varicellovirus* ("Herpesvirales," 2017). Wiriony herpeswirusów składają się z rdzenia, kapsydu i otoczki. Rdzeń z kolei zbudowany jest z genomu wirusowego upakowanego jako pojedyncza, liniowa cząsteczka dsDNA w białkowym kapsydzie. Genom DNA jest powiązany z białkami i ściśle upakowany. Wokół kapsydu znajduje się typowa otoczka lipoproteinowa z licznymi kolcami glikoproteinowymi. Ze względu na zmienny rozmiar otoczki, średnica wirionów może wynosić od 200 do 300 nm (MacLachlan i wsp., 2017). CHV-1 jest filogenetycznie spokrewniony z α-herpeswirusami atakującymi inne gatunki zwierząt, ale receptory powierzchniowe ograniczają zakres żywicieli CHV-1 do psów domowych i innych psowatych. Wiriony herpeswirusów łatwo ulegają inaktywacji w środowisku (MacLachlan i wsp., 2017). Wirus ten stabilny przy pH 6,5-7,5 i w niskich temperaturach, do około -70ºC. Niemniej jednak łatwo ulega zniszczeniu przy pH poniżej 5 lub w temperaturze powyżej 40ºC (Davidson i wsp., 2021).

Częstość występowania CHV-1 w populacji polskich psów nie została jeszcze oszacowana, ale dane z literatury pokazujące seroprewalencję wirusa w poszczególnych krajach sugerują, że występuje on powszechnie. Seroprewalencja herpeswirusa u psów z problemami reprodukcyjnymi wynosiła 50,3% we Włoszech (Rota i wsp., 2020), 22% w RPA (Decaro i wsp., 2008), 39%-62,1% w Turcji (Yeşilbağ i wsp., 2012) i 81,5% w Finlandii (Dahlbom i wsp., 2009). Gracin i wsp. porównali sero- i prewalencję u psów z infekcjami układu rozrodczego, z których 32,02% wykazało dodatnie wyniki w serologicznych metodach diagnostycznych, podczas gdy wszystkie próbki były ujemne w badaniu PCR (Gracin i wsp., 2023). Wynik ten może sugerować, że badanie PCR jest bardziej adekwatne niż badania serologiczne, gdyż podwyższone miano przeciwciał może występować po kontakcie z wirusem lub po szczepieniu. Warto również mieć na uwadze fakt, iż miano przeciwciał spada stosunkowo szybko po aktywnym zakażeniu CHV-1 i tym samym nie utrzymuje się na wykrywalnych poziomach u wszystkich latentnie zakażonych psów, a badania seroprewalencji prawdopodobnie zaniżają rzeczywisty wskaźnik zakażeń w populacjach psów (Evermann i wsp., 2011).

Ze względu na przerywany charakter fazy siewstwa i względną niestabilność CHV-1 poza komórkami żywiciela, możliwości przenoszenia wirusa są ograniczone. Transmisja zwykle obejmuje przejście z matki na potomstwo śródmacicznie, podczas porodu, przez bezpośredni kontakt ustno-nosowy lub podczas krycia między zakażonymi i niezakażonymi zwierzętami (Morresey, 2004). CHV-1 rozprzestrzenia się po organizmie żywiciela przez fuzję lub transport komórek. Po replikacji nabłonkowej w miejscu wniknięcia, następuje wiremia i wirus dociera do tkanki nerwowej i limfatycznej. W ostrej fazie zakażenia wszystkie psy wydalają wirusa przez błonę śluzową nosa, niezależnie od drogi zakażenia (Miyoshi i wsp., 1999). Po ostrej lub podklinicznej fazie infekcji psy stają się utajonymi nosicielami wirusa, a CHV-1 można wyizolować ze zwojów nerwu trójdzielnego i lędźwiowo-krzyżowego, ślinianek, migdałków i wątroby, nawet przy braku objawów klinicznych. Reaktywacja utajonego wirusa może być związana ze stresem lub jeśli podawane są leki immunosupresyjne, lub surowice antylimfocytarne (Pratelli i wsp., 2014).

Objawy kliniczne herpeswirozy zależą od wieku, w którym doszło do zakażenia. U szczeniąt w wieku poniżej pierwszego miesiąca życia, zakażenie CHV-1 może mieć przebieg śmiertelny (Evermann i wsp., 2011). Zakażenie psów w wieku trzech miesięcy lub starszych wirusem CHV-1 może prowadzić do rozwoju łagodnego nieżytu nosa i zapalenia gardła oraz tchawicy.

W przypadku immunosupresji może dojść do uogólnionego zakażenia o przebiegu śmiertelnym (Ronsse i wsp., 2004). Częste objawy kliniczne obserwowane w przypadku zakażenia oczu CHV-1 obejmują zaczerwienienie spojówek, światłowstręt, świąd i wypływ z worka spojówkowego (Ledbetter i wsp., 2009). Łzawienie jest jednym z najwcześniejszych objawów choroby oczu wywołanej przez CHV-1, ale wraz z postępem zakażenia wydzielina z oczu przybiera charakter śluzowy, śluzowo-ropny lub surowiczy (Ledbetter i wsp., 2009).

Wpływ CHV-1 na układ rozrodczy suk został dobrze poznany i opisany. Zakażenie może powodować resorpcje zarodków, poronienia oraz rodzenie martwych i słabych szczeniąt (Ronsse i wsp., 2004). Zarówno u suk jak i u psów, podczas infekcji obserwuje się występowanie grudkowo-pęcherzykowych zmian na powierzchni błony śluzowej przedsionka pochwy oraz u podstawy prącia (Hashimoto i wsp., 1983). Wpływ zakażenia CHV-1 na jakość nasienia psów nie został jeszcze wyjaśniony, jednakże obniżona płodność spowodowana zakażeniem herpeswirusem u innych gatunków sugeruje, że podobne zjawisko może występować u psów (El-Mohamady i wsp., 2020).

Zakażenie wirusem CHV-1 może być diagnozowane w oparciu o badanie hodowlane, metody immunologiczne i molekularne (PCR) (Castro i wsp., 2022). Herpesvirus canis rozwija się wyłącznie w hodowanych komórkach pochodzenia psiego, głównie w komórkach nerek psa w optymalnym zakresie temperatur od 35°C do 37°C. Zainfekowane komórki stają się zaokrąglone i odrywają się od powierzchni, pozostawiając wyraźne płytki otoczone nekrotycznymi komórkami. CHV-1 wytwarza wewnątrzjądrowe wtręty Cowdry'ego typu A, które mogą być trudne do wykazania i najlepiej ujawniają się w tkankach utrwalonych w płynie Bouina (Davidson i wsp., 2021).

Testy serologiczne do niedawna były rutynową metodą diagnozowania CHV-1 (Pratelli i wsp., 2014). Podejście to może być mylące, ponieważ wirus jest słabo immunogenny. Po ekspozycji psa na CHV-1 miana przeciwciał szybko rosną, a następnie spadają w ciągu 4 do 8 tygodni (Botinelli i wsp., 2016). Ponadto szacuje się, że utrzymują się one nie dłużej niż 60 dni po ekspozycji (Bottinelli i wsp., 2016). Miana przeciwciał przeciwko CHV-1 są niskie i wahają się od 1:2 do 1:32. W przypadku braku objawów klinicznych, wynik seropozytywny jest wiarygodnym wskazaniem jedynie ekspozycji. Aby ułatwić diagnozę za pomocą tej metody, pary surowic powinny być pobierane w odstępie 10 do 14 dni i przechowywane w lodówce do czasu oceny. Wykazano, że czterokrotny wzrost miana przeciwciał wskazuje na aktywne zakażenie (Morresey, 2004).

Ze względu na ryzyko uzyskania fałszywie dodatnich lub fałszywie ujemnych wyników badań serologicznych, złotym standardem diagnostyki jest badanie PCR. Badanie to charakteryzuje większa czułość oraz swoistość, ponieważ wykrywa ono obecność określonego fragmentu DNA wirusa, a w każdym cyklu generowany jest wykładniczy wzrost produktu (Poulet i wsp., 2001).

Leczenie ogólnoustrojowego zakażenia CHV-1 lekami przeciwwirusowymi jest nieskuteczne (Davidson i wsp., 2021). Z tego względu, niezwykle istotnym jest zapobieganie zakażeniom.

U psów występuje minimalny przezłożyskowy transfer odporności. Zatem odpowiednie spożycie siary powinno nastąpić natychmiast po porodzie, aby szczenięta nabyły odporność bierną (Chastant & Mila, 2019). Przeniesienie ochronnej odporności biernej (przeciwciała łożyskowe lub siarowe) między suką a jej szczeniętami zależy od wcześniejszego istnienia odpowiednich przeciwciał matczynych w surowicy. Dlatego też suki hodowlane z ekspozycją na CHV-1 we wcześniejszym okresie życia mają najlepszą możliwość serokonwersji i wytworzenia przeciwciał ochronnych (Davidson, 2014). Suki,

które są wrażliwe na zakażenia wirusem CHV-1, muszą być ściśle izolowane od potencjalnego narażenia podczas ciąży i przez co najmniej 6 tygodni po porodzie, aby zapobiec przeniesieniu wirusa na płody lub noworodki (Davidson, 2014). Kolejne ciąże zakażonej suki przebiegają zwykle fizjologicznie ze względu na obecność przeciwciał przeciwko CHV-1. Na rynku dostępna jest również szczepionka przeciwko herpeswirusowi, jednakże zgodnie z zaleceniami producenta może być ona stosowana jedynie u suk. Obecnie brak jest możliwości immunizacji psów-samców (Poulet i wsp., 2001).

Podsumowując, wpływ CHV-1 na płodność samców nie został do tej pory określony. Ponadto, nieznana jest częstość występowania psiego herpeswirusa w populacji psów-samców w Polsce. Najlepszą metodą diagnostyki zakażeń CHV-1 wydaje się być badanie PCR. Ze względu na trudności leczenia herpeswirozy, niezwykle istotnym jest zapobieganie rozprzestrzeniania się wirusa.

5.3 *Chlamydia* (*Chlamydophila*) spp.

Chlamydie są Gram-ujemnymi, wewnątrzkomórkowymi patogenami i symbiontami różnych organizmów, od ludzi po ameby (Elwell i wsp., 2016). Przez ostatnie 10 lat nastąpił gwałtowny wzrost liczby poznanych mikroorganizmów w obrębie tego rzędu, w tym bakterii pochodzących z wielu źródeł zwierzęcych i środowiskowych. Doprowadziło to do proponowanego podziału rodziny *Chlamydiaceae* na dwa rodzaje, *Chlamydia* i *Chlamydophila* (C) (Abdel-Rahman & Belland, 2005). Taksonomia *Chlamydiales* jest obecnie kontrowersyjna i wielu autorów uważa, że podział na rodzaje jest niepotrzebny. U zwierząt najczęściej diagnozowanymi gatunkami Chlamydii są: *C. trachomatis, C. muridarum, C. suis, C. psittaci, C. abortus, C. caviae, C. felis, C. pneumoniae, C. pecorum, C. avium, C. gallinacea, C. serpentis, C. poikilothermis, Candidatus* (Ca*) C. ibidis, Ca. C. corallus i Ca. C. sanzinia* (Bommana & Polkinghorne, 2019). Nie wiadomo czy wszystkie z nich występują u psa domowego, ale dotychczas opisano występowanie: *C. psittaci* (Fukushi i wsp., 1985, Gresham i wsp., 1996), *C. abortus* (Hoelzle i wsp., 2005), *C. felis* (Pantchev i wsp., 2010) i *C. caviae* (Pantchev i wsp., 2010).

Chlamydie różnią się od innych bakterii wewnątrzkomórkowych, tym, że ich namnażanie jest związane z dwufazowym cyklem rozwojowym, w którym zmieniają się między zewnątrzkomórkowym, zakaźnym ciałem elementarnym a wewnątrzkomórkowym, niezakaźnym ciałem siateczkowatym. Ciała elementarne dostają się do komórek błony śluzowej i różnicują się w ciała siateczkowate. Po kilku cyklach replikacji ciała siateczkowate ponownie różnicują się w ciała elementarne i są uwalniane z komórki gospodarza, gotowe do infekowania sąsiednich komórek (Abdel-Rahman & Belland, 2005; Elwell i wsp., 2016).

Ciała elementarne są cząstkami zakaźnymi, które mogą być przenoszone z zakażonych tkanek do niezakażonych tkanek u tego samego osobnika lub od chorego do zdrowego (Becker, 1996). U zakażonych, *Chlamydia/Chlamydophila* spp. powoduje uszkodzenie tkanek i wydzielanie interleukiny-1α (IL-1α), interleukiny-1ß (IL-1 ß) i czynnika martwicy nowotworów alfa (TNF-α), które są cytokinami biorącymi udział w procesie zapalnym (Becker, 1996). Do czynników zjadliwości chlamydii zalicza się zdolność do stymulacji apoptozy, produkcja antygenów powierzchniowych takich jak

białka błonowe, lipopolisacharydy (LPS) oraz białka szoku termicznego (Pawlikowska-Warych i wsp., 2017).

Doniesienia o chlamydiozie psów są rzadkie, prawdopodobnie dlatego, że chlamydie nie są często opisywane jako czynniki chorobotwórcze u psów. Psy wydają się być nietypowymi żywicielami chlamydii (Sprague i wsp., 2009). Objawy kliniczne zakażenia chlamydiami u psów są zróżnicowane, a choroba może mieć przebieg ostry, podostry lub przewlekły. Objawy te obejmują gorączkę (do 41°C), odoskrzelowe zapalenie płuc, kaszel (Gresham i wsp., 1996), zapalenie rogówki lub rogówki i spojówek (Gresham i wsp., 1996; Hoelzle i wsp., 2005), apatię, wymioty, biegunkę i objawy neurologiczne (drgawki toniczno-kloniczne) (Gresham i wsp., 1996). Ponadto opisano septyczne zapalenie wielostawowe wywołane przez chlamydie (Lambrechts i wsp., 1999) oraz zmiany miażdżycowe (Sako i wsp., 2002).

Dotychczas nie opisano związku przyczynowego między zakażeniem chlamydiami a chorobami układu moczowo-płciowego u psów, chociaż odnotowano małe liczebności miotów w hodowli psów, w której wykryto *C. psittaci* w próbce pobranej od jednego z psów (Sprague i wsp., 2009). W warunkach eksperymentalnych zmiany zapalne w gruczole krokowym zostały wywołane przez wstrzyknięcie *C. trachomatis* bezpośrednio do gruczołu krokowego (Nielsen i wsp., 1982). Możliwe również, że bakterie te mogą być przyczyną aborcji u suk (Romagnoli, 2002). Liutkeviciene i wsp. (2009) zasugerowali, że *Chlamydophila* spp. może być główną przyczyną niepłodności psów.

Ze względu na fakt, iż bakterie te są obligatoryjnymi bakteriami wewnątrzkomórkowymi, które do rozmnażania wymagają wzrostu wewnątrz komórki gospodarza, nie mogą być hodowane na konwencjonalnych podłożach bakteriologicznych (Scidmore, 2006).

Do 1965 r. pasaż w woreczku żółtkowym żółtka jaja kurzego był jedynym sposobem izolacji i namnażania tego mikroorganizmu (Whitman i wsp., 2010; Scidmore, 2006). Obecnie stosuje się system hodowli tkankowej, który umożliwia łatwiejszą hodowlę laboratoryjną i utrzymanie gatunku *Chlamydia/Chlamydophila* spp.

Diagnostyka serologiczna zakażeń *Chlamydia/Chlamydophila* spp. nadal opiera się głównie na mikroimmunofluorescencji i testach immunoenzymatycznych (ELISA) (Sachse i wsp., 2018). Biorąc pod uwagę fakt, iż opisywane bakterie mogą powodować infekcje nie tylko w obrębie układu rozrodczego, uzyskanie wyniku z wysokim mianem przeciwciał nie jest przydatne w diagnostyce chlamydiozy przenoszonej drogą płciową,

ponieważ testy serologiczne nie są specyficzne dla diagnozowania zakażeń układu rozrodczego (Domrazek & Jurka, 2024). Z tego względu reakcja łańcuchowa polimerazy w czasie rzeczywistym (real-time PCR) jest dokładniejsza niż serologia, ponieważ wyniki wskazują na obecność patogenu w badanym narządzie lub układzie (García Coca i wsp., 2019; Pantchev i wsp., 2010).

Wzrost chlamydii jest hamowany przez antybiotyki z grupy tetracyklin, makrolidów, azalidów i fluorochinolonów oraz chloramfenikol i ryfampinę. Namnażanie chlamydii nie jest blokowane przez aminoglikozydy, bacytracynę lub wankomycynę. *C. trachomatis, C. muridarum* i *C. suis* są wrażliwe na sulfonamidy, podczas gdy inne gatunki, z wyjątkiem szczepu 6BC *C. psittaci*, są oporne (Whitman i wsp., 2010) . Zaleca się długotrwałe leczenie zakażeń chlamydiami (3-4 tygodnie). Do tej pory opracowano kilka schematów antybiotykoterapii do leczenia chlamydiozy u psów, w tym terapię amoksycyliną z kwasem klawulanowym, doksycykliną, tetracykliną, erytromycyną/azytromycyną lub enrofloksacyną (McAnaney, 2022).

Podsumowując, wpływ *Chlamydophila (Chlamydia)* spp. na płodność samców nie został dotychczas określony. Ponadto, nie wiadomo jaka jest częstość występowania tej bakterii w układzie rozrodczym psów. Ze względu na możliwość zakażeń tymi bakteriami wielu układów bądź narządów, PCR wydaje się być lepszym rozwiązaniem w przypadku diagnostyki zakażeń układu rozrodczego.

6. Cel pracy

Dane dotyczące wpływu mikroorganizmów *Mycoplasma* spp., Herpesvirus canis i *Chlamydia* (*Chlamydophila*) spp. na płodność psów są sprzeczne. Ocena płodności nosicieli tych patogenów jest istotna zarówno z poznawczego, jak i praktycznego punktu widzenia, ponieważ może się przyczynić do opracowania skutecznych i bezpiecznych protokołów postępowania z psami o dodatnich wynikach badań w kierunku *Mycoplasma* spp., CHV-1 i *Chlamydia* (*Chlamydophila*) spp.

Wyznaczono następujące cele:

1. **Oszacowanie częstości występowania rożnych gatunków *Mycoplasma* spp., CHV-1 i *Chlamydia* (*Chlamydophila*) spp. w napletku i nasieniu psa domowego**
2. **Ocena wpływu tych drobnoustrojów na jakość nasienia psa domowego**

Realizacja w/w celów obejmowała wykonanie następujących zadań badawczych:

I. **Diagnostykę PCR *Mycoplasma* spp. i jej rożnych gatunków, *Chlamydia* (*Chlamydophila)* spp. i Herpesvirus canis występujących w napletku i nasieniu psów**

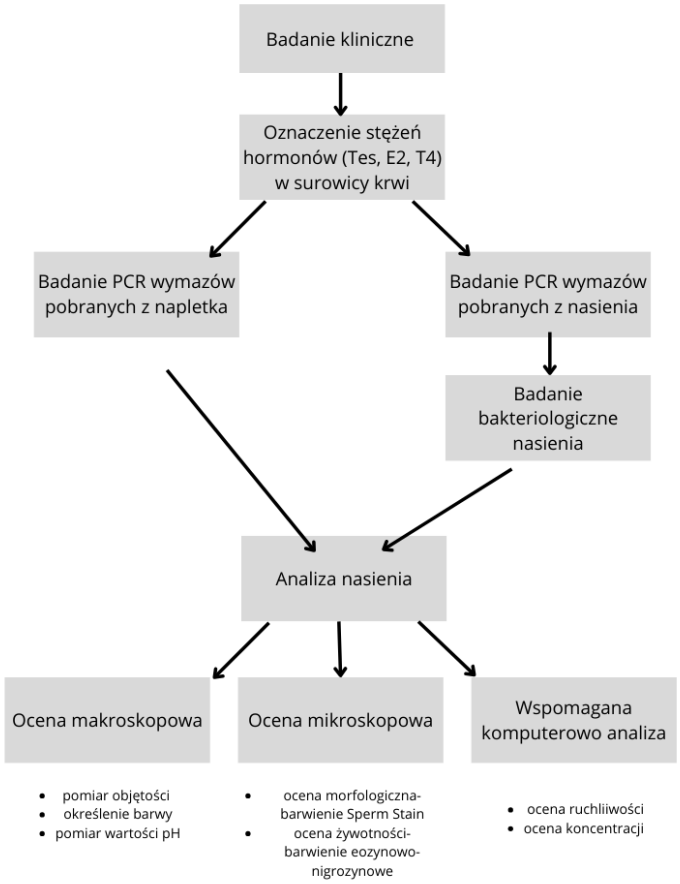
oraz

II. **Ocenę i porównanie jakości nasienia psów-nosicieli *Mycoplasma* spp. (rożnych gatunków), Herpesvirus canis i *Chlamydia* spp. oraz psów, u których nie stwierdzono obecności tych drobnoustrojów w napletku lub nasieniu**

7. Materiał i metody

Z uwagi na rosnący problem niepłodności u psów, niezwykle istotna jest kontrola ich jakości nasienia i zapobieganie infekcjom mogącym mieć potencjalny wpływ na ich jakość nasienia. Zwykle badanie andrologiczne psów opiera się na badaniu klinicznym ze szczególnym uwzględnieniem układu rozrodczego, analizie stężeń hormonów płciowych, ocenie jakości nasienia i pobraniu próbek do badań mikrobiologicznych oraz PCR. Systematyczne badania pozwalają na ogólną ocenę zdrowia psa oraz oszacowanie jego zdolności reprodukcyjnych. Ponadto regularne badania mikrobiologiczne pozwalają na wczesną diagnostykę infekcji układu rozrodczego i tym samym umożliwiają szybkie rozpoczęcie terapii, co może uchronić psa przed trwałą utratą zdolności reprodukcyjnych. Badania stanowiące przedmiot niniejszej pracy stanowiły uzupełnienie rutynowej kontroli płodności o diagnostykę gatunkową mykoplazm. Ponadto, rutynowo próbki do badania PCR pobiera się z powierzchni napletka. W wykonanych doświadczeniach pobierano próbki zarówno z napletka jak i z nasienia.

Rycina 1. Schemat doświadczenia



Badania przeprowadzono na psach samcach w wieku 1-8 lat. Zwierzęta pochodziły z hodowli zrzeszonych w Międzynarodowej Federacji Kynologicznej (FCI), do której należą Związek Kynologiczny w Polsce (ZKwP), American *Kennel Club* (AKC) oraz *International Sheep Dog Society* **(**ISDS**),** a także psy ze schroniska dla bezdomnych zwierząt. Pozyskanie materiału miało miejsce w latach 2021-2023.

W każdym etapie badań wykorzystano próbki od różnej liczby osobników. W tabeli 1 przedstawiono liczbę psów, od których próbki (nasienie i wymazy z napletka) wykorzystano w poszczególnych etapach badań.

Tabela 1. Liczba psów i rodzaj próbek pobranych do diagnostyki PCR

Rodzaj pobranej próbki	Badany drobnoustrój		
	Mycoplasma spp.	CHV-1	*Chlamydia/Chlamydophila* spp.
Wymaz z napletka	78	78	78
Wymaz z nasienia	63	52	52

Każdy pies był poddawany rutynowemu badaniu klinicznemu oraz oznaczeniu stężeń hormonów (testosteron, estradiol i tyroksyna) w surowicy krwi, przy użyciu analizatora MiniVidas (biomerieux, Francja). Procedura ta miała za zadanie wykluczenie z badania osobników chorych oraz z zaburzeniami hormonalnymi mogącymi wpływać na płodność. Od każdego psa nasienie pobierano metodą manualnej stymulacji opuszki prącia do kalibrowanych, wysterylizowanych zbiorniczków z płaszczem wodnym o temperaturze 37°C. Materiał pozyskiwano w sposób frakcjonowany, a w doświadczeniu wykorzystywano tylko frakcję nasienną ejakulatu. Po pobraniu materiału poddawano go ocenie makroskopowej, mikroskopowej oraz analizie w systemie CASA. Do oceny makroskopowej zaliczała się ocena objętości, barwy oraz pH badanej próbki.

Rycina 2. Kalibrowany pojemnik z płaszczem wodnym ogrzany do temperatury 37^{O}C

Analizę wspomaganą komputerowo przeprowadzono przy użyciu oprogramowania Sperm Class Analyzer (SCA wersja 6.5.0.67, MICROPTIC, Hiszpania) w połączeniu z mikroskopem NIKON ECLIPSE E 200 i kamerą. Stół termostabilny analizatora podgrzewano do temperatury 37 ℃. W zależności od gęstości ejakulatu, badaną próbkę rozcieńczano w soli fizjologicznej buforowanej fosforanami (PBS, Sigma Aldrich) w stosunku od 1:1 do 1:5, a następnie inkubowano 5 minut w temperaturze 37℃ przed oceną. Analizę przeprowadzono przy użyciu komór 20 µm GoldCyto 4 chamber Slide (Goldcyto Biotechcorp, Chiny).

Zastosowano ustawienia producenta dla materiału pochodzącego od psów. Poszczególne ustawienia zostały przedstawione w tabeli 2.

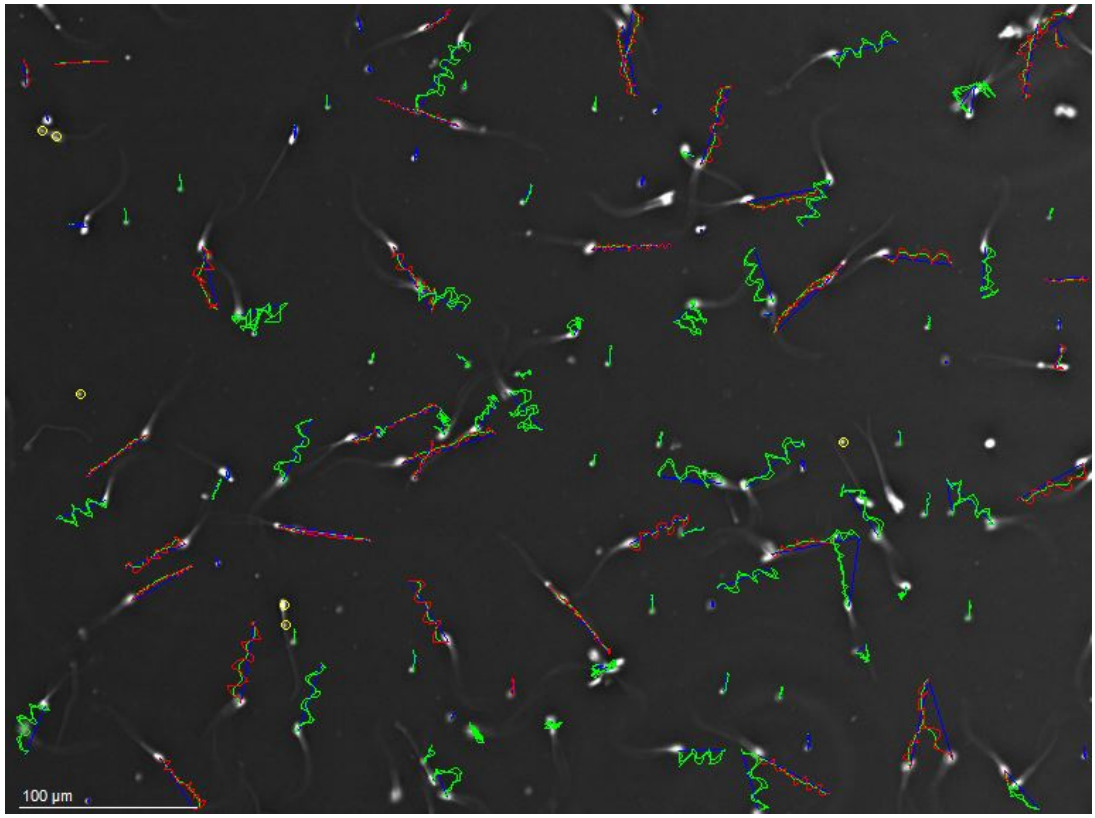
Tabela 2. Ustawienia do komputerowo wspomaganej analizy nasienia psa (SCA, wersja 6.5.0.67, MICROPTIC**, Hiszpania)**

Parametr	Ustawienie
Obiektyw	Powiększenie 10x
Metoda obserwacji	Kontrast fazowy
Filtr	Zielony
Ruch postępowy	>25 µm/s
Ruch średnio postępowy	5-25 µm/s
Ruch niepostępowy	<5 µm/s
Średnia powierzchnia główki	20 µm
VCL szybkiego ruchu	165 µm/s
Lin szybkiego ruchu	55%
Minimalna liczba ocenianych plemników	500

Określano koncentrację gamet w jednostce objętości ($x10^6$/ml), całkowitą liczbę plemników w ejakulacie ($x10^6$), odsetek plemników ruchomych, koncentrację okrągłych

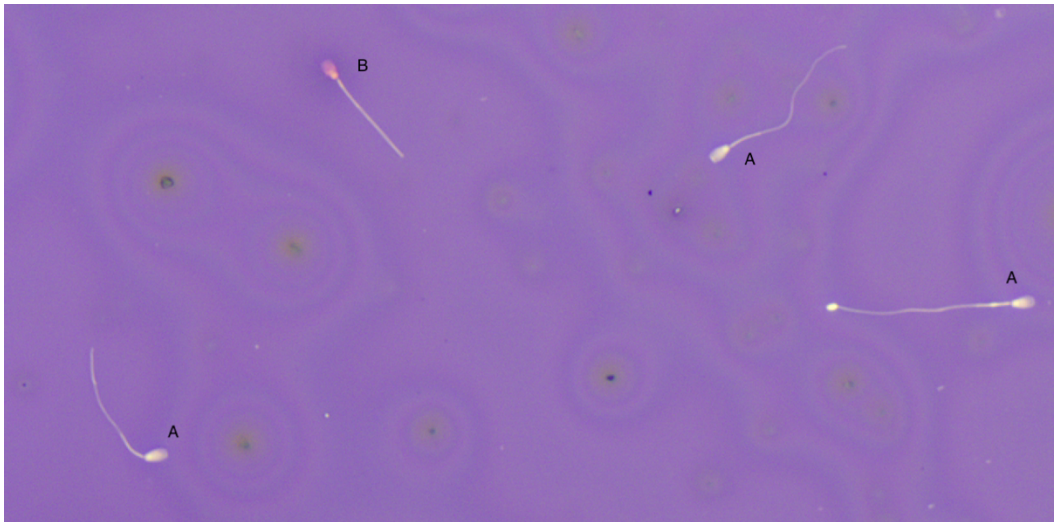
komórek i penetrację śluzu. Ponadto plemniki dzielono na subpopulacje o szybkim (RAPID), umiarkowanym (MEDIUM), wolnym (SLOW) i braku ruchu, a także analizowano 6 parametrów charakteryzujących ich ruch: VCL (*Track Speed Velocity*) - średnia prędkości plemnika po rzeczywistej ścieżce ruchu (µm/s); VAP (*Average Path Velocity*) - średnia prędkość plemników po przybliżonej ścieżce ruchu (µm/s); VSL (*Straight Line Velocty*) – średnia prędkość plemników wzdłuż linii prostej wyznaczonej między początkowym a końcowym położeniem gamety (µm/s); LIN (*Linearity*) - iniowość ruchu plemnika wyrażona stosunkiem VSL/VCL w %; STR (*Straghtness*) - prostość ruchu plemnika wyrażona stosunkiem VSL/VAP w %; WOB (*Wobble*) - indeks oscylacji.

Rycina 3. Komputerowo wspomagana analiza nasienia (SCA, wersja 6.5.0.67, MICROPTIC, Hiszpania) - plemniki z zaznaczonymi torami ruchu.
Czerwony – ruch postępowy; Zielony – ruch średniopostępowy; Niebieski - ruch niepostępowy; Żółty - plemniki nieruchome



Ocena mikroskopowa nasienia polegała na analizie żywotności oraz morfologii plemników. Do oceny plemników żywych (z nieuszkodzoną błoną komórkową) oraz martwych (z uszkodzoną błoną komórkową) zastosowano barwienie eozynowo-nigrozynowe. Na ogrzane szkiełko podstawowe nanoszono za pomocą pipety po 2 µl nasienia oraz eozyny i nigrozyny podgrzanych do temperatury 37°C. Preparaty pozostawiono do wyschnięcia na stoliku grzewczym, a następnie poddawano ocenie w mikroskopie świetlnym (Nikon ECLIPSE E2000) przy 1000-krotnym powiększeniu z użyciem olejku immersyjnego. Każdorazowo ocenie poddawano 200 plemników. Eozyna, w plemnikach o uszkodzonej błonie komórkowej wnikała do ich wnętrza barwiąc je na różowo. Takie plemniki uznawano za martwe. Te, które pozostawały niezabarwione (białe), klasyfikowano jako żywe. Wyniki przedstawiano jako odsetek plemników żywych oraz martwych.

Rycina 4. Ocena żywotności plemników, barwienie eozynowo- nigrozynowe.
A – niezabarwione, żywe plemniki; B – zabarwione, martwe plemniki
Nikon ECLIPSE E2000, powiększenie 20x

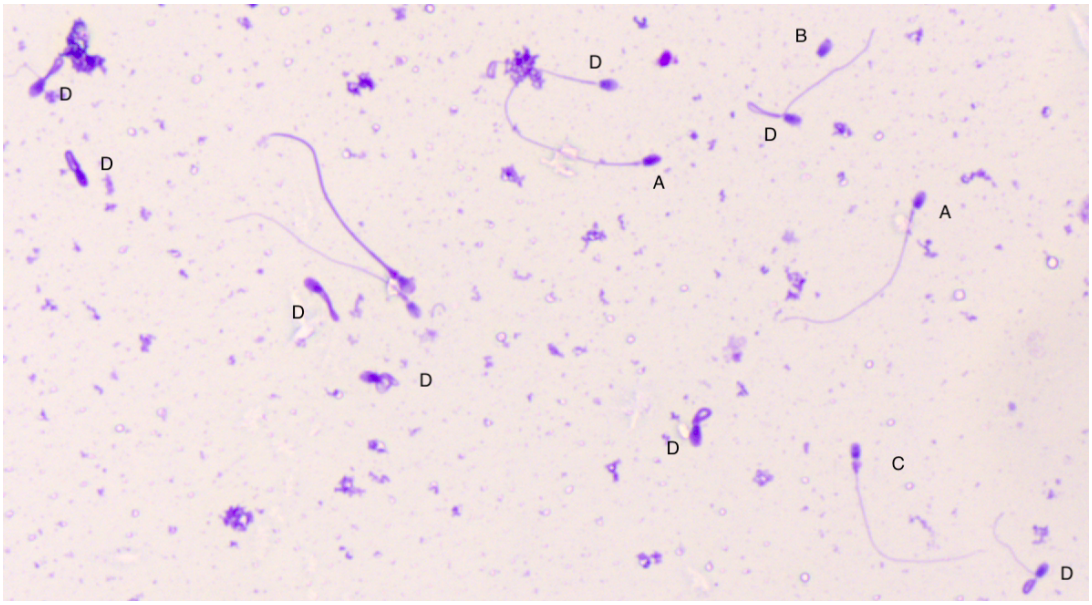


Do przeprowadzenia analizy mikroskopowej morfologii gamet wykonywano rozmaz na szkiełku podstawowym, który pozostawiano do wyschnięcia na 24h. Następnie preparaty utrwalano i barwiono przy użyciu dostępnego komercyjnie zestawu Sperm Stain (MICROPTIC, Hiszpania) zgodnie z zaleceniami producenta. Po wykonaniu barwienia, preparat pozostawiano do wyschnięcia na wolnym powietrzu. Tak przygotowane rozmazy poddawano ocenie w mikroskopie świetlnym (Nikon ECLIPSE E2000) przy 1000-krotnym powiększeniu z użyciem olejku immersyjnego. Każdorazowo

ocenie poddawano 200 plemników, a wyniki przedstawiano jako odsetek plemników prawidłowych, oraz nieprawidłowych z rozróżnieniem na wady główki, witki oraz wstawki.

Rycina 5. Ocena morfologii plemników, barwienie Sperm Stain (MICROPTIC, Hiszpania).

A – plemnik prawidłowy; B – wada główki; C – wada wstawki; D – wada witki Nikon ECLIPSE E2000, powiększenie 20x



Od psów pobierane były również próbki do badań mikrobiologicznych oraz oznaczeń PCR i były to wymazy z napletka – grupa 1 (N=78) oraz wymazy z nasienia - grupa 2 (N=63). Od każdego z psów pobierano 3 suche wymazy, dodatkowo od psów z grupy 2 pobierano wymaz do badania bakteriologicznego. Jeden z nich był wysyłany do komercyjnego laboratorium w celu potwierdzenia lub wykluczenia obecności mikroorganizmów: *Mycoplasma* spp., Herpesvirus canis oraz *Chlamydia* (*Chlamydophila*) spp. Metodyka dotycząca diagnostyki PCR i Real-Time PCR powyższych mikroorganizmów została szczegółowo opisana w publikacjach:

1. **Domrazek K,** Jurka P. Prevalence of Chlamydophila spp. and Canid herpesvirus-1 in Polish dogs. Veterinary World. 2024; 17(1): 226-232. DOI: 10.14202/vetworld.2024.226-232

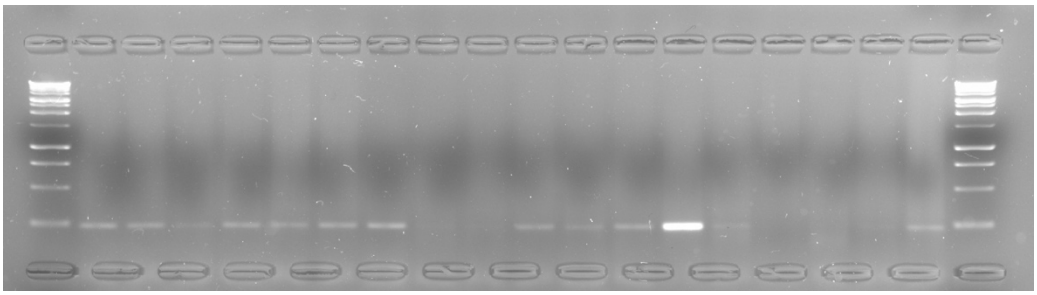
2. **Domrazek K**, Konieczny P, Majka M, Czopowicz M, Cywińska A, Jurka P. The lack of the influence of various species of Mycoplasma spp. on canine semen quality. Theriogenology. 2024; 219: 86-93. DOI: 10.1016/j.theriogenology.2024.02.018

Pozostałe wymazy były przechowywane do dalszych oznaczeń. W przypadku uzyskania dodatniego wyniku w kierunku *Mycoplasma* spp., przeprowadzano identyfikację gatunkową.

Izolacja materiału genetycznego z próbek uzyskanych od psów o dodatnich wynikach w kierunku *Mycoplasma* spp. była wykonana przy użyciu komercyjnie dostępnego zestawu Swab-Extract DNA Purification Kit (Eurx, Polska) zgodnie z instrukcją producenta. Do reakcji PCR użyto starterów opisanych w pracy przeglądowej: **Domrazek, K,** Kaszak I, Kanafa S, Sacharczuk M, Jurka, P. The Influence of Mycoplasma Species on Human and Canine Semen Quality: A Review. *Asian J Androl* **2023**, *25*, 29, doi:10.4103/aja2021124.

Produkty otrzymane w reakcjach zostały poddane elektroforezie w 2% żelu agarozowym. Uzyskane wyniki analizowano używając markera 100bp DNA ladder. Szczegółowy opis metodyki wykorzystanej do identyfikacji gatunkowej mykopkazm został opisany w artykule: **Domrazek K**, Konieczny P, Majka M, Czopowicz M, Cywińska A, Jurka P. The lack of the influence of various species of Mycoplasma spp. on canine semen quality. Theriogenology. 2024; 219: 86-93. DOI: 10.1016/j.theriogenology.2024.02.018

Rycina 6. Wynik PCR dla *M. canis* (wielkość produktu 247 bp) dla 18 próbek. Próbki pochodzą z tego samego doświadczenia, a żele/bloty były przetwarzane równolegle. Użyty marker wielkości molekularnej: Perfect Plus 1 kb DNA Ladder (Eurx, Polska).



Próbki nasienia były pobierane do badań bakteriologicznych przy użyciu wymazówek z podłożem agarowym i niezwłocznie wysyłane do laboratorium komercyjnego (Vetlab, Polska). Próbki hodowano na następujących podłożach mikrobiologicznych: Columbia Agar z 5% krwią owiec, MacConkey Agar, Columbia CNA Agar z 5% krwią owiec oraz Agar czekoladowy. Warunki inkubacji obejmowały utrzymywanie temperatury 35-37°C przez 48 godzin w atmosferze tlenowej (Columbia Agar z 5% krwią owiec, MacConkey Agar i Columbia CNA Agar z 5% krwią owiec) lub w atmosferze o podwyższonym stężeniu CO_2 (Agar czekoladowy), wspomaganej przez generator atmosfery CO_2 (Gen Compact, bioMérieux, Francja). Wzrost bakterii był kontrolowany 24 i 48 godzinach po inkubacji. Następnie uzyskane kolonie bakterii poddawano analizie w aparacie MALDI TOF Biotyper Sirius IV (Billerica, MA, USA).

W opisanych powyżej badaniach analiza statystyczna wyników została przeprowadzona z wykorzystaniem programu TIBCO Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). Ze względu na brak rozkładu normalnego danych ilościowych, wykazany za pomocą testu Shapiro-Wilka, ich opisu dokonano z użyciem mediany, zakresu międzykwartylowego (ang. interquartile range, IQR) i zakresu. W analizie statystycznej danych ilościowych wykorzystano nieparametryczne testy istotności takie jak test U Manna-Whitneya, współczynnik korelacji rang Spearmana. Zmienne jakościowe przedstawiono w postaci rozkładów liczebności i częstości, a analizowano z zastosowaniem testu G najwyższej wiarygodności i dokładnego testu

Fishera. Przedziały ufności dla 95% poziomu ufności (95% PU) obliczano z zastosowaniem metody Wilsona (Altman i wsp., 2000). Szczegółowy opis przeprowadzonych analiz statystycznych dla każdego z badań został opisany w artykułach:

1. **Domrazek K**, Konieczny P, Majka M, Czopowicz M, Cywińska A, Jurka P. The lack of the influence of various species of Mycoplasma spp. on canine semen quality. Theriogenology. 2024; 219: 86-93. DOI: 10.1016/j.theriogenology.2024.02.018
2. **Domrazek K**, Konieczny P, Majka M, Czopowicz M, Jurka P. The impact of microorganisms on semen quality. Animals 2024, 14, 1267. DOI: 10.3390/ ani14091267

8. Omówienie uzyskanych wyników i najważniejszych obserwacji

8.1 Ocena wpływu różnych gatunków *Mycoplasma* spp. występujących w napletku psów na jakość nasienia

Znaczenie badań dotyczących wpływu bakterii *Mycoplasma* spp. na jakość nasienia psów opisano w pracy przeglądowej: **Domrazek K**, Kaszak I, Kanafa S, Sacharczuk M, Jurka, P. The Influence of Mycoplasma Species on Human and Canine Semen Quality: A Review. *Asian J Androl* **2023**, *25*, 29, DOI:10.4103/aja2021124.

W pracy tej przedstawiono również startery PCR wykorzystane do identyfikacji gatunkowej mykoplazm. Wyniki badań własnych dotyczących wykrywania *Mycoplasma* spp. i jej poszczególnych gatunków w napletku psów przedstawiono w artykule: Domrazek **K**, Konieczny P, Majka M, Czopowicz M, Cywińska A, Jurka P. The lack of the influence of various species of Mycoplasma spp. on canine semen quality. Theriogenology. 2024; 219: 86-93.
DOI: 10.1016/j.theriogenology.2024.02.018.

Mediana wieku badanych psów wynosiła 2,5 roku. Ich masa ciała wahała się od 1,5 do 80 kg, z medianą wynoszącą 24 kg. Większość psów była w dobrej kondycji fizycznej, z oceną kondycji ciała (BCS) na poziomie 3/5, chociaż charty nieznacznie odbiegały od normy z BCS na poziomie 2,5/5 ze względu na ich charakterystyczną budowę anatomiczną.

Wśród badanych psów, 83,3% miało dodatnie wyniki badań PCR w kierunku *Mycoplasma* spp. Przeprowadzono identyfikację gatunkową tej bakterii, która wykazała jedenaście różnych gatunków, przy czym *M. cynos* i *Mycoplasma* HRC 689 były najczęściej diagnozowane. Nie stwierdzono jednak istotnego związku między obecnością *Mycoplasma* spp. a azoospermią, którą zaobserwowano u 7,7% psów. W dostępnej literaturze brak jest wyników przedstawiających taką różnorodność gatunkową tej bakterii w obrębie psiego układu rozrodczego. Maksimovic i wsp. (2018) przeprowadzili identyfikację gatunkową *Mycoplasma* spp. u 60 suk. Badanie to pozwoliło na wykrycie tylko 3 gatunków: *M. canis, M. spumans* i *M. edwardii* (Maksimović i wsp., 2018). Z kolei Lechner i wsp. (2023) wykazali obecność

M. arginini, M. canis, M. cynos, M. edwardii i *M. spumans* w ejakulatach psów, ale w tym badaniu nie wykorzystano PCR. Obecnie metody molekularne są uważane za złoty standard w diagnostyce mykoplazm. Zgodnie z naszą najlepszą wiedzą, występowanie *M. molare*, *Mycoplasma* HRC 689, *M*. VJC 358 oraz *M. feliminutum* nie zostało dotychczas opisane w układzie rozrodczym psów.

Szczególną uwagę zwraca fakt, że u większości nosicieli *Mycoplasma* spp. (72,3%) wykryto więcej niż jeden gatunek tej bakterii. Najczęściej wykrywano współistnienie gatunków *M. cynos* i *Mycoplasma* HRC 689 - 8 psów. Pięć kombinacji wykryto u 2 psów: *M. molare* i *M. felis; M. cynos*, *M. canis* i *Mycoplasma* HRC 689; *M. cynos, M. canis* i *M. maculosum*; *M. cynos, M. feliminutum* i *Mycoplasma* HRC689, a także *M. cynos, M. molare* i *Mycolasma* HRC689.

Nie stwierdzono istotnego statystycznie związku między obecnością *Mycoplasma* spp. a azoospermią. Nie stwierdzono również korelacji między parametrami jakości nasienia a liczbą wykrytych gatunków tej bakterii. Całkowita liczba plemników w ejakulacie była podobna w grupach psów o dodatnich i ujemnych wynikach badań w kierunku *Mycoplasma* spp. Całkowita ruchliwość, ruchliwość postępowa i odsetek plemników o ruchu szybkim były nieznacznie wyższe u psów wolnych od *Mycoplasma* spp., ale różnica ta nie była istotna statystycznie. Ani poszczególne gatunki, ani ich liczba nie miały istotnego wpływu na parametry morfologiczne, jak również na żywotność plemników.

Uzyskane wyniki mają charakter aplikacyjny, gdyż w codziennej praktyce lekarsko-weterynaryjnej rutynowo pobiera się wymazy z napletka w celu diagnostyki niepłodności spowodowanej chorobami zakaźnymi przenoszonymi drogą płciową. Obecność *Mycoplasma* spp. w napletku nie jest związana z występowaniem niepłodności, w związku z czym metoda ta wydaje się być nieadekwatna. Jednakże warto mieć na uwadze, iż podczas krycia naturalnego układ rozrodczy suki ma bezpośredni kontakt z patogenami zasiedlającymi powierzchnię prącia i napletka samca, co może skutkować zakażeniem. Kluczową rolę w rozwoju infekcji odgrywa układ immunologiczny, w związku z tym konieczne jest przeprowadzenie dalszych badań pozwalających na ocenę ryzyka zakażenia suki bakteriami *Mycoplasma* spp. zasiedlającymi napletek psów oraz ich potencjalnego wpływu na zdrowie i płodność samicy.

8.2 Ocena wpływu różnych gatunków *Mycoplasma* spp. występujących w nasieniu na jego jakość

Wyniki badań przeprowadzonych w celu określenia częstości występowania różnych gatunków bakterii *Mycoplasma* spp. i wpływu tej bakterii na jakość psiego nasienia opisano w publikacji: **Domrazek K**, Konieczny P, Majka M, Czopowicz M, Jurka P. The impact of microorganisms on semen quality. Animals 2024, 14, 1267. DOI: 10.3390/ ani14091267

Mediana wieku badanych psów wynosiła 3 lata. Ich masa ciała wahała się od 3 do 120 kg, z medianą wynoszącą 24 kg. Oprócz badania PCR w kierunku *Mycoplasma* spp. wykonano badanie bakteriologiczne nasienia w celu wykluczenia infekcji układu rozrodczego mogącego mieć wpływ na jakość badanego nasienia. U ośmiu psów z nasienia wyhodowano następujące bakterie tlenowe: *Staphylococcus pseudintermedius* u 3 psów, *Streptococcus canis* u 2 psów oraz *Staphylococcus vitulinus*, *E. coli* i *Pseudomonas* sp. u 1 psa. Nie wykazano zależności pomiędzy tymi bakteriami jakością badanego nasienia.

Badanie PCR w kierunku *Mycoplasma* spp. wykazało obecność tej bakterii w nasieniu 38 (60,3%) psów. Podobne wyniki uzyskano w badaniach prowadzonych przez Schafer-Somi i wsp. (2009), w których wykryto obecność tych bakterii w 55% próbek. Wyniki niniejszego badania wykazały większą częstość występowania *Mycoplasma* spp. na powierzchni napletka. Wynik ten może sugerować, iż bakterie występują z malejącą częstością w poszczególnych częściach układu rozrodczego tak jak ma to miejsce w drogach oddechowych (Ericsson i wsp., 2016). W celu potwierdzenia tej tezy, niezbędne jest przeprowadzenie diagnostyki PCR z różnych miejsc układu rozrodczego jednego osobnika.

Dalsza identyfikacja pozwoliła na określenie dokładnego gatunku w 28 próbkach. Szczególną uwagę zwraca fakt, iż u 10 psów - nosicieli *Mycoplasma* spp. użycie standardowych starterów nie pozwoliło na określenie gatunku tej bakterii. Wynik ten sugeruje, iż w nasieniu mogą bytować inne, nieopisane dotychczas gatunki lub szczepy *Mycoplasma* spp. W celu ich identyfikacji niezbędne jest przeprowadzenie dalszych badań przy użyciu bardziej zaawansowanych metod diagnostycznych np. sekwencjonowania nowej generacji (Next Generation Sequencing-NGS).

U 28 psów zidentyfikowano 12 gatunków *Mycoplasma* spp. - 1 gatunek u 11 psów (39,3%), 2 gatunki u 10 psów (35,7%), 3 gatunki u 5 psów (17,9%) i 4 gatunki u 2 psów (7,1%), podczas gdy, badania dotyczące izolacji mykoplazm z napletka wykazały, iż psy najczęściej są nosicielami więcej niż jednego gatunku tej bakterii. Najczęściej wykrywanymi gatunkami tej bakterii były *Mycoplasma* HRC689 oraz *M. canis*, jednakże wyniki uzyskane przez innych autorów sugerują, że *M. canis* oraz *M. cynos* są najczęściej izolowanymi gatunkami z psiego nasienia (Lechner i wsp., 2023; Schäfer-Somi i wsp., 2009).

Tylko u jednego psa stwierdzono azoospermię, a u pięciu oligospermię ($<200\times10^6$ plemników). Nieprawidłową morfologię plemników (≤70% prawidłowych plemników w nasieniu) stwierdzono u trzech psów. Nie wykazano istotnej statystycznie zależności pomiędzy występowaniem *Mycoplasma* spp. w nasieniu a jego jakością.

Opisywane wyniki zostały pozyskane przy użyciu metod jakościowych a nie ilościowych. Na przebieg kliniczny zakażenia może mieć wpływ ilość drobnoustrojów. Istnieje możliwość, iż zastosowanie ilościowych metod diagnostycznych pozwoliłoby na opisanie negatywnego wpływu *Mycoplasma* spp. na płodność psów.

8.3 Diagnostyka PCR *Chlamydia* (*Chlamydophila)* spp. i Herpesvirus canis występujących w napletku i nasieniu psów oraz ocena jakości nasienia nosicieli tych drobnoustrojów

Dotychczas większość badań dotyczących występowania *Chlamydia* (*Chlamydophila*) spp. oraz CHV-1 była prowadzona przy użyciu metod immunologicznych. Biorąc pod uwagę możliwość występowania latencji CHV-1 oraz chlamydiozy innego układu niż układ rozrodczy, zdecydowano się przeprowadzić badania dotyczące prewalencji tych drobnoustrojów przy użyciu diagnostyki Real- Time PCR. Pozytywny wynik w kierunku *Chlamydia/Chlamydophila* spp. uzyskano tylko w jednej próbce pochodzącej z napletka (1/78). Nie wykryto tej bakterii w żadnej z próbek pobranych z nasienia.

Oceniono jakość nasienia psa-nosiciela *Chlamydia/Chlamydophila* spp., a uzyskane wyniki porównano z normami parametrów opisujących jakość nasienia. Poszczególne parametry opisujące jakość nasienia psa-nosiciela *Chlamydia/Chlamydophila* spp. zostały przedstawione w publikacji: **Domrazek K,** Jurka P. Prevalence of Chlamydophila spp. and Canid herpesvirus-1 in Polish dogs. Veterinary World. 2024; 17(1): 226-232. DOI:10.14202/vetworld.2024.226-232.

Wszystkie parametry opisujące jakość nasienia, z wyjątkiem ruchu postępowego mieściły się w granicach normy. U psa-nosiciela *Chlamydia/Chlamydophila* spp. dokładny gatunek tej bakterii nie został określony. Ze względu na fakt, iż chlamydioza jest zoonozą, wprowadzono antybiotykoterapię. Zastosowano doksycyklinę w dawce 10 mg/kg masy ciała, raz dziennie przez 4 tygodnie. Po leczeniu właściciel odmówił ponownego zbadania zwierzęcia, w związku z czym nie możliwa była ocena ewentualnej poprawy jego jakości nasienia.

Na podstawie powyższego przypadku klinicznego nie można jednoznacznie ocenić wpływu zakażenia *Chlamydia*/*Chlamydophila* spp. na jakość psiego nasienia. W tym celu konieczne jest przeprowadzenie doświadczenia na większej liczbie psów.

W żadnej z badanych próbek nie wykryto obecności CHV-1. Seroprewalencja herpeswirusa u psów wykorzystywanych do rozrodu wynosiła 50,3%

we Włoszech (Rota i wsp., 2020), 22% w RPA (Gadsden i wsp., 2012), 39%-62,1% w Turcji (Yeşilbaĝ i wsp., 2012) i 81,5% w Finlandii (Dahlbom i wsp., 2009). W badaniach własnych nie wykonywano badań serologicznych, w związku z czym seroprewalencja nie jest możliwa do oszacowania i porównania z wynikami innych autorów. Z drugiej jednak strony Gracin i wsp. (2023) porównali sero- i prewalencję u psów. U 32,02% badanych psów wykazano dodatnie wyniki w serologicznych metodach diagnostycznych, podczas gdy wszystkie wymazy dały wynik ujemny w badaniu PCR.

Istnieje ryzyko, że w niniejszym badaniu uzyskano wyniki fałszywie ujemne, gdyż niektórzy autorzy sugerują, iż siewstwo wirusa w stanie latencji jest niewielkie. Z drugiej jednak strony, diagnostyka metodami molekularnymi jest niezwykle czuła i zgodnie z aktualnym stanem wiedzy, ryzyko uzyskania wyników fałszywie ujemnych jest niezwykle małe.

9. Wnioski

W niniejszej pracy przedstawione zostały wyniki badań dotyczących częstości występowania *Mycoplasma* spp. (i różnych gatunków), CHV-1 oraz *Chlamydia* (*Chlamydophila*) spp. w obrębie układu rozrodczego psów oraz wypływu tych drobnoustrojów na jakość psiego nasienia.

Na podstawie przeprowadzonych analiz oraz uzyskanych wyników sformułowano następujące wnioski:

1. *Mycoplasma* spp. występuje powszechnie w napletku i nasieniu psów. Najczęściej izolowanymi z napletka gatunkami są *M. cynos* i *Mycoplasma* HRC 689, a z nasienia *Mycoplasma* HRC689 oraz *M. canis.*
2. Napletek psów najczęściej zasiedlany jest przez wiele gatunków *Mycoplasma* spp., w przeciwieństwie do nasienia, z którego najczęściej izoluje się pojedyncze gatunki.
3. Poznane startery dla *Mycoplasma* spp. nie umożliwiają pełnej diagnostyki gatunkowej tej bakterii. Konieczne jest przeprowadzenie dalszych badań w tym zakresie.
4. Obecność *Mycoplasma* spp. w napletku oraz nasieniu nie wpływa na jakość frakcji nasiennej ejakulatu
5. Występowanie CHV-1 i *Chlamydia* (*Chlamydophila*) spp. w populacji polskich psów-samców jest sporadyczne.

10. Piśmiennictwo

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11. Publikacje wchodzące w skład dysertacji i oświadczenia o współautorstwie

Asian Journal of Andrology (2023) 25, 29–37
www.asiaandro.com; www.ajandrology.com

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REVIEW

Semen Analysis

The influence of *Mycoplasma* species on human and canine semen quality: a review

Kinga Domrazek[1], Ilona Kaszak[1], Szymon Kanafa[1], Mariusz Sacharczuk[2], Piotr Jurka[1]

Mycoplasma **species (spp.) are bacteria that are difficult to detect. Currently, the polymerase chain reaction (PCR) is considered the most effective diagnostic tool to detect these microorganisms in both human and veterinary medicine. There are 13 known species of human *Mycoplasma* and 15 species of canine *Mycoplasma*. Owing to the difficulties in identifying the individual species of *Mycoplasma*, there is a lack of information regarding which species are saprophytic and which are pathogenic. The prevalence of the individual species is also unknown. In addition, in both humans and dogs, the results of some studies on the impact of *Mycoplasma* are conflicting. The presence of *Mycoplasma* spp. on the epithelium of reproductive tract is often associated with infertility, although they are also detected in healthy individuals. The occurrence of *Mycoplasma* spp. is more common in dogs (even 89%) than in humans (1.3%–4%). This is probably because the pH of a dog's genital is more conducive to the growth of *Mycoplasma* spp. than that of humans. Phylogenetically, human and canine *Mycoplasma* are related, and majority of them belong to the same taxonomic group. Furthermore, 40% of canine *Mycoplasma* spp. are placed in common clusters with those of human. This suggests that species from the same cluster can play a similar role in the canine and human reproductive tracts. This review summarizes the current state of knowledge about the impact of *Mycoplasma* on canine and human male fertility as well as the prospects of further development in this field.**

Asian Journal of Andrology (2023) **25**, 29–37; doi: 10.4103/aja2021124; published online: 04 March 2022

Keywords: *Mycoplasma*; sperm morphology; sperm motility

INTRODUCTION

In human medicine, infertility is defined as a failure to conceive after 12 months of regular intercourse without contraception,[1] and it affects 8%–12% of couples.[2] Infectious organisms in the reproductive tract may affect male fertility. Although some researchers suggested a correlation between *Mycoplasma* and infertility in humans and dogs, this phenomenon has not been proved in other studies.[3] It is suspected that these bacteria may be commensals, although it is difficult to estimate their role. This article summarizes the current state of knowledge about the impact of *Mycoplasma* species (spp.) on fertility in dogs and men.

Mycoplasma spp. are the smallest self-replicating organisms, belonging to the *Mycoplasmataceae* family, and are detectable in humans, animals, as well as in plants.[4] There is a theory that *Mycoplasma* spp. evolved from Gram-positive bacteria, and phylogenetically they are close to *Clostridia*.[4] Morphologically, *Mycoplasma* spp. stand out because of the total lack of a cell wall, and because they are included in the *Mollicutes* class (from Latin: *mollis* means soft, *cutis* means skin). The *Mycoplasma* cell contains only the organelles that are essential for growth and replication.[4] Taxonomically, *Mycoplasma* spp. are divided into the following groups: *anaeroplasma*, *asteroleplasma*, *hominis*, *pneumoniae,* and *spiroplasma*.[5] The majority of both canine (Ca) and human (Ho) genital *Mycoplasma* belong to the hominis group, which shows that they are relatively closely related. In the *hominis* group, among others, there are three clusters: *hominis*, *bovis,* and *synoviae*, in which both human and canine *Mycoplasma* are placed. The *hominis* cluster includes *Mycoplasma* (*M.*) *arginini* (Ca), *M. gateae* (Ca), *M. spumans* (Ca), *M. buccale* (Ho), *M. faucium* (Ho), *M. hominis* (Ho), and *M. orale* (Ho); the *bovis* cluster includes *M. bovigenitalium* (Ca), *M. maculosum* (Ca), *M. opalescens* (Ca), *M. fermentans* (Ho), *M. primatum* (Ho), and *M. spermatofilum* (Ho); and the *synoviae* cluster includes *M. cynos* (Ca), *M. edwardii* (Ca), *M. felis* (Ca), and *M. canis* (Ca).[6] Therefore, on the basis of *Mycoplasmataceae* taxonomy, it has been estimated that 40% of canine species are in the same cluster as human (not published).

All phylogenetic are shown in **Figure 1**. The 16S ribosomal DNA sequences of *Mycoplasma* species were retrieved from GenBank (NCBI), as shown in **Table 1**. Alignment of the sequences was constructed using GeneDoc using Blosum62 matrix (gap open cost: 8, gap extend cost: 4). Aligned sequences were trimmed to the longest overlapping region and sequences of *M. primatum, M. haemocanis,* and *M. arginini* were rejected due to small overlapping region, and rest of the sequences were aligned again using aforementioned parameters. An evolutionary tree was constructed with Molecular Evolutionary Genetic Analysis (MEGA) software using the maximum likelihood method and Tamura-Nei model with bootstrap consensus inferred from 10 000 replicates.

This affinity between species of human and canine *Mycoplasma* suggests that they could influence semen quality similarly. Accordingly, the dog can probably be treated as a model organism for research on

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Received: 04 October 2021; Accepted: 20 December 2021

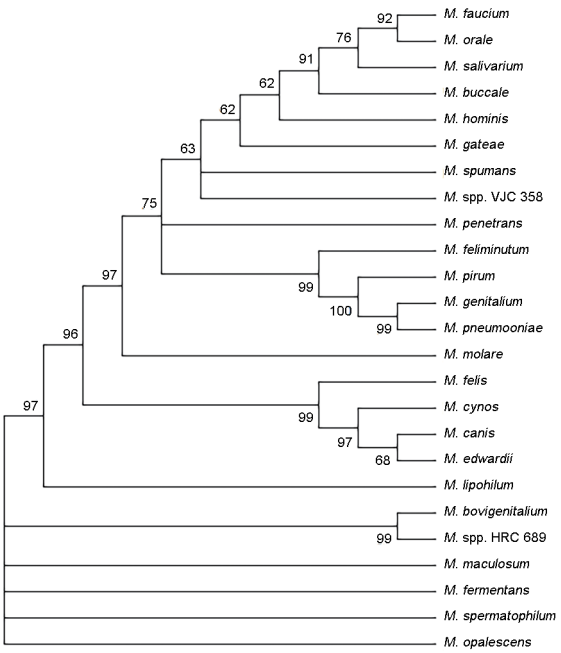

Figure 1: The evolutionary tree of 16S ribosomal DNA sequences of canine and human species of *Mycoplasma*. Numbers above the branches show the percentage of probability of the result. *M.*: *Mycoplasma*.

mycoplasmosis of the genital tract. In addition, it is possible for canine *Mycoplasma* to colonize the human body. Klein *et al*.[7] have isolated *M. canis* from human tissue after a dog bite.

The primary habitats of human and canine *Mycoplasma* are the mucous surfaces of the respiratory and urogenital tracts, eyes, digestive system, mammary glands, and joints.[4] In addition, there is a report about their occurrence in pathological canine brain tissues.[8] As well as other mollicutes, *Mycoplasma* spp. can be present intracellularly in the host's cells. In both humans and animals, *Mycoplasma* is taken up by leukocytes and macrophages, but the mechanism of entry into the cells is still unclear. However, it has been described that this invasion may affect cell function.[4] Díaz-García *et al*.[9] demonstrated that *M. hominis* can also infect spermatozoa.[9]

Mycoplasma adheres to the surface of the epithelium in the reproductive tract, and this process is strong enough to prevent their elimination in their secretions or urine.[4] It is also known that *M. genitalium* has a major surface adhesion complex known as the nucleoid-associated protein (NAP) on its surface, and because of this, it can adhere to surfaces and remains motile.[10] Furthermore, no specific toxins or virulence factors of *M. genitalium* have been described, and it is suspected that the lipoproteins exposed on their surface can stimulate local inflammatory response in the reproductive tract.[11] There is limited knowledge about the virulence factors of canine *Mycoplasma* spp. However, some species can cause hemolysis during culturing; therefore, it has been suggested that some of them can synthesize hemolytic enzymes.[12] Genital *Mycoplasma* in humans and possibly in veterinary patients are natural inhabitants of the male urethra, and therefore, they can be present in spermatozoa during ejaculation.[13] There are 13 known species of human *Mycoplasma* which occur in the genital tract including *M. buccale*, *M. faucium*, *M. fermentans*, *M. genitalium*, *M. hominis*, *M. lipophilum*, *M. orale*, *M. penetrans*, *M. pirum*, *M. pneumoniae*, *M. primatum*, *M. salivarium*, and *M. spermatophilum*,[14] but the more common are *M. genitalium* and *M. hominis*.[15]

Table 1: List of species of *Mycoplasma* and their numbers in the GenBank used to create a phylogenetic tree

Species of Mycoplasma	*GenBank number*
Mycoplasma facium	NR_024983.1
Mycoplasma orale	NR_043199.1
Mycoplasma salivarium	NR_041745.1
Mycoplasma hominis	NR_041881.1
Mycoplasma gateae	NR_029180.1
Mycoplasma spumans	NR_24980.1
Mycoplasma spp. VJC 358	AY246564.1
Mycoplasma penetrans	RCH401000003.1
Mycoplasma feliminutum	NR_029181.1
Mycoplasma pirum	NR_029165.1
Mycoplasma genitalium	NR_026155.1
Mycoplasma pneumooniae	NR_041751.1
Mycoplasma molare	NR_041931.1
Mycoplasma felis	U09787.1
Mycoplasma cynos	NR_025181.1
Mycoplasma canis	AB680678.1
Mycoplasma lipohilum	AB680693.1
Mycoplasma bovigenitalium	AB680692.1
Mycoplasma spp. HRC 689	AF527624.1
Mycoplasma maculosum	AB680679.1
Mycoplasma fermentans	NR_044666.2
Mycoplasma spermatophilum	NR_025069.1
Mycoplasma opalescens	NR_025067.1

In the canine reproductive tract, *M. arginini*, *M. bovigenitalium*, *M. canis*, *M. cynos*, *M. edwardii*, *M. feliminutum*, *M. felis*, *M. gateae*, *M. haemocanis*, *M. maculosum*, *M. molare*, *M. opalescens*, *Mycoplasma* spp. HRC 689, *Mycoplasma* spp. VJC 358, and *M. spumans* can be detected, and the more common are *M. canis*, *M. spumans*, and *M. maculosum*.[16] Both canine and human *Mycoplasma* are shown in **Figure 2**.

It has been estimated that their prevalence in the human reproductive tract in countries with high levels of development is 1.3%, while it is almost 4% in countries with lower levels of development.[17] In veterinary medicine, the occurrence of *Mycoplasma* spp. in animals is more common. It has been estimated that among dogs, up to 89% can be *Mycoplasma* positive.[18] There are possible reasons that *Mycoplasma* spp. is more common in dogs than in humans. On the one hand, dogs have more different sexual partners than humans, and in addition, people are using safeguards against contracting venereal diseases. On the other hand, *Mycoplasma* spp. may be present in the prepuce of some dogs before the first mating. The pH value of the canine reproductive tract may be potentially more suitable for the growth of this microorganism. The best pH conducive for *Mycoplasma* growth is between 7.8 and 8.[19] In canine females, the pH value in the vagina is 7.4–8.3[20] and 6.3–6.7 in prepuce of males,[21] as opposed to humans who have lower values of 5.71 in men's prepuce[22] and 3.8–4.5 in women's vagina.[23] The pH values of canine semen are as follows; first fraction: 6.37, second fraction: 6.37, and the third one is 7.2;[24] and human semen pH values are between 7.2 and 8.[25] The most important factor seems to be pH in the place of arising the *Mycoplasma*. In the tunica mucosa of the human reproductive tract, the pH is inappropriate for growth and development of these bacteria. This phenomenon can be a reason that *Mycoplasma*-positive results are more common in the dog than in the human reproductive tract. In a few publications, the presence of *Mycoplasma* was in semen, not the prepuce.[26,27] Ultimately, the

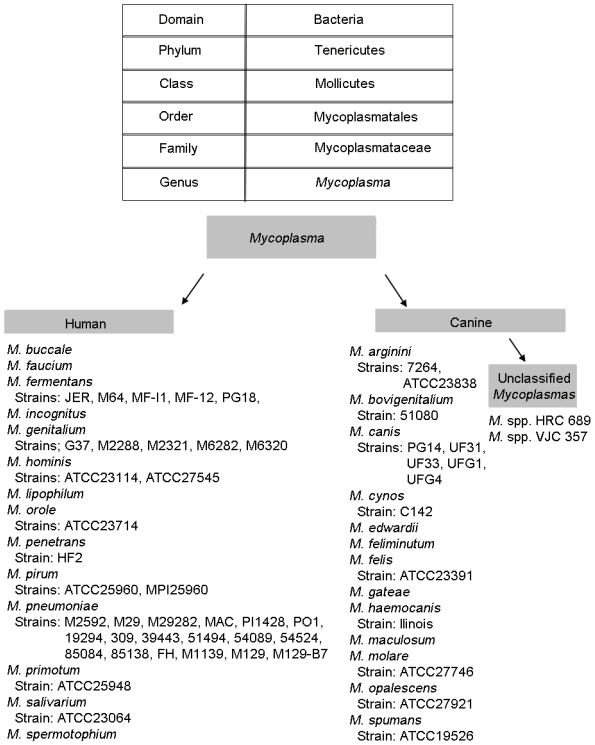

Figure 2: Scientific classification of human and canine genital *Mycoplasmas*, based on: ncbi.nlm.nih.gov (strains) and patricbrc.org/view/Taxonomy/ (taxonomy). *M.*: *Mycoplasma*.

hypothesis is that in the canine reproductive tract, the environmental conditions are better for *Mycoplasma* spp. can be given. However, more research is needed to confirm this theory. Moreover, the prevalence of *Mycoplasma* in the respiratory tract is higher in dogs than that in man; in humans, it ranges from 2% to 35%,[27] while in dogs, it ranges from 86% to 90%.[28] Nevertheless, a study performed in mice showed that those infected by *Mycoplasma* intranasally were more resistant to *Mycoplasma* infections of the reproductive tract than the noninfected.[29] Probably, a similar phenomenon can be observed in dogs and humans; however, further studies are required to confirm this suggestion.

Similar to *Mycoplasma* spp. are *Ureaplasma* (*U.*) spp. which reside in the urogenital tract. These bacteria, by evolution, have also lost their cell wall. In humans, there are two known species: *U. urealyticum* and *U. parvum*. Like *Mycoplasma* spp., *Ureaplasma* spp. are also considered to be a cause of infertility, but it has also been suggested that they could be a part of the normal genital flora.[30] Since *Mycoplasma* and *Ureaplasma* are related and very similar, some researchers have named them together as "*Mycoplasmas*", and their effect on the semen is examined together in studies.

SPECIFYING THE *MYCOPLASMA*

In the past, the main method of detecting *Mycoplasma* spp. was by culturing them, but owing to the high requirements of these bacteria, this method is not used nowadays in commercial laboratories. The polymerase chain reaction (PCR) is now the most commonly used method in both veterinary and human medicine. Peerayeh and Samimi[31] have shown that the PCR method enables a higher rate of detection of *Mycoplasma* than standard microbiologic cultures.

The ribosomal 16S gene sequence is frequently used in molecular techniques owing to its universal presence among bacteria. The 16S rRNA gene contains nine hypervariable regions (V1–V9) that show differences among bacteria. These specific sequences are useful for diagnostic assays, *e.g.*, V6 helps to distinguish among most bacterial species except *Enterobacteriaceae*. In the case of 16S rRNA analysis, identification of the bacteria is easier when the entire gene can be sequenced. Unfortunately, this technique is not rapid, so it is not common. A faster and commonly used method is based on assays that combine nucleic acid amplification with a sequence-specific probe of the amplified product. In this technique, there is a possibility to query short DNA sequences. Therefore, the identification of the regions within the target gene is important.[32]

In human medicine, there are primers which are capable of detecting *M. hominis*, *M. genitalium*, and *U. urealyticum* simultaneously.[33] In addition, highly specific primers have been developed for the detection of *M. hominis*, *U. urealyticum*, and two others reproductive tract pathogens,[34] and they are based on the ribosomal 16S gene. There are also commercial biochemical assay-based kits available for the identification of *M. hominis*, but the PCR method is faster, more reliable, and more sensitive.[35] The primers which can be used for the identification of human *Mycoplasma* are shown in **Table 2.**

The current knowledge regarding their molecular nature is very limited. Chalker and Brownlie[5] revealed that most canine *Mycoplasma* have a variable phylogenetic origin, but a great part of them lies in a variety of clusters within the hominis group of *Mycoplasma*. Owing to the similarity between the 16S rRNA genes of canine *Mycoplasma*, PCR tests have been created to identify the species-specific regions in the 16S/23S rRNA intergenic spacer region.[36] **Table 3** shows the primers that can be used in the PCR assay to detect canine *Mycoplasma*.

Recently, a novel quantitative qPCR to monitor *Mycoplasma* infection in dogs has been developed by Hemmatzadeh *et al*.[37] A single band of bacterial 16S ribosomal DNA was amplified by using universal *Mycoplasma* primers. The band was excised from the gel, and the purified DNA was submitted to the Australian Genome Research Facility Ltd. for Sanger sequencing. This sequence was used to search GenBank using BLAST for matching a sequence. Thereafter, the prepared DNA was used as a standard for qPCR reactions. The number of copies of the *Mycoplasma* plasmid was calculated on an online calculator. This method was developed because conventional PCR fails to detect less than 100–200 genomes per μl.[37]

INFLUENCE OF INFECTION ON SEMEN QUALITY

The influence of human and canine *Mycoplasma* on the quality of the semen seems to be similar. Infections of the reproductive tract in both humans and animals play an important role in infertility. It is suggested that bacterial and viral infections are two of the factors responsible for male infertility.[38] However, this correlation and the underlying pathogenesis remain unclear. It has been suggested that decreased effectiveness of spermatogenesis, obstruction of the seminal tract, and dysfunction of the spermatozoa are among the adverse effects of bacterial infections.[39] *In vitro* studies have shown that bacterial infection can affect sperm function, in addition to inducing sperm agglutination and apoptosis.[40,41]

The role of *Mycoplasma* infection in both dogs and humans remains unclear. In veterinary medicine, this issue is even more complicated than in human medicine because not all veterinary laboratories specify the species of *Mycoplasma* because of difficulty in their recognition. Previously, the identification of canine *Mycoplasma* was by serological methods which were dependent on specific antisera for each species. However, cross-reactions were also observed; consequently, antisera are not readily available in laboratories.[42] Moreover, owing to the

Table 2: Polymerase chain reaction primers for specifying human *Mycoplasmas*

Mycoplasma spp.	*Mycoplasma primer sequence (5'–3')*	*Source*
Mycoplasma buccale	Forward: ATGCATGTCGAGCGGAAGTA Reverse: AATCCGAAGACCGTCATCATGC	GenBank: AF125586.1[a]
Mycoplasma faucium	Forward: CATGTCGAGCGGAAGTAGCA Reverse: TTAGCTGCGTCAGTGGCTC	GenBank: NR_024983.1[a]
Mycoplasma fermentans	Forward: GGACTATTGTCTAAACAATTTCCC Reverse: GGTTATTCGATTTCTAAATCGCCT	Vojdani and Franco[87] 1999
Mycoplasma genitalium	Forward: TACATGCAAGTCGATCGGAAGTAGC Reverse: AAACTCCAGCCATTGCCTGCTAG	Jensen *et al*.[88] 2003
Mycoplasma hominis	Forward: GGAAGA-TATGTAACAAAAGAAGGTGCTG Reverse: TTTATCTTCTGGCGTAATGATATCTTCG	Baczynska *et al*.[89] 2004
Mycoplasma lipophilum	Forward: CAATATTTAACCGCCGCGCA Reverse: AGCACCCATTAAAGCACGGT	GenBank: DQ112177.1[a]
Mycoplasma orale	Forward: AAGCTTGATGGAGCGACACA Reverse: GCGTTAGCTGCGTCAGTAGT	GenBank: NR_043199.1[a]
Mycoplasma penetrans	Forward: CATGCAAGTCGGACGAAGCA Reverse: AGCATTTCCTCTTCTTACAA	Vojdani and Franco[87] 1999
Mycoplasma pirum	Forward: TACATGCAAGTCGATCG-GAT Reverse: CATCCTATAGCGGTC-CAAAC	Grau *et al*.[90] 1993
Mycoplasma pneumoniae	Forward: CAAGCCAAACACGAGCTCCGGCC Reverse: CAGTGTCAGCTGTTTGTCCTTCCCC	Chaudhry *et al*.[91] 2013
Mycoplasma primatium	In the GenBank, there is no sequence based on which the primer designing could be possible.	-
Mycoplasma salivarium	Forward: ATGATGCTAACCGTGCGCT Reverse: CCATCTTGTCGCCGACTCT	GenBank: EU797448.1[a]
Mycoplasma spermatophilum	Forward: TGACGCTAACCGTGCATTGA Reverse: TGTTACCGTGACGACCTGAC	GenBank: DQ219487.1[a]

[a]Primers not published previously. Parts of the data from the table are cited from the articles and other part of the data are primers not published previously. They are designed based on the sequence from GenBank (ncbi.nlm.nih.gov/genbank). -: no data

Table 3: Polymerase chain reaction primers to specifying canine *Mycoplasma*

Mycoplasma spp.	*Mycoplasma primer sequence*	*Source*
Mycoplasma arginini	Forward: CA-CCGCCCGTCACACCA Reverse: GTTGTATGACCTATTGTTGTC	Chalker[36] 2004
Mycoplasma bovigenitalium	Forward: CGTAGATGCCGCATGGCATTTACGG Reverse: CATTCAATATAGTGGCATTTCCTAC	Kobayashi *et al*.[92] 1998
Mycoplasma canis	Forward: CA-CCGCCCGTCACACCA Reverse: CTGTCGGGGTTATCTCGAC	Chalker[36] 2004
Mycoplasma cynos	Forward: CA-CCGCCCGTCACACCA Reverse: GATACATAAACACAACATTATAATATTG	Chalker[36] 2004
Mycoplasma edwardii	Forward: CA-CCGCCCGTCACACCA Reverse: CTGTCGGGTTATCATGCGAC	Chalker[36] 2004
Mycoplasma feliminutum	Forward: AAGGTCCGTTTGGATCGCTT Reverse: TTTTGGAGCGGGACATGGTT	GenBank: U16758.1[a]
Mycoplasma felis	Forward: CA-CCGCCCGTCACACCA Reverse: GGACTATTATCAAAAGCACATAAC	Chalker[36] 2004
Mycoplasma gateae	Forward: CA-CCGCCCGTCACACCA Reverse: GTTGTATGACCTATTGTTGTC	Chalker[36] 2004
Mycoplasma haemocanis	Forward: GTGCTACAATGGCGAACACA Reverse: TCCTATCCGAACTGAGACGAA	Barker *et al*.[93] 2010
Mycoplasma maculosum	Forward: CA-CCGCCCGTCACACCA Reverse: CCTATGATTGTTACAGATG	Chalker[36] 2004
Mycoplasma molare	Forward: CA-CCGCCCGTCACACCA Reverse: AGCCTATTGTTTTTGATTTG	Chalker[36] 2004
Mycoplasma opalescens	Forward: CA-CCGCCCGTCACACCA Reverse: TAAGCTTTGTAGACCATAA	Chalker[36] 2004
Mycoplasma spp. HRC 689	Forward: CA-CCGCCCGTCACACCA Reverse: CTTGCGACCTAACAAGTCC	Chalker[36] 2004
Mycoplasma spp. VJC 358	Forward: AGGGAGACTGCCCGAGTAAT Reverse: TCGGGTTATCTCGACACATGAC	GenBank: AY246564.1[a]
Mycoplasma spumans	Forward: CA-CCGCCCGTCACACCA Reverse: GTTGTATGACCTATTGTTGTC	Chalker[36] 2004

[a]Primers not published previously. Parts of the data from the table are cited from the articles and other part of the data are primers not published previously. They are designed based on the sequence from GenBank (ncbi.nlm.nih.gov/genbank)

high similarity between the 16S rRNA genes of canine *Mycoplasma*, diagnosis by PCR is also challenging.[12] This is the reason that *Mycoplasma* spp. associated with negative changes in the semen are still unknown.

In human medicine, a meta-analysis has suggested that the presence of *M. hominis*, rather than *M. genitalium*, correlates with male infertility.[43] This indicates that some *Mycoplasma* spp. may also affect male fertility in dogs and some may not. The impact of *Mycoplasma* spp. on the basic semen parameters is described below.[26,44,45]

IMPACT ON BASIC SEMEN PARAMETER VALUES

Volume of the ejaculate

Following the World Health Organization (WHO) guidelines, the volume of the ejaculate should be measured in all semen evaluations. The influence of *Mycoplasma* on the semen volume is not clear. Gdoura *et al*.[44] did not find a significant influence on the semen volume in *Mycoplasma*-positive patients. On the other hand, a study by Ahmadi *et al*.[46] showed a significant increase in the semen volume after treatment of *Mycoplasma* infection. Owing to these contradictory study results, it is not possible to evaluate the impact of *Mycoplasma* on the semen volume, and more studies on this issue are needed.

Progressive sperm motility and sperm concentration

The effect of both canine and human *Mycoplasma* infection on sperm concentration and motility remains unclear. However, a study performed by Gdoura *et al*.[44] showed a negative correlation between the sperm concentration and detection of *M. genitalium* in the semen. Furthermore, semen was analyzed in a Greek study performed to investigate the influence of *Chlamydia* spp., *Ureaplasma* spp., and *Mycoplasma* spp. on sperm concentration, total motility, and progressive motility. No correlation was found between these bacteria and sperm parameter values.[47] However, it has been demonstrated

that *M. genitalium* can influence semen quality by adhering to the sperm heads, midpieces, and tails, owing to which the spermatozoa become immotile.[48] Similarly, the research by Köhn *et al.*[49] showed that spermatozoa incubated with *M. hominis* are less motile than spermatozoa from the control group. In addition, it revealed that for men who were *M. hominis*-positive group, the sperm concentration and motility were significantly lower.[50]

In veterinary medicine, studies on the impact of *Mycoplasma* on dog semen are very limited and many of them are old. In one study from 1977, the researchers tried to infect the reproductive tracts of male dogs. In this study, the *M. canis* isolates were inoculated into the ductus deferens via vasotomy in three dogs (examined group). The control was one dog who received uninoculated broth. All dogs were clinically healthy during this experiment. An increase in the scrotal temperature as well as changes in the testes and epididymides was noticed in two animals (from the examined group) on days 23 and 29. In all dogs in the study group, a significant increase in abnormal spermatozoa and a decrease in the sperm motility were reported, although *Mycoplasma canis* were detected in only one dog.[16] It may be suggested that the abnormalities in the sperm morphology occurred because of the inflammation caused by manipulations during vasotomy, and not because of the *Mycoplasma* infection. In addition, the examined group of dogs was too small to draw final conclusions. There is also a case report of a male dog which was found to be positive for *M. spumans* and *M. maculosum*, and of which seminal sperm concentration was low (1.5×10^6 ml^{-1}) and the spermatozoa were immotile. After *Mycoplasma* treatment, semen quality improved.[51] To confirm the negative effect of those two species of *Mycoplasma* on the semen quality, more research is needed.

In a study by Schäfer-Somi *et al.*[26] andrological examination was correlated with the presence of *Mycoplasma* spp. and other bacteria in the reproductive tract and semen of dogs. *M. canis* was isolated from the semen samples of 18% of dogs whose semen was collected for cryopreservation, 40% of infertile dogs, and 45% of dogs with benign prostatic hyperplasia (BPH). This study showed that these bacteria may be present even in the high-quality semen of a young dog. The authors suggested that the number of the microorganisms is not a decisive factor, but the duration of the infection, degree of epithelial damage, or local immune response may be important. In addition, it has been suggested that the concentration of the spermatozoa may be lower after germinal epithelium damage.[26] To confirm this hypothesis and estimate the real impact of *Mycoplasma* spp. on the morphology of dog spermatozoa and sperm concentration, further studies are needed.

Effect on sperm morphology

A normal human sperm tail should be without cytoplasmic residues and should have a length of approximately 45–50 μm.[52] In veterinary medicine, the assessment of sperm morphology is more difficult owing to the lack of morphometry information.[53] Only a few of the more popular breeds of dogs have been evaluated by morphometrical examination,[54] and this is not enough to define the standard values for all dogs. In male dogs, during the evaluation of the morphology of the spermatozoa, mainly the cytoplasmic residues and tail are considered.

In humans, the lower reference limit for normal forms of spermatozoa is 4%,[55] while in dogs, it should be greater than or equal to 80%.[18] In the past, the reference limit of this parameter was different for men. It was 30% in 1992 and 14% in 1999. The reference values are based on the sperm parameters of fertile men in the fifth percentile in the percentile distribution of results of pregnancy rates. The discrepancy in the lower reference limit is probably because in humans, the sperm counts fall with every decade of life.[56]

Rose *et al.*[45] investigated the influence of *Mycoplasma* spp. on the morphology of spermatozoa. After semen incubation with *Mycoplasma*, there was a significant increase in abnormal midpieces and tails compared with the control group, which suggests that *in vivo Mycoplasma* spp. can have an influence on sperm morphology. Moreover, older reviews have suggested that ejaculates contaminated by *Mycoplasma* spp. contain coiled forms as well as swollen necks of the spermatozoa.[57] An electron microscopical study showed that the spermatozoa from *Mycoplasma*-positive ejaculates had several distinctive features. *Mycoplasma* was attached to the sperm cells by interlacing fibrils of variable diameter, and was associated with spherules. Another characteristic feature was numerous sperms with coiled tails.[58] In addition, a study investigated the real influence of *Mycoplasma* on sperm morphology. In this research, *Mycoplasma* were detected by a *Mycoplasma* IST kit (BioMerieux, Marcy-l'Étoile, France), and the changes in the sperm morphology were found to be as follows: abnormalities in the head's shape, disrupted nuclear membrane, vacuoles within the nuclear chromatin, protuberances in acrosomes, cytoplasmic residues, and vacuoles inside the chromatin.[59]

Since the effect of *Mycoplasma* on sperm morphology remains unclear, and because of limited publications, new studies are needed on this issue. Owing to the similarity between *Mycoplasma* spp. and *Ureaplasma* spp., the impact on the sperm quality of these two bacteria could also be comparable. In one study on the influence of *Ureaplasma* on sperm morphology, it was shown that the *U. urealyticum*-positive group had a higher proportion of abnormal spermatozoa than the control group.[60] This indicates that both *Ureaplasma* spp. and *Mycoplasma* spp. can influence sperm morphology. However, another study showed that *U. urealyticum* had a more significant impact on sperm morphology than *Mycoplasma* and four other pathogens.[61]

IMPACT ON CELLS OTHER THAN SPERMATOZOA AND SPERM AGGLUTINATION

The ejaculate contains cells other than spermatozoa, including epithelial cells, leukocytes, and immature germ cells. All of them can be identified by examining a stained smear.[55] There is a controversial report suggesting that epithelial cells can phagocytose the spermatozoa, which possibly acts as a removal process for abnormal spermatozoa. This phenomenon was noted in men infected by *Chlamydia trachomatis* and *Mycoplasma* spp.[62]

Leukocytes in the ejaculate

The occurrence of leukocytes in the ejaculate is due to infections of the male reproductive tract. This process can be divided into three stages. The first stage occurs shortly after infection, and is not associated with a significant number of leukocytes. During the second stage, it is assumed that the leukocytes take part in the immune response, and therefore, activated leukocytes appear in the semen. During the third stage, the bacteria are eliminated by the immune system, but the leukocytes persist in the ejaculate.[63]

A study has revealed that the presence of *Mycoplasma* in the semen is not correlated with leukocytospermia in humans.[64] In dogs, there was a similar study in which the semen cytology was investigated. Only in 15 of 41 *Mycoplasma*-positive dogs did the cytology show a higher amount of leukocytes than noninflammatory samples.[65] These two studies suggest that *Mycoplasma* spp. may not be related with infections of the male reproductive tract. However, one report has claimed that leukocytes are present in the ejaculate of *Mycoplasma*-

and *Chlamydia*-positive men, and they could phagocytose abnormal spermatozoa. The researchers described a process in which, during the early stages, the sperm head adheres to the surface of the leukocyte, and in the later stages, it is surrounded by the leukocytic pseudopodia. They also found that the leukocytes contained spermatozoa.[59] This study did not comment on the amount of leukocytes in the ejaculate.

Agglutination and aggregation of spermatozoa

Aggregation is the adherence of spermatozoa to other cells or debris,[66] it has been suggested that in *Mycoplasma*-positive men, the number of cells other than spermatozoa was not increased. The phenomenon of the motile spermatozoa sticking to each other is called agglutination.[55] It can be positively correlated not only with anti-sperm antibodies but also with other causes such as genital tract infections and ascorbic acid deficiency.[67] There are two reports on the effect of *Mycoplasma* on sperm agglutination. Both of them involved humans, and did not find a relationship between the presence of anti-sperm antibodies and *Mycoplasma* spp.[68,69] This may indicate that *Mycoplasma* have no influence on sperm agglutination.

IMPACT ON THE FUNCTIONAL PROPERTIES OF SPERMATOZOA

Sperm DNA fragmentation

Any abnormalities in the sperm chromatin or damage to the DNA can cause infertility because the sperm DNA must decondense during fertilization.[70] In a study performed on 143 infertile patients with diagnosed genitourinary infection with *Chlamydia* spp. and *Mycoplasma* spp., sperm DNA fragmentation was examined by the sperm chromatin dispersion (SCD) method. The result showed that the mean percentage of spermatozoa with fragmented DNA in the infertile patient group was 3.2 times higher than that in the control fertile group. After antibiotic and anti-inflammatory treatment, the frequency of the sperm cells with fragmented DNA decreased from 37.7% to 24.2%.[71] This suggests that *Mycoplasma* spp. can influence sperm DNA fragmentation, which is associated with infertility in men. In another study in which flow cytometry was performed after staining with acridine orange (AO), the chromatin integrity, measured by the presence of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) breaks in the sperm chromatin in men with semen positive for *Ureaplasma* and *Mycoplasma* strains, was not disturbed.[72] However, in these studies, as the *Mycoplasma* spp. were not specified, it could not be determined which *Mycoplasma* spp. could affect DNA fragmentation.

Acrosome reaction

There are only two studies in this field. In the first study, the spermatozoa were incubated with *Mycoplasma* (*M. hominis* and *U. urealitycum*). The authors showed that spermatozoa from the experimental group were less likely to undergo an acrosome reaction in response to calcium ionophore treatment than the control cells.[45] The second study also showed that *M. hominis* can reduce the inducibility of human sperm acrosome reaction.[49] However, no similar studies have been performed on dogs.

SPERM VITALITY

In both veterinary and human medicine, the most common method to assess the sperm vitality is a test using eosin-nigrosin. This method is based on the principle that the damaged plasma membrane (in dead spermatozoa) allows the entry of membrane-impermeant stains.[73]

In the flow cytometry method, the most common stain used is SYBR-14 with propidine iodine (PI). SYBR-14 penetrates undamaged cell membranes to cause light green fluorescence. Damaged cell membranes allow PI penetration, which displaces SYBR-14, causing red fluorescence. This double staining shows three subpopulations of spermatozoa: live cells (SYBR-14$^+$, PI$^-$), dead cells (SYBR-14$^-$, PI$^+$), and moribund cells (SYBR-14$^+$, PI$^+$).[74,75]

Gallegos *et al.*[71] found no significant impact of these bacteria on sperm vitality. Andrade-Roha[64] also investigated the influence of *Mycoplasma* on this parameter. Sperm vitality was lowest in semen with more than 10^3 colony-forming units per ml of semen (cfu ml^{-1}), but it was not statistically significant. In another study in which the intracellular location of *M. hominis* was investigated, it was noted that this species of *Mycoplasma* does not affect sperm viability.[9]

Although the influence of canine *Mycoplasma* on sperm vitality is unknown, in a dog which was a carrier of *M. spumans* and *M. maculosum*, 100% of the spermatozoa were dead.[51]

EFFECT OF *MYCOPLASMA* SPP. ON PROSTATE FUNCTIONS

In men, the seminal vesicles are the main accessory gland of the male reproductive system,[76] while in dogs, the prostate is the only accessory sex gland.[18] In humans, acute bacterial prostatitis is not associated with infertility in contrast to chronic prostatitis. This phenomenon can be attributed to the impairment of the secretory capacity of the prostate, which might have a negative effect on all semen parameters.[76] It has been suggested that *M. genitalium* is associated with chronic prostatitis in humans, because it is detected more frequently in patients with prostatitis than in healthy ones.[77] However, Mändar *et al.*[78] reported that both *Mycoplasma* and *Ureaplasma* occurred more frequently in the semen of men with prostatitis than in healthy ones, and the most frequently occurring species was *U. parvum*. In another research, *M. hominis* was detected in 13% of men with prostate cancer, while these bacteria was not detected in any of the men with BPH.[79]

In dogs, the correlation between prostate diseases and infertility has not been proven. However, in a study performed on nine stud dogs who presented with infertility, five had prostatitis and one had BPH.[18] In a study by Schäfer-Somi *et al.*[26], *M. canis* was detected in 83.3% of the dogs that were diagnosed with BPH, although it remains unknown if these bacteria play a role in the pathogenesis of this disease.

IMPROVEMENT IN SEMEN QUALITY AFTER TREATMENT OF *MYCOPLASMA* INFECTION

Treatment of *Mycoplasma* infection is based on antibiotic therapy, but because of the lack of a cell wall, these bacteria are resistant to β-lactam antibiotics. Some species are also resistant to macrolides, sulfonamides with trimethoprim and rifampicin.[80] Doxycycline is widely used to treat infections by *Mycoplasma* spp.[81] Treatment with doxycycline (twice daily, for 7 days) in patients with *Mycoplasma* infection results in a significant improvement in all semen parameter values except for volume, pH, and nonprogressive sperm motility.[82,83] However, in another study, 3 months after antibiotic treatment, only 55.3% of men were free from microorganisms, and no significant improvement in any of the investigated semen parameters was noted.[72] It should be noted that doxycycline is a drug that stops bacterial protein synthesis; therefore, the duration of doxycycline therapy should be longer than bactericidal antibiotics. In dogs, the most common drug used for treatment is also doxycycline. Successful treatment has also been reported by the use of doxycycline for 15 days, followed by azithromycin for 9 days.[51] In this case, although the semen quality improved after therapy, a control PCR test was not performed.[51]

In case of low-grade infections with no changes in the semen quality parameters, it has been suggested that preputial irrigation

Table 4: Influence of human and canine *Mycoplasmas* on semen parameters and prostate diseases

Andrological finding	*Human Mycoplasmas*			*Canine Mycoplasmas*	
	Mycoplasma spp.	*Mycoplasma hominis*	*Mycoplasma genitalium*	*Mycoplasma spp.*	*Mycoplasma canis*
Prevalence	1.3%–4%[17]	No data	No data	89%[18]	No data
Infertility	No data	Negative influence[43]	No influence[43]	No data	Present in 17.8% high quality ejaculates and in 40.4% poor semen quality[16]
Volume	Conflicting results[44,82]	No influence[39]	Conflicting results[44,82]	The ejaculate volume is not so important as in human patients	
Concentration	No data	Conflicting results[44,82]	Conflicting results[44,82]	No data	No data
Motility	No data	Conflicting results[44,82]	Conflicting results[44,82]	No data	Temporary decreased spermatozoa motility[16]
Morphology	Negative influence[45]	No influence[63]	No influence[63]	No data	Temporary increased numbers of abnormal spermatozoa[16]
Number of leukocytes	No data	No influence[63]	No influence[63]	No data	In 15 of 41 dogs, the semen cytology showed a higher amount of leukocytes[64]
Sperm agglutination	No influence[67,68]	No data	No data	No data	No data
DNA fragmentation	Conflicting results[71,94]	No data	No data	No data	No data
Acrosomal reaction	No data	Negative influence[45,49]	No data	No data	No data
Viability	Negative influence[63]	No influence[9]	No data	No data	No data
Prostate diseases	Prostatitis[77]	Cancer, BPH[78]	Prostatitis[78]	No data	Prostatitis, BPH[26]

BPH: benign prostatic hyperplasia

with 2.5% marbofloxacin can be a form of therapy,[26] but there is no report about the effectiveness of this method. After treatment, it is recommended that stud dogs should have an 8-week break in mating in order to regenerate and improve the quality of the semen from new germ cells formed during spermatogenesis. Supplementation with vitamin E for 10 weeks has also been suggested to regenerate the epithelium of the seminal tubules.[26]

CONCLUSIONS

Mycoplasma spp. occur on mucosal surfaces in both humans and dogs. Previous studies have described their effect on pelvic diseases in women,[84,85] reproductive tract of female canines,[86] respiratory tract in dogs,[36] and fertility in men.[69,49] In these studies, bacteria were detected in both healthy and diseased study participants; consequently, the impact of *Mycoplasma* remains unclear. A summary of current state of the knowledge about influence of *Mycoplasma* spp. on fertility is shown in **Table 4**.

Almost 89% of the dog population has been reported to be *Mycoplasma* positive,[18] suggesting that not all species or strains are pathogenic, or their virulence is low. Some authors have identified which bacterial species can cause infertility in dogs.[51] However, the knowledge about all strains is still limited.

Further research is required to compare the mechanisms underlying mycoplasmosis in the genital tract in both humans and dogs, especially in close phylogenetic species. It is also necessary to investigate if antibodies induced by *Mycoplasma* infection of the respiratory tract can potentially protect the genital tract during contact with pathogenic species of *Mycoplasma*. Importantly, there is a need to identify which *Mycoplasma* species and strains are pathogenic and which are not.

AUTHOR CONTRIBUTIONS

KD reviewed the literature and wrote the main body of the manuscript. IK helped with designing the manuscript, and made linguistic and stylistic corrections. PJ, SK, and MS critically reviewed and substantially contributed to the final draft of the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENTS

All authors acknowledge Dr. Ricardo Faúndez (InviMed Fertility Clinics, Warsaw, Poland) who provided critical insights into the reviewed topic during manuscript preparation. We also thank Dr. Piotr Bąska (Warsaw University of Life Sciences, Institute of Veterinary Medicine, Department of Preclinical Sciences, Division of Pharmacology and Toxicology, Warsaw, Poland) for designing primers used in **Table 2** and **3**, and **Figure 2**.

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Załącznik nr 2 – Wzór oświadczenia o współautorstwie

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Jurka Piotr

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Podpis

Veterinary World, EISSN: 2231-0916
Available at www.veterinaryworld.org/Vol.17/January-2024/26.pdf

RESEARCH ARTICLE
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Prevalence of *Chlamydophila* spp. and Canid herpesvirus-1 in Polish dogs

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Received: 18-09-2023, **Accepted:** 08-01-2024, **Published online:** 25-01-2024

doi: www.doi.org/10.14202/vetworld.2024.226-232 **How to cite this article:** Domrazek K and Jurka P (2024) Prevalence of *Chlamydophila* spp. and Canid herpesvirus-1 in Polish dogs, *Veterinary World*, 17(1): 226–232.

Abstract

Background and Aim: *Chlamydophila* spp. affect Leydig and Sertoli cells by dysregulating spermatogenesis, inducing apoptosis and sperm DNA fragmentation, as well as benign prostate hyperplasia. Canid herpes virus 1 (CHV-1) infection in male dogs is manifested by lesions on the base of the penis and foreskin. There is a lack of information on the influence of these microorganisms on the quality of canine semen. Seroprevalence of *Chlamydophila* spp. (55%–61%) and CHV-1 (22%–81%) in Europe is high. The prevalence of *Chlamydophila* spp. and CHV-1 has been evaluated using polymerase chain reaction (PCR) only in Sweden and Croatia, respectively. No positive samples were detected in either case. The aim of this study was to evaluate the epidemiological situation in Polish male dogs (PMDs) to provide a solution to limit the spread of these microorganisms using assisted reproduction techniques or elimination from the reproduction of CHV-1 carriers. In addition, we assessed the semen quality of *Chlamydophila* spp. carriers and CHV-1 carriers.

Materials and Methods: Cotton swabs were collected from prepuce or semen from each dog (n = 130). Real-time PCR for *Chlamydophila* spp. and CHV-1, as well as semen analysis, was performed using the computer-assisted semen analysis system.

Results: To the best of our knowledge, this is the first report of *Chlamydophila* spp. infection in PMD confirmed by real-time PCR. All parameters, except progressive movement in *Chlamydophila* semen carriers, were normal.

Conclusion: The average velocity values for a dog with *Chlamydia* are detailed. No CHV-1 was detected. The results achieved should be verified on the basis of a larger number of studies. However, the high prevalence of these pathogens in the PMD population has not been established.

Keywords: *Chlamydia*, canid herpes virus 1, dog, herpesvirus, semen.

Introduction

The effects of *Chlamydophila* spp. and Canid herpes virus 1 (CHV-1) on male canine fertility remain unclear. In addition, there are limited data on its prevalence in canine populations. Several studies have been published on the seroprevalence of these pathogens. Studies based on polymerase chain reaction (PCR) have been performed for *Chlamydophila* spp. only in Sweden [1] and for CHV-1 in Croatia [2]. However, similar studies have not been conducted in Central Europe.

Chlamydiaceae are Gram-negative intracellular bacteria that attack eukaryotes. The *Chlamydiaceae* family contains 11 species and is the best-known group in the *Chlamydiales* phylum [3]. *Chlamydia* spp. and *Chlamydophila* spp. have been identified in humans, mammals (mainly koala, cattle, and cats) [4, 5], and birds [6].

However, the effects of chlamydiosis on the canine reproductive system remain poorly understood. Bitches carrying *Chlamydophila psittaci* genotype C with symptoms such as recurrent keratoconjunctivitis, respiratory disorders, and a reduction in the number of puppies in litters have been reported by Sprague *et al*. [7]. It has also been suggested that these bacteria are involved in the pathogenesis of abortion in bitches [8]. Liutkeviciene *et al*. [9] suggested that *Chlamydophila* spp. may be the main cause of canine infertility.

The pathogenesis of male reproductive tract chlamydial infection has been studied in rodents [10, 11]. These bacteria affect Leydig and Sertoli cells by dysregulating spermatogenesis and inducing apoptosis and DNA fragmentation [10]. Nielsen *et al*. [12] directly inoculated *Chlamydia trachomatis* into the prostate glands. All participants in the experiment developed benign prostatic hyperplasia.

Studies on canine chlamydiosis in Swedish kennels suggest that these infections are of little importance in terms of fertility and ocular disease since *Chlamydiaceae* bacteria have not been detected in any dog showing clinical signs [1]. However, contradictory results have also been reported by Lithuanian authors. According to published data, 61% of dogs with genitourinary signs tested positive for *Chlamydophila* spp. [9]. A case of azoospermia in

a dog in which *Chlamydia* spp. was detected in the ejaculate has been described in Austria [13]. To date, reports are insufficient for clarifying the effects of *Chlamydophila* spp. on canine fertility. However, further studies are required in this field.

Previously, the diagnosis of chlamydiosis was based on cell culture, serology, and microimmunofluorescence. PCR and real-time PCR are currently the most commonly used diagnostic methods [14]. Long-term treatment of chlamydial infections (3–4 weeks) is recommended. To date, several antibiotic regimens have been developed to treat chlamydiosis, including amoxicillin with clavulanic acid (15–25 mg/kg), doxycycline (5–10 mg/kg), tetracycline (22 mg/kg), erythromycin/azithromycin (10–15 mg/kg), and enrofloxacin [15].

Herpesvirus infections of the reproductive tract have been reported in dogs, cattle, and humans. CHV-1 may infect domesticated dogs and other canines, such as foxes [16]. It was first described as a cause of puppy mortality in dogs. Its prevalence in the canine population has not been estimated, but data from the literature showing seroprevalence in individual countries suggest that the virus is not sporadic [17].

CHV-1 belongs to the *Herpesviridae* family, *Alphaherpesvirinae* subfamily, and *Varicellovirus* genus [18]. Hematopoietic cells mediate the development of viremia following CHV-1 infection [16]. Similar to other herpes virus, CHV-1 can infect the respiratory and reproductive systems and cause conjunctivitis and keratitis [19]. It can cause clinical signs of upper respiratory tract infection in young animals, whereas in older animals, respiratory infection is subclinical [18].

The effects of CHV-1 on puppies are well understood. Herpes virus infection in neonates is generally fatal. Puppies may be infected during passage through the bitch's birth canal or during contact with maternal secretions and excretions [20]. The most commonly observed signs include vocalization, anorexia, dyspnea, abdominal pain, incoordination, abnormal excrement consistency, hemorrhages, and petechiae on the mucous membrane. The mortality rate was 100% in the litter. Infections in older puppies are usually asymptomatic, although cases of neurological signs, blindness, and deafness in older puppies have been reported [21]. In addition, one case of fatal herpes virus infection in an adult dog has been reported [22]. CHV-1 infections are estimated to be the cause of death in 22.8% of infant dogs [23].

Reproductive problems caused by herpes virus infections are well understood in bitches; however, the effect of CHV-1 on male dogs is not well understood. Primary genital infections in adult bitches can manifest as vesicular lesions and congestion of the reproductive tract mucous membranes. Similar lesions at the bases of the penis and foreskin have been observed in dogs [21]. The immunization of bitches intended for breeding is recommended due to the high risk of abortion.

The effect of CHV-1 infection on the quality of canine semen has not yet been clarified; however, the reduced fertility caused by herpes virus infection in other species suggests that a similar phenomenon may occur in dogs. Bovine herpesvirus 1 (BHV1) also belongs to α-herpesviruses and is responsible for rhinotracheitis, vesicular vulvovaginal lesions, vesicular penile and preputial inflammation, and abortion and infertility [20]. BHV1 infection also negatively affects the quality of semen by decreasing the motility, viability, concentration, and percentage of morphological defects in sperm [24].

CHV-1 infection is diagnosed based on immunological (immunofluorescence, hemagglutination inhibition, and enzyme-linked immune sorbent assay) and molecular (PCR) methods [25]. To date, no method has been developed to treat herpesvirus infection. Therefore, the prevention of infection is essential. Inactivated vaccines are currently available in Europe. The vaccine should be administered to bitches during the initial stages of pregnancy and again at 6 weeks of gestation [21]. Passive immunity comes from the mother. Puppies nursed from CHV-1-seronegative bitches usually develop fatal diseases, whereas puppies from CHV-1-seropositive mothers remain asymptomatic but can still be infected [20].

The aim of this study was to estimate the prevalence of *Chlamydia* spp. and CHV-1 infection in Polish male dogs (PMD). However, there is a lack of data on the prevalence of these pathogens in Poland or their impact on the fertility of male dogs. In addition, it is necessary to clarify many discrepancies between prevalence and seroprevalence. The results of the present study could enable the future development of a protocol for use in the reproduction of *Chlamydia* spp. and CHV-1-positive dogs. This aspect appears to be of particular importance since chlamydiosis is a zoonosis. Herpesvirus is an incurable disease, and knowledge of its prevalence in dogs will help reduce its spread in the environment. The second aim was to determine whether dogs can be the primary reservoir of CHV-1, which infects bitches during mating, leading to abortion or stillbirth of puppies. The third aim was to evaluate the usefulness of PCR as a diagnostic tool for sexually transmitted diseases.

Materials and Methods

Ethical approval and Informed consent

The study was performed in line with the general recommendations [European Code of Good Veterinary Practice (FVE)]. Some recommendations (reduction of stress) have been adopted from the regulations (Act 15 January). All examination procedures were performed as part of the health examination at the owners' request. According to the European Directive EU/2010/63 and Polish regulations, ethics committee approval was not required for the described procedures, which qualified as non-experimental clinical veterinary practices and were excluded from the

Directive (Act of January 15, 2015, on the protection of animals used for scientific or educational purposes). All dog owners have been informed that the data obtained in this study could be used for publication.

Study period and location

The study was conducted from March 2021 to February 2023 at Institute of Veterinary Medicine, Warsaw University of Life Sciences, Poland.

Dogs

We enrolled 130 privately owned, intact PMDs of 61 different breeds and six mixed-breed dogs of reproductive age (1–8 years). The most common breeds were golden retriever (4), border collie (4), Bernese Mountain Retriever (3), and Nova Scotia Duck Tolling Retriever (3). Fifty-five (42.3%) of the examined dogs had previously reproduced. Body weight ranged from 1.5 kg to 80 kg, with a median (interquartile range) of 24 kg (range, 13–32 kg).

The aim of the first stage of the study was to select non-castrated dogs. Data regarding breeding history and reproductive disorders, medical history, and any medication or supplements administered over at least the previous 6 months [26] were collected for each dog. The dogs examined were not vaccinated against CHV-1 or *Chlamydophila* spp. Each dog underwent a routine clinical examination including heart and respiratory rate, rectal temperature, and lymph node palpation. Additional tests (blood count, blood biochemistry, echocardiography, or X-ray) were ordered in dogs with a questionable clinical status. These tests aimed to exclude diseases affecting semen test results (n = 12). The potential inclusion of these data does not alter the merits of this publication. Only patients with PMD without systemic diseases were included. The level of libido was evaluated using scales L1–L4, and L3 values were considered normal. Samples were obtained using cotton swabs without any transport or culture medium. Samples from the prepuce (n = 78) were obtained by rolling swabs against the mucosa or dipping swabs in the Eppendorf with semen samples (n = 52).

Real-time PCR

Immediately after collection, the samples were stored in a refrigerator. Cotton swabs were sent to a commercial veterinary laboratory (VETLAB, Poland) on the same day to diagnose CHV-1 and *Chlamydia* spp. infection using real-time PCR.

Isolation of genetic material

Genetic material was isolated from cell-rich swabs using an IndiMag 48s robot (Indical, Leipzig, Germany) and dedicated DNA/RNA isolation kits (IndiMag Pathogen Kit, Indical catalog number SP947654P224), according to the manufacturer's recommendations. The swabs were transferred to sterile Eppendorf tubes containing 400 μL of sterile 0.9% NaCl and shaken at 64× *g* for 15 min. Subsequently, 200 μL of the resulting lysate was collected and transferred to a plate using a DNA/RNA isolation kit (with the addition of proteinase K). Subsequently, 500 μL of the lysis buffer included in the kit was added.

Real-time PCR

Real-time PCR for *Chlamydia* spp. and CHV-1 was performed using commercial *Chlamydia*/*Chlamydophila* (Amplicon) and CHV (Amplicon) PCR kits according to the manufacturer's instructions. For this reaction, 5 μL of isolated DNA template was obtained. The final volume of the reaction mixture was 20 μL for each reaction.

Real-time PCR conditions for *Chlamydia*/*Chlamydophila* and CHV were as follows: (1) 95°C, 5 min; (2) 95°C, 10 s; (3) 58°C, 25 s; and (4) 40°C, 30 s. Stages 2–4 were repeated in 45 cycles.

A duplicated fragment of the *Chlamydia* spp. or CHV genome was used as the positive control. With the exception of *Chlamydia*, we dealt with a putative "*Chlamydia* spp.," which is not the exact species for positive controls. A negative control was performed on DNA-free water. The primers used in the reactions were proprietary to Amplicon sp. z o.o. (Poland) and are listed in Table-1.

Semen analysis

Semen was collected by manually stimulating the bulb of the penis. The level of libido was analyzed during masturbation. Three fractions were collected. Second and third fractions were evaluated. Macroscopic examination was performed at the first stage of semen evaluation. We measured and recorded the volumes of the sperm-rich fraction. The semen color and density were evaluated by the same observer. Sperm morphology was assessed by second fraction smears, followed by drying, immersion for 5 min in Sperm Stain (Microoptics, Spain), and air drying. We evaluated the samples under a light microscope (ECLIPSE E 200, NIKON, Tokyo, Japan) at 100-fold magnification. Vitality was evaluated using eosin-nigrosine staining [27]. Computer-assisted analysis was performed using a Sperm Class Analyzer (SCA version 6.5.0.67, Microoptic) combined with a Nikon Eclipse E 200 microscope (Nikon, Tokyo, Japan) and camera. The thermostable analyzer table was heated to 37°C [28]. The spermatozoa-rich fluid was diluted in phosphate-buffered saline (PBS, Sigma-Aldrich) and incubated for 5 min at 37°C before evaluation. Analyses were performed using a 20-micron

Table-1: Sequences of primers used to perform PCR reactions.

Name	Primer sequence
Chfelis-F	CTTGCCTGTAGGGAATCC
Chfelis-R	TCTCCGTAGAATCCTGCA
CHV-F	GATTGATAGAAGAGGTATGTG
CHV-R	CCAGAATGATAAAATCCAGAA

PCR=Polymerase chain reaction, CHV-F=Canid herpes virus F, CHV-R=Canid herpes virus R

GoldCyto 4 chamber slide (Goldcyto Biotechcorp, China). Curvilinear Velocity Rapid, 165 μm/s; Lin Rapid, 55%; and average head area, 20 μm^2 were used by the manufacturer. A minimum of 500 sperm were counted and assessed for each analysis. Sperm concentration, sperm motility, round cell concentration, and mucus penetration were determined. In addition, the sperm was divided into the following subpopulations: Fast (Rapid), moderate (Medium), slow (Slow), and no movement.

Statistical analysis

We calculated 95% confidence intervals (CI 95%) for the proportions using the Wilson score method [29].

Results

All of the examined dogs were in good body condition with normal mentation. The dogs were not dehydrated. The rectal temperature ranged from 37.5% to 38.9% and the heart rate ranged from 80 to 100 beats/min. The respiratory rate ranged between 15 and 30 beats/min, pink and shiny mucous membranes. No abnormalities were detected in the auscultation of the heart and airways in the dogs examined. Abdominal palpation did not reveal any clinically significant symptoms. Redness in the mucus membrane of the penis or prepuce was observed in 18 dogs. Discharge in the prepuce was observed in all 14 dogs. Palpable testicles in the scrotum were detected in all dogs. No abnormalities were noted during abdominal palpation. Semen collection was performed with (45/130, 34.6%) or without females (85/130, 65.4%). The level of libido during masturbation was as follows: L1, 10 dogs; L2, 22 dogs; L3, 83 dogs; and L4, 15 dogs.

The volume of semen varies between 0.3 mL and 3 mL. The color ranged from "watered down milk" to beige. Color was correlated with ejaculate density. Clinically healthy dogs with normospermia (n = 124) or azoospermia (n = 6) were categorized.

Chlamydia was detected in 1/130 dogs with a prevalence of 0.8% (95% CI: 0.1%, 4.2%).

CHV was not detected in any of the 130 dogs; therefore, the prevalence was 0% (95% CI: 0%, 2.9%).

Chlamydia positive dog

A 13-month-old Golden Retriever dog was brought to the Small Animal Clinic, Warsaw University of Life Sciences, Warsaw University of Life Sciences, Warsaw University of Life Sciences, Warsaw University of Life Sciences. Clinical examination did not reveal any pathology. Preliminary *Chlamydophila* spp. and CHV-1 swabs were collected and sent to the laboratory for analysis. The dog showed *Chlamydophila* spp.-positive results. Because the dog has not yet been used for reproduction, he cannot be sexually infected. There were no birds or dogs in the house. The most likely source of infection was wild birds used for hunting, or it could have been infected by other dogs in the kennel where it was born.

The semen analysis results are shown in Tables-2 [26, 30, 31] and 3. To the best of our knowledge, this is the first report of the analysis of semen from a *Chlamydophill*a spp.-positive dog using a reference method such as the computer-assisted semen analysis (CASA) system. Due to the lack of data in this area, basic semen analysis (sperm count, motility, viability, and morphology) results were compared with semen standards. The following values were considered normal: motility >70%, 200–300 million sperms per ejaculation, and morphologically normal sperm >70% [26]. All semen parameters, except progressive movement, were considered normal. Parameters such as round cells, circular tracks, mucus penetration, or the division of progressive movement into general, progressive, and moderate progressive movements, are not routinely tested in dogs. Due to this fact, a comparison with the standard could not be performed. Further research in this field is required. The trajectories of spermatozoa are shown in Figure-1.

It is unknown which species we are dealing with, some of which are zoonotic [32]. Therefore, antibiotic therapy was applied. We prescribed 10 mg/kg of doxycycline daily for 4 weeks. After treatment, the owner refused to re-examine the animal.

Discussion

To the best of our knowledge, this is the first study to estimate the prevalence of *Chlamydophila* spp. in PMD. There is only one similar publication on the prevalence of *Chlamydophila* detected using PCR [1]. However, many studies of these pathogens in dogs have been based on serology [9]. The seroprevalence of this bacterium was estimated at 17.6% in China [33], 20% in Germany [34], and 5.5% in Slovakia [35]. This wide heterogeneity of seroprevalence makes it interesting for owners and breeders to travel all over the world with their dogs. Some authors have also suggested that the elevated titers of these bacteria may be caused by subclinical infections [36]. *Chlamydophila* spp. may inhabit parts of the body other than the reproductive system in these dogs.

*Chlamydophil*a spp. are commonly found in reptiles [4], bovines [5], birds [6] and humans [36]. Infection with *Chlamydophila* spp. in animals and humans occurs through direct contact with infected animals. Feces, urine, respiratory secretions, birth fluids, and placentas of infected animals may be sources of contamination [33]. Therefore, there is a high risk that the described case of a dog with *Chlamydophila* may have been infected while hunting birds. It should be noted that contact between dogs and other *Chlamydophila*-positive animals does not necessarily lead to infection, but may increase antibody titers against *Chlamydophila* spp.

To date, only one real-time PCR result for the prevalence of *Chlamydophila* in the canine reproductive tract has been published. Holst *et al*. [1] have estimated the presence of this bacteria in Swedish

Table-2: Semen parameters of *Chlamydia*-positive dog.

Semen parameter	Obtained value (%)	Reference value
Semen volume	1.9 mL	1–30 mL [30]
Concentration	145.25 million/mL	100–300 mln/mL [31]
Total concentration	275.96 million	>200–300 mln [26]
Motility	97.80	>90% [31]
Immotile	2.20	<30% [26]
General progressive motility	42.91	>70% [26]
Progressive motility	29.14	Standards are not described.
Moderate progressive motility	13.77	Standards are not described.
Non-progressive motility	54.89	Standards are not described.
Fast motility	31.94	Standards are not described.
Moderate motility	15.37	Standards are not described.
Slow motility	50.50	Standards are not described.
Round cells	0.67 million/mL	Standards are not described.
Circular tracks	56.29	Standards are not described.
Mucus penetration	43.06	Standards are not described.

Table-3: Average sperm velocity values for different subpopulations of *Chlamydia*-positive dog's spermatozoa.

Average velocity value	Motile	Non-progressive motility	Moderate progressive motility	Progressive motility	Units
Curvilinear Velocity	66.93	34.04	91.47	117.28	µm/s
Average path velocity	35.67	15.13	66.17	84.26	µm/s
Linear Velocity	42.92	7.73	55	79.16	µm/s
Straightness Coefficient	38.26	35.34	84.15	93.37	%
Linearity Coefficient	59.5	16.84	62.12	67.33	%
Wobble Coefficient	52.91	37.95	72.77	71.69	%

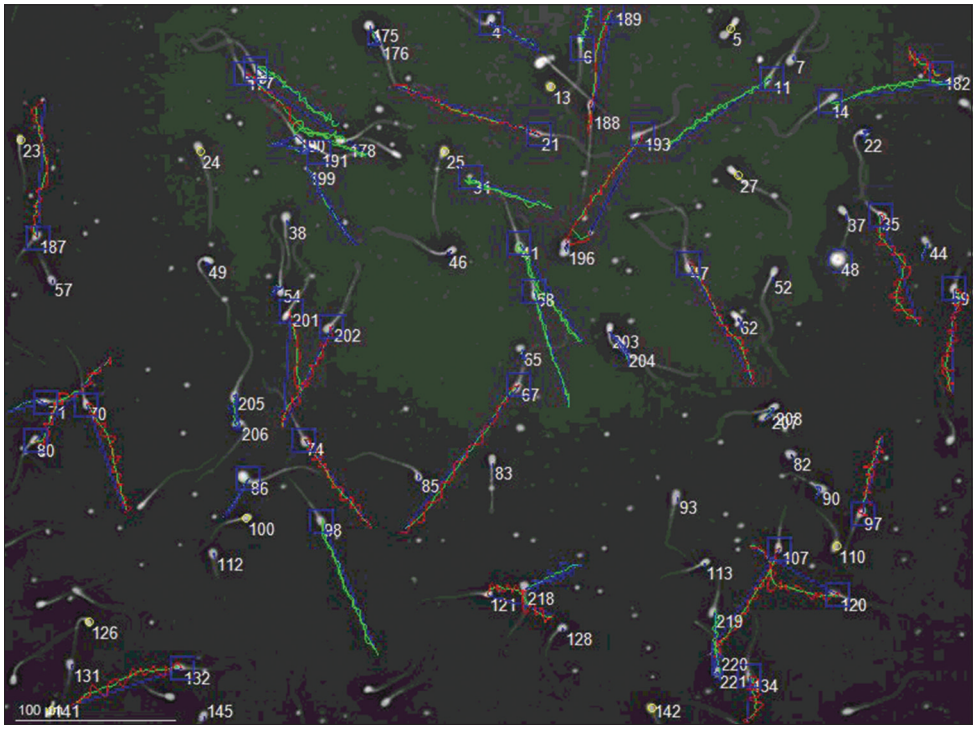

Figure-1: Spermatozoa of a *Chlamydia* spp.-positive dog with marked trajectories.

dogs. *Chlamydiaceae* were not detected in any of the 79 dogs. One *Chlamydophilla* spp.-positive sample was obtained in our study from 130 samples. In our opinion, real-time PCR is more accurate than serology because the results indicate the presence of the pathogen at the site under investigation [2]. A small amount of material is a weakness of the PCR test because the result may be false negative. In addition, in the case of a recent infection, the presence of dead pathogens which have not yet been eliminated may give false positive results. *Chlamydophila* may occur not only in the reproductive system. It has also been described in patients with coronary arteriosclerotic lesions, arthritis, pleural effusion, conjunctivitis, encephalic syndrome, bronchopneumonia, rhinitis, urethritis, and enteritis [33]. Therefore, obtaining a result with a high

level of antibodies is not useful in the diagnosis of these sexually transmitted diseases because serology tests are not specific for the diagnosis of infections in the reproductive tract.

The prevalence of CHV-1 in the canine population has not yet been estimated, but data from the literature showing the seroprevalence of the virus in individual countries suggest that it occurs widely. The seroprevalence of herpesvirus in dogs with a reproduction history was 50.3% in Italy [17], 22% in South Africa [37], 39%–62.1% in Turkey [38], and 81.5% in Finland [39]. Gracin *et al*. [40] compared the seroprevalence and prevalence in dogs with Croatian infections, 32.02% of which showed positive results in the serological diagnostic methods, while all swabs tested negative for PCR.

To the best of our knowledge, Rezaei *et al*. [20] conducted the first molecular study of canine herpesvirus 1 in reproductive specimens of adult dogs in southeast Iran. In addition, Gracin *et al*.'s [40] study is based on quantitative PCR from reproductive tract swabs. Larsen *et al*. [23] performed PCR but used dead puppy tissues rather than cotton swabs. The prevalence in dead puppies was high (22.8%). There are several possible reasons why the prevalence is low in adult dogs. First, as suggested by some authors, the possibility of virus detection is very low due to brief viral excretion [40]. The virus establishes latency following viremia. PCR performed during the latent stage may result in false negative results. However, PCR has been demonstrated to be a reliable diagnostic tool even in the latent state of the virus [17]. In addition, the immune system may react quickly after contact with the virus so that infection does not occur and the antibody titers increase. In addition, CHV-1 is poorly immunogenic. Antibody concentrations may remain high for only 2 months [17]. This phenomenon suggests a high risk of false positive results. From a practical point of view, it is important to perform serological tests to decide on preventive measures, such as vaccination of pregnant bitches. Third, none of the cited studies were 3 years, and the prevalence of these viruses in the environment is dynamic. Since breeders attach great importance to bioassurance, prevention, and vaccination, the spread of the virus among breeding dogs may decrease.

Limitation

A limitation of our study was the fact that each dog was tested only once. Therefore, the shedding phase of the virus may not have occurred in the case of CHV-1 carriers. It is well known that CHV-1 remains in the latency phase most of the time. Reactivation and shedding of CHV-1 occurs during a decline in immunity caused, for example, by stress. In future studies, we plan to test the same individual several times to capture the shedding phase of the microorganism. However, further studies are required in this field.

Conclusion

We confirmed low prevalence of CHV-1 and *Chlamydophila* spp. in the PMD population. To the best of our knowledge, this publication is the first report of semen evaluation in the CASA system of *Chlamydophila* spp. carriers. Trajectory of sperm motility in carriers of *Chlamydophila* spp. was also described for the first time. These results should be verified on a larger number of dogs.

Authors' Contributions

KD and PJ: Conceptualization. KD and PJ Methodology. KD: Data analysis and writing-original draft preparation. PJ: Writing-review and editing and supervision. All the authors have read, reviewed, and approved the final manuscript.

Acknowledgment

No external funding was received for this study. All authors acknowledge Janusz Piechota (Amplicon sp. z o. o.) who shared the primer sequences with us. We would also like to express our gratitude to Filip Tobolewski for his invaluable linguistic corrections to the manuscript.

Competing Interests

The authors declare that they have no competing interests.

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Załącznik nr 2 – Wzór oświadczenia o współautorstwie

Warszawa, 17.06.2024

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Szkoły Głównej Gospodarstwa Wiejskiego w Warszawie

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Domrazek K, Jurka P. Prevalence of *Chlamydophila* spp. and Canid herpesvirus-1 in Polish dogs. Veterinary World. 2024; 17(1): 226-232 mój indywidualny udział w jej powstaniu polegał na opracowaniu koncepcji badań, pozyskiwaniu materiału do badań, pisaniu tekstu publikacji oraz pełnieniu funkcji autora korespondencyjnego. Swój wkład w powstanie publikacji oceniam na 80%.

.....Domrazek.....

Podpis

Załącznik nr 2 – Wzór oświadczenia o współautorstwie

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.....Jurka Piotr.....

Podpis

Theriogenology 219 (2024) 86–93

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

The lack of the influence of various species of *Mycoplasma* spp. on canine semen quality

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ARTICLE INFO

Keywords:
Mycoplasma
Dog
Semen
CASA-System
PCR

ABSTRACT

Mycoplasmas colonize fish, reptiles, birds and mammals, being commensals or causing diseases, sometimes severe in ruminants, swine, poultry, or wildlife animals. So far, 15 species of canine *Mycoplasma* spp. have been described. Conflicting results have been presented regarding the pathogenicity of *Mycoplasma* spp. Although many virulence factors of these bacteria have been described, they still require attention. The main aim of our study was to evaluate the presence of known canine *Mycoplasmas* in the male reproductive tract of clinically healthy dogs. The second aim was to check if *Mycoplasma* spp. cause any abnormalities in semen quality that could have further consequences and to propose the schemes for managing the carriers.

83.3% of examined dogs were *Mycoplasma* spp. -positive dogs, and most of them were the carriers of more than one species. Six dogs had azoospermic ejaculates. The total spermatozoa numbers were similar in *Mycoplasma* -positive and negative groups. Motility was slightly higher in *Mycoplasma* spp.-negative group, but the difference was not statistically significant. There was no significant difference in semen characteristics between the carriers and *Mycoplasma* spp.-negative dogs. Neither the individual species nor the number of species strains had a significant effect on sperm morphological parameters as well as viability.

Semen quality parameters are not correlated with the species found on the prepuce. Over 70% *Mycoplasma* spp.- positive dogs have more than one species of this bacteria. Despite finding mycoplasmas in azoospermic dogs, we suggest that they were not the cause of infertility. *Mycoplasma* spp. could be a part of normal microbiota in canine prepuce in individuals without any clinical signs.

1. Introduction

The first original report regarding *Mycoplasma* spp., was published in 1898 [1]. Mycoplasmas are the smallest and simplest self-replicating bacteria organisms [2,3]. During the evolution, they have lost the cell wall, so that their cells are built of the cell membrane, ribosomes and circular double-stranded DNA [4]. Mycoplasmas are pleomorphic, 300–800 nm in diameter [5]. Mycoplasmas are usually nonmotile, but gliding motility has been noted in some species. The optimum for their growth is 37 °C. The colonies are usually less than 1 mm in diameter, with a fried-egg appearance. The genome size ranges from 580 kbp to about 1350 kbp [5].

Taxonomically, the *Mycoplasma* spp. pose the separate class named *Mollicutes* [4] and are divided into the following groups: *anaeroplasma*, *asteroleplasma*, *hominis*, *pneumoniae,* and *spiroplasma* [5]

Mycoplasmas colonize fish, reptiles, birds and mammals, being commensals or causing diseases, sometimes severe in ruminants, swine, poultry, or wildlife animals [6]. So far, 15 species of canine *Mycoplasma* (M.) have been described: *M. arginini, M. bovigenitalium, M. canis, M. cynos, M. edwardii, M. feliminutum, M. felis, M. gateae, M. haemocanis, M. maculosum, M.molare, M. opalescens, Mycoplasma sp. HRC689, Mycoplasma sp. VJC358, M. spumans* [7].

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https://doi.org/10.1016/j.theriogenology.2024.02.018
Received 27 November 2023; Received in revised form 19 February 2024; Accepted 19 February 2024
Available online 22 February 2024

Conflicting results have been presented regarding the pathogenicity of *Mycoplasma* species (spp.). It is known that they are a part of the physiological microbiota in canine upper respiratory tract [8]. Some authors indicate that *Mycoplasma* spp. also colonize lungs causing pneumonia[9]; however, their studies reported that it can be detected in the lungs of healthy dogs [10]. On the other hand, *M. haemocanis* can cause anemia [11], and *Mycoplasma* sp. *HRC689* can be the cause of colitis[12]. It has been estimated that 30–89% of dogs can have *Mycoplasma* spp. in the reproductive tract [13,14], but their role in canine infertility is largely unknown. Some authors suggested that *Mycoplasma* spp. could be related to decreased canine fertility [15], but the cases of fertile dogs with mycoplasmas in their reproductive tract have also been described [14,16]. Additionally, some authors have described this bacteria as an etiological agent of orchitis, epididymitis and prostatitis [13]. It has also been isolated from dogs with benign prostate hyperplasia [17] and haematuria [18].

Although many virulence factors of *Mycoplasma* spp. have been described, they still require attention. It is known that some species possess multiple types of virulence factors: adhesins and accessory proteins, extracellular polysaccharide structures, and pro-inflammatory or pro-apoptotic membrane lipoproteins; they can also excrete potentially toxic by-products of intermediary metabolism (hydrogen peroxide, superoxide radicals, or ammonia). In addition, virulence mechanisms, such as motility, biofilm formation, or facultative intracellular invasion have also been shown in some species of *Mycoplasma* spp. They can also suppress or inappropriately stimulate host immune cells via receptors and affect cytokine production [19].

Mycoplasma spp. infection can be diagnosed by: microbial culture, serology and polymerase chain reaction (PCR) [20]. PCR is a highly useful method of detecting *Mycoplasma* spp. and is routinely performed in commercial and non-commercial laboratories [21]. Nowadays, it allows precise identification of *Mycoplasma* species [22]. The main limitation of this method is detecting both live and dead pathogens.

Specifying canine mycoplasmas may be difficult; thus, most studies have focused on the presence or absence of *Mycoplasma* spp. in a clinical sample collected from dogs. In addition, there are few studies conducted on small groups of animals with regard to mycoplasmas in the male reproductive tract, so the pathogenicity of the bacteria is not well understood.

The main aim of our study was to evaluate the presence of known canine *Mycoplasmas* in male reproductive tract in clinically healthy dogs. The second aim was to check if *Mycoplasma* spp. cause any abnormalities in semen quality that could have further consequences and to propose the schemes for managing the carriers. Our aim was also to assess the prevalence of this bacteria in the foreskin of dogs living in central Poland.

2. Materials and methods

2.1. Animals

Seventy-eight male dogs were included in this study. The dogs were housed in the kennels affiliated with the Polish Kennel Club, as well as in the shelters for homeless animals. Only male dogs in reproductive age (from 12 moths to 8 years) were selected for sample collection. All examination procedures were performed as a part of health examination, upon the owners' request. According to the European directive EU/2010/63 and Polish regulations, there was no need for the approval of Ethical Committee for the described procedures, qualified as non-experimental clinical veterinary practices, excluded from the directive (Act of January 15, 2015 on the protection of animals used for scientific or educational purposes).

2.2. Study design

The first stage of study aimed to select clinically healthy, non-castrated male dogs. Data regarding breeding history, medical history, and any medication or supplements administered over at least previous 6 months were collected from each dog. Subsequently, each dog underwent routine clinical examination. Only dogs without systemic diseases were included in the study. From each dog classified as healthy, blood samples were collected to measure the concentration of hormones: testosterone, oestradiol and thyroxin. Blood samples were taken from the cephalic vein into a 2 ml plain tubes. This step aimed to rule out possible effects of abnormal hormone concentrations on semen quality. Finally, 78 male dogs with hormone levels within reference ranges were selected. Semen was collected by the masturbation, and the sperm-rich fraction of the ejaculate was analysed immediately after collection. The obtained semen analysis results were compared with semen standards. The following values were considered normal: motility over 70%, 200–300 million sperm per ejaculate, and morphologically normal sperms over 70% [23].

From each dog, 3 cotton swabs from the prepuce were taken. One swab was sent to the commercial veterinary laboratory (VETLAB, Poland) for diagnostics of *Mycoplasma* spp., canine herpesvirus type 1 (CHV-1) and *Chlamydia* spp. infection using PCR. In the commercial veterinary laboratory the genetic material was isolated from cell-rich swabs by an automated method using an IndiMag 48s robot (Indical) and dedicated DNA/RNA isolation kits (IndiMag Pathogen Kit, Indical), according to the manufacturer's recommendations. Then PCR reactions were performed. The positive control was a duplicated fragment of the *Mycoplasma* spp. genome. In dogs positive for *Mycoplasma* spp., an exact species of this bacteria was identified as described in section 'PCR analyses'. Following swab collection, semen was obtained by masturbation and the sperm-rich fraction of the ejaculate was immediately examined (Fig. 1). Based on results of PCR for *Mycoplasma* spp., dogs were classified into *Mycoplasma*-positive or *Mycoplasma*-negative groups. The study design is presented in Fig. 1.

2.3. Hormones measurements

Blood, after clotting was centrifuged 2057×*g* for 5 min. Serum samples were tested using an enzyme immunoassay competition method with a final fluorescent detection (ELFA) kits accordance with the manufacturers' instructions for: testosterone (TES), oestradiol (E2) and thyroxin (T4) (MINI, VIDAS, bioMérieux, France). The following values were assumed to be physiological: testosterone >1 ng/ml [24], oestradiol <115 pg/ml [25] and thyroxin 15–50 nmol/l [26]. Due to manufacturer recommendations, the quality controls are performed every 2 weeks, and the equipment underwent annual technical inspections. The results obtained are reliable.

2.4. PCR analyses

Both of the remaining swabs were stored for further tests. The swabs from *Mycoplasma* spp.-positive dogs were air-dried. One swab was used for PCR reactions, and the second one was collected as a back-up sample in case the reaction did not yield results and the test had to be repeated. DNA was isolated using Swab-Extract DNA Purification Kit (Eurx, Poland) according to the manufacturer's recommendations. Recently published primers for species-specific of Mycoplasma were used for PCR reactions along with Taq PCR Master Mix (2x) (Eurx, Poland) [27]. Protocols *for M. arginine, M., canis, M. cynos, M. edwardii, M. felis, M. gateae, M. maculosum, M. molare, M. opalescens, M. spumans, Mycoplasma HRC689* for PCR reactions were designed and standardised by Chalker [22,28,28]. Conditions for *M. bovigenitalium, M. haemocanis, M. felisminutum* and *M. VJC 358* have been designed for this research. The cycle conditions are shown in Table 1.

The products were analysed by electrophoresis in 2% agarose gel, and the approximate length of amplicons were assessed with a molecular-weight size marker (100 bp DNA ladder) as a reference.

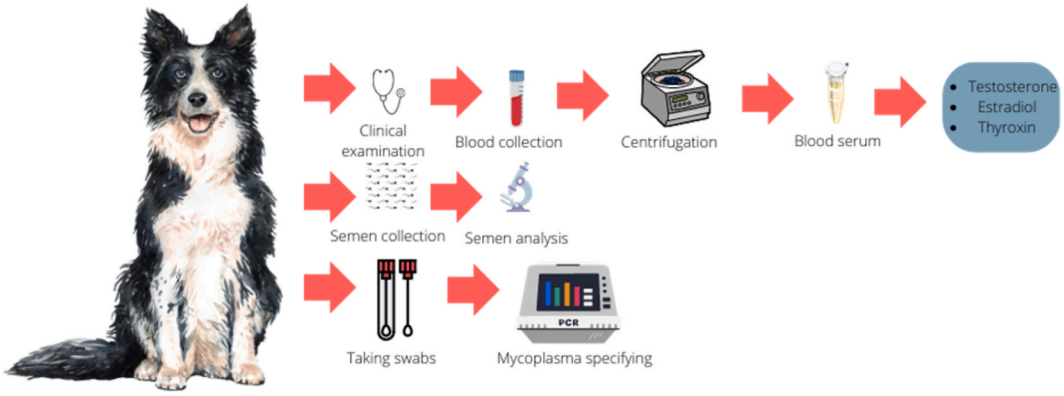

Fig. 1. Study design.

Table 1
Mycoplasma species PCR reaction conditions and products size.

Species	Cycle conditions x40	Product size (bp)
M. bovigenitalium	95 °C 5min, 95 °C 45s,60 °C 45s, 72 °C	312 [47]
M. haemocanis	1min, 72°C5min	1458 [48]
M. feliminutum	95 °C 5min, 95 °C 45s, 55 °C 30s,	150
M. VJC 358	72°C1min 72 °C 5 min	489
M. canis	95 °C 1 min, 55 °C 30 s, 72 °C 1 min	247 [29]
M. molare		397 [29]
M. arginini	95 °C 1 min, 50 °C 30 s, 72 °C 1 min	312 [29]
M. gateae		312 [29]
M. spumans		312 [29]
M. maculosum		432 [29]
M. opalescens		236 [29]
M. cynos	95 °C 45 s, 55 °C 30 s, 72 °C 20 s	227 [29]
M. edwardii		250 [29]
M. felis	95 °C 45 s, 51 °C 30 s, 72 °C 20 s	238 [29]
Mycoplasma sp. *HRC 689*		227 [29]

2.5. Semen quality evaluation

2.5.1. Macroscopic evaluation

The volume of sperm-rich fraction was measured and recorded. Then, the colour of the semen was evaluated by the same observer. Cloudy or milky opacity were considered as normal [29].

2.5.2. Morphology evaluation

A morphology of sperm was assessed by performing second fraction smears, dried and immersed for 5 min in Sperm Stain (microptic, Spain), and air dried. The samples were evaluated in a light microscope (ECLIPSE E 200, NIKON, Japan) at 100-fold magnification.

Spermatozoa were classified according to Freshman [23] as normal or possessing head abnormalities, midpiece defects, or tail abnormalities [23]. At least 200 cells were assessed each time. More than 70% of normal spermatozoa were accepted as physiological value.

2.5.3. Viability evaluation

The traditional microscopic evaluation of the percentage of alive (with preserved cell membrane continuity) and dead (with damaged cell membrane) spermatozoa was performed with the use of nigrosine-eosin stain [30,31]. On a heated basic slide the warm stain was mixed with semen (3 μl of eosin, 3 μl of nigrosine and 3 μl of semen) [32]. Then, the smear was performed and allowed to air dry. The samples were evaluated in a light microscope (Nikon ECLIPSE E 200), 100-fold magnification. At least 200 cells were assessed each time and the results were presented as a percentage of viable spermatozoa [14].

2.5.4. Computer-assisted sperm analysis (CASA)

The computer assisted analysis was performed using Sperm Class Analyzer (SCA version 6.5.0.67, microptic, Spain) combined with microscope NIKON ECLIPSE E 200 and the camera. The thermostable table of the analyser was heated to temperature of 37 °C [33]. The spermatozoa-rich fluid was diluted in phosphate buffered saline (PBS, Sigma Aldrich), incubated 5 min at 37^{O}C before evaluation. The analysis was carried out using 20 μm GoldCyto 4 chamber Slide (Goldcyto Biotechcorp, China). The manufacturer settings for dogs were used: VLC Rapid 165 μm/s, Lin Rapid 55%, average head area 20 μm^2. For each analysis, a minimum of 500 spermatozoa were counted and assessed. The sperm concentration, motility, round cells concentration and mucus penetration were determined. In addition, the spermatozoa were divided into subpopulations with fast (RAPID), moderate (MEDIUM), slow (SLOW) and no movement. As normal total sperm count more than 200×10^6 were accepted [29]. The percentage of progressively motile spermatozoa 70% or greater was accepted as normal [29]. To control the reliability of the results, each semen was microscopically assessed by a qualified member of staff. To avoid errors, all samples were examined by the same person.

2.5.5. Statistical methods

Categorical variables were presented as counts and percentages in groups and compared between groups using the likelihood ratio G test or Fisher exact test (if the any expected cell count in the contingency table was <5). The 95% confidence interval (CI 95%) for proportions was calculated using the Wilson score method [34]. Numerical variables were tested for normality of distribution by inspection of the normal probability quantile-quantile (Q-Q) plots and using the Shapiro-Wilk test. As normality of distribution was violated in most of cases the numerical variables were expressed as the median, interquartile range (IQR), and range and compared between groups using the Mann-Whitney *U* test. Their correlations were tested using the Spearman's rank correlation coefficient (R_s). Strength of correlation was classified as follows [35]: R_s = 0.00 to 0.19, very weak; 0.20 to 0.49, weak; 0.50 to 0.69, moderate; 0.70 to 0.89, strong; and 0.90 to 1.00, very strong. CI 95% for R_s was calculated using the method described by Altman et al. [34]. All statistical tests were 2-tailed and the significance level (α) was set at 0.05. Statistical analysis was performed using TIBCO Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA).

3. Results

3.1. Study population

Seventy-eight dogs met the study criteria: intact clinically healthy, aged from 1 to 8 years with the median (IQR) of 2.5 (1.5–4) years. Six dogs (7.7%) were cross-breeds, and remaining 72 dogs belonged to 47

breeds (Table 2). Body weight ranged from 1.5 to 80 kg with the median (IQR) of 24 (13–32) kg. As the animals studied were mainly dogs, taking an active part in dog shows, all were in good physical condition. Their BCS was 3/5, with the exception of the Greyhounds, which were rated 2.5/5 on the BCS scale due to their characteristic body structure. The demographic and hormonal characteristic of dogs are shown in Table 3.

3.2. PCR results

Twenty-one out of fifty nine *Mycoplasma* spp.-positive dogs (35.6%) and 4 of 13 *Mycoplasma* spp.-negative dogs (30.8%) were used in reproduction previously. Eleven different species of *Mycoplasma* spp. were detected in the collected samples. The most common were *M. cynos* (40 samples) and *Mycoplasma HRC 689* (33 samples). The prevalence of all detected species is shown in Table 4. There was no association between the presence of *Mycoplasma* spp. and azoospermia.

Only 5 out of 11 species of *Mycoplasma* spp. were detected as the single species and it was as follows: *Mycoplasma HRC 689* (in 8 dogs), *M. cynos* (in 5 dogs), *M. canis* (in 3 dogs), *M. maculosum* (in 1 dog) and *Mycoplasma VJC 358* (in 1 dog). A sample gel photography has been shown in Fig. 2.

Most of the examined dogs (72.3%) were the carriers of more than one species (Fig. 3). The most common combination was *M. cynos & HRC 689* – detected in 8 dogs. Five combinations were detected in 2 dogs: *M. molare & M. felis, M. cynos & M. canis & HRC689, M. cynos & M. canis & M. maculosum, M. cynos & M. feliminutum & HRC689* as well as *M. cynos & M. molare & HRC689*. The observed combinations of *Mycoplasma* species in examined dogs are shown in Fig. 3.

Table 2
Breeds of dogs which took part in the study.

Breed	No. of dogs	%
Crossbreed	6	7.7
Pure breed	72	92.3
Golden Retriever	4	5.6
Border Collie	4	5.6
Bernese Mountain Dog	3	4.2
Nova Scotia Duck Tolling Retriever	3	4.2
American Bully	3	4.2
Tibetan Mastiff	3	4.2
Whippet	3	4.2
American Staffordshire Terrier	3	4.2
Polish Lowland Sheepdog	2	2.8
Jack Russel Terrier	2	2.8
Eurasier	2	2.8
Shiba Inu	2	2.8
Samoyed	2	2.8
Small Münsterländer	2	2.8
Labrador Retriever	2	2.8
Boston Terrier	1	1.4
Corgi Pembroke	1	1.4
Miniature Poodle	1	1.4
Continental Bulldog	1	1.4
Boxer	1	1.4
Cairn Terrier	1	1.4
German Shepperd	1	1.4
Medium Schnauzer	1	1.4
Brussels griffon	1	1.4
Chihuahua	1	1.4
Jack Russel Terrier	1	1.4
French Shepperd Briard	1	1.4
Chow-chow	1	1.4
Pug	1	1.4
French Bulldog	1	1.4
Polish Greyhound	1	1.4
White Swiss Shepperd	1	1.4
Coker Spaniel	1	1.4
English Bulldog	1	1.4
Hungarian Pointed Dog	1	1.4
Malinois	1	1.4
Australian Shepperd	1	1.4
Boston Terrier	1	1.4
Medium Poodle	1	1.4
Great Dane	1	1.4
Doberman	1	1.4
Miniature Bullterrier	1	1.4
Scottish Collie Sheepdog	1	1.4
Shih Tzu	1	1.4
Spanish Water Dog	1	1.4
Pettit Basset Griffon Vendeen	1	1.4
Flat Coated Retriever	1	1.4

Table 3
Demographic and hormonal characteristics of *Mycoplasma* spp.-positive and negative dogs.

Demographic characteristic	*Mycoplasma* spp.-positive (n = 65)[a]	*Mycoplasma* spp.-negative (n = 13)[a]	p-value
Age [years]	2.5, 1.5–4.0 (1.0–8.0)	3.0, 1.5–3.0 (1.0–6.0)	0.882
Body weight [kg]	23, 12–32 (1.5–63)	25, 17–34 (6.5–80)	0.555
Hormone			
Estradiol (E2) [pg/ml]	14.4, 9.7–22.5 (<9–92.7)	11.6, <9–22.5 (<9–64.1)	0.408
Testosterone (T) [ng/ml]	4.2, 1.9–6.5 (1.0–13.5)	2.4, 1.0–5.4 (1.0–8.2)	0.123
Thyroxin (T4) [nmol/l]	22.9, 17.0–29.0 (6.4–40.0)	25.6, 21.4–31.2 (16.3–38.9)	0.112
T/E2 ratio	0.29, 0.18–0.45 (0.03–2.0)	0.20, 0.16–0.33 (0.06–0.66)	0.305
Previous mating[b]	21/59 (35.6%)[b]	4/13 (30.8%)[b]	0.739

[a] Presented as the median, interquartile range (IQR), and range unless otherwise stated.
[b] Presented as the number and percentage of dogs in a group.

Table 4
The prevalence of species of *Mycoplasma* in canine prepuce.

Mycoplasma species	No. of dogs	Prevalence (CI 95%) [%]
M. cynos	40	61.5 (49.4–72.4)
HRC *689*	33	50.8 (38.9–62.5)
M. canis	18	27.7 (18.3–39.6)
VJC *358*	16	24.6 (15.8–36.3)
M. maculosum	14	21.5 (13.3–33.0)
M. molare	12	18.5 (10.9–29.6)
M. feliminutum	5	7.7 (3.3–16.8)
M. arginini	4	6.2 (2.4–14.8)
M. felis	4	6.2 (2.4–14.8)
M. edwardii	1	1.5 (0.3–8.2)
M. opalescens	1	1.5 (0.3–8.2)

3.3. Semen analysis

Six dogs (7.7%; CI 95%: 3.6%–15.8%) had azoospermic ejaculates. Neither the age (median 3.5 years, range 2–5 years; $p = 0.130$) nor the body weight (median 12 kg, range 9–40 kg; $p = 0.237$) of 6 azoospermic dogs differed significantly when compared with 72 dogs with spermatozoa in the semen. There was no significant association between the presence of *Mycoplasma* spp. and azoospermia ($p = 0.582$). Semen volume did not significantly differ between 6 azoospermic dogs (median 3.5 ml, range 2.0–7.5 ml) and 72 dogs with spermatozoa in the semen (median 2.0 ml, range 0.3–7.5 ml) ($p = 0.099$), and was weakly positively, correlated with the body weight ($R_s = 0.49$, CI 95%: 0.30–0.64; $p < 0.001$). The general characteristics of semen of 72 clinically healthy dogs with spermatozoa in the semen are shown in Table 5. There was no significant difference in semen characteristics between *Mycoplasma* spp.-positive and *Mycoplasma* spp.-negative dogs (Table 6).

The semen volume [ml] ranged from 0.3 to 7.5 ml in *Mycoplasma* spp.-positive and from 0.8 to 7.0 ml in *Mycoplasma* spp.-negative dogs.

There were no correlations between semen quality parameters and the number of species of *Mycoplasma* (Table S2 in supplementary file). The total spermatozoa number was similar in *Mycoplasma* spp. -positive and *Mycoplasma* spp.-negative groups and it was averagely 383.5 × 10^6

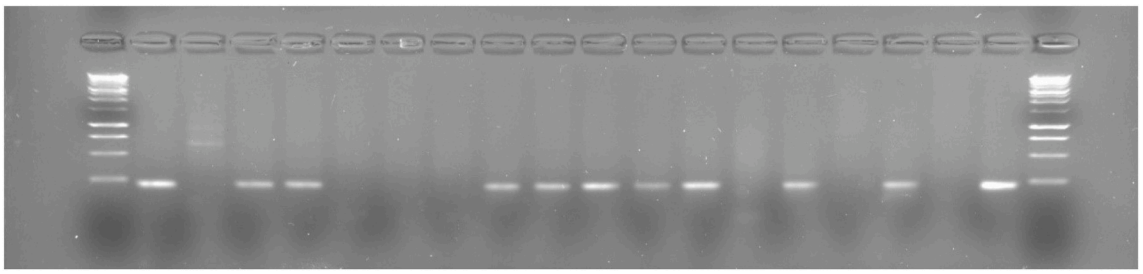

Fig. 2. The result of PCR for *M. cynos* (product size 227 kbp) for 18 samples. The samples derive from the same experiment and that gels/blots were processed in parallel. Used molecular-weight size marker (DNA ladder) was 100 kbp.

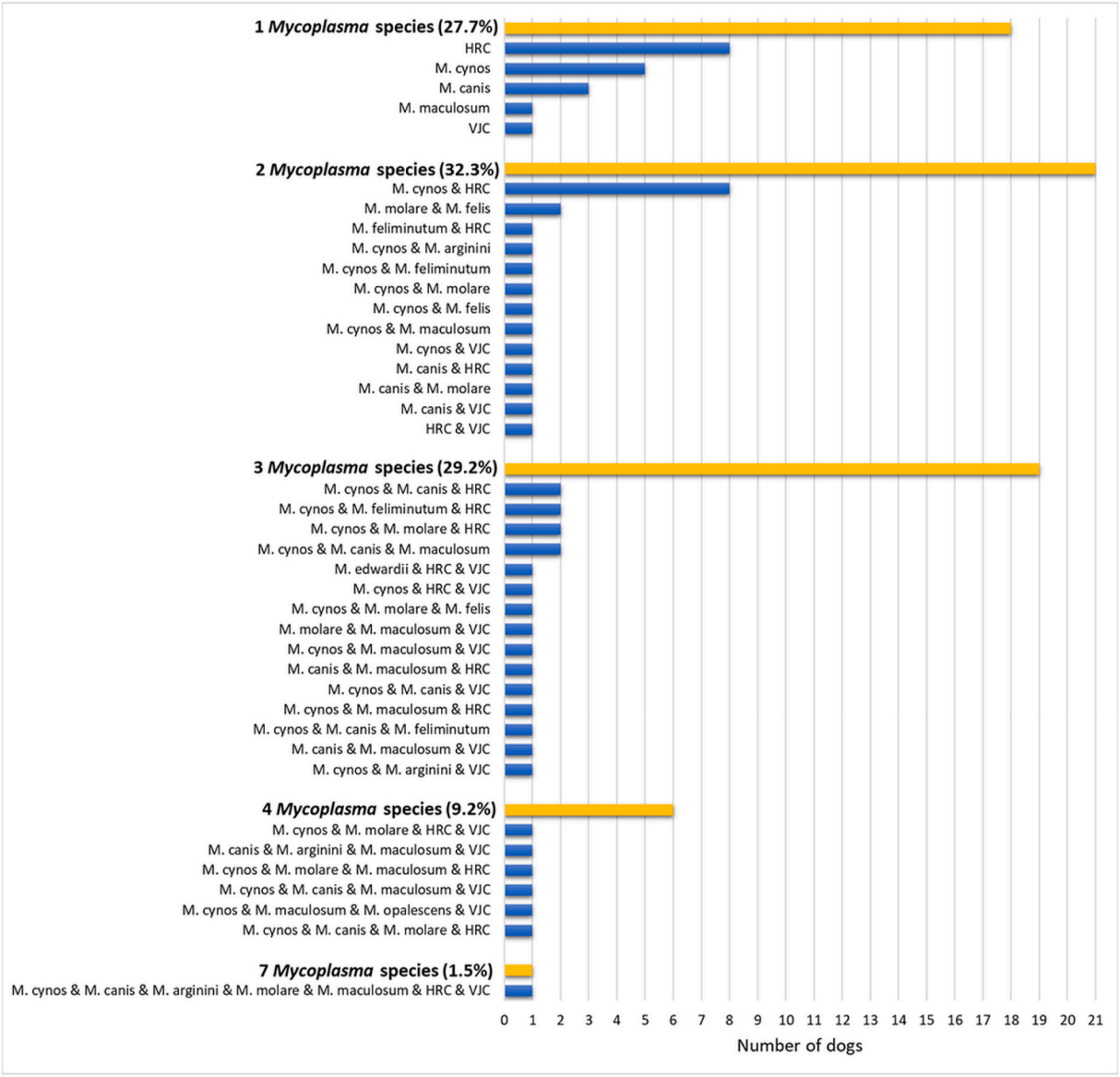

Fig. 3. Combinations of different *Mycoplasma* species present in microcolonies in the canine prepuce and its prevalence (confirmed by PCR).

and 322.8 × 10^6 respectively. In *Mycoplasma* spp.-positive dogs the percentage of normal spermatozoa ranged from 60.0% to 97.0% and in *Mycoplasma* spp.-negative it was from 51.0% to 97.5%. The total motility, progressive motility and rapid motility were slightly higher in *Mycoplasma* spp.-negative group, but the difference was not statistically significant. There was no significant difference in semen characteristics between the carriers and *Mycoplasma* spp.-negative dogs (Table 6).

Neither the individual species nor the number of species strains had a significant effect on sperm morphological parameters as well as viability (Table S2).

4. Discussion

To our knowledge, this is the first study to examine the presence of

Table 5
General characteristics of semen of 72 clinically healthy dogs with spermatozoa in the ejaculate.

Semen characteristics	Median (IQR)	Range
Semen volume [ml]	2.0 (1.5–3.9)	0.3–7.5
Sperm concentration [× 10^6/ml]	177.3 (108.7–357.4)	9.4–976.8
Total sperm number [× 10^6]	360.0 (214.6–696.3)	4.7–2197.4
Number of oval cells [× 10^6/ml]	2.0 (0.7–4.3)	0–56.0
Sperm morphology:		
Normal spermatozoa [%]	92.0 (88.8–94.8)	51.0–97.5
Head abnormalities [%]	2.0 (1.0–3.0)	0–10.0
Midpiece abnormalities [%]	2.5 (1.5–4.0)	0–23.0
Tail abnormalities [%]	2.5 (1.5–4.0)	0–28.0
Abnormal spermatozoa [%]	8.0 (5.3–11.3)	2.5–49.0
Sperm motility:		
Total motility[%]	96.5 (93.6–98.9)	65.4–99.8
Progressive motility [%]	37.1 (22.6–47.6)	3–69.3
Medium-progressive motility [%]	25.7 (15.5–33.3)	5.1–63.1
Non-progressive motility [%]	28.6 (20.0–42.8)	11–62.7
Spherical tracks [%]	37.1 (23.0–49.7)	7.5–91.2
Rapid motility [%]	57.1 (32.5–68.1)	3.8–82.6
Medium motility [%]	15.9 (9.7–24.3)	3.2–54
Slow motility [%]	21.9 (14.2–36.3)	2.5–57.4
Mucus penetration [%]	50.1 (40.4–64.0)	0–76.6
Viability [%]	90.0 (86.0–92.8)	60.0–98.0

Table 6
Influence of *Mycoplasma* spp. infection on semen characteristics.

Semen characteristic[a]	*Mycoplasma* spp.– positive (n = 65)	*Mycoplasma* spp. negative (n = 13)	p-value
Volume [ml]	2.0, 1.5–3.8 (0.3–7.5)	3.5, 1.5–5.0 (0.8–7.0)	0.149
Azoospermia[b]	6/65 (9.2%)	0/13 (0%)	0.582
72 dogs with spermatozoa in the ejaculate			
	***Mycoplasma* spp.– positive (n = 59)**	***Mycoplasma* spp. negative (n = 13)**	**p-value**
Volume [ml]	2.0, 1.5–3.6 (0.3–7.5)	3.5, 1.5–5.0 (0.8–7.0)	0.095
Concentration [× 10^6/ml]	182.5, 120.4–364.5 (9.4–976.8)	96.5, 48.7–239.9 (29.7–536.7)	0.135
Total sperm number [× 10^6]	383.5, 216.8–735.6 (4.7–2197.4)	322.8, 208.2–482.6 (153.8–839.5)	0.341
Oval cell count [× 10^6/ml]	2.4, 0.7–4.5 (0–56.0)	1.6, 0.8–3.8 (0.1–6.4)	0.534
Normal spermatozoa [%]	92.0, 88.5–94.5 (60.0–97.0)	92.0, 89.0–95.5 (51.0–97.5)	0.533
Head abnormalities [%]	2.0, 1.0–3.0 (0–10.0)	1.5, 1.0–2.0 (0–6.5)	0.601
Midpiece abnormalities [%]	2.5, 1.5–4.0 (0–23.0)	2.0, 1.5–4.0 (0.5–22.0)	0.900
Tail abnormalities [%]	2.5, 1.5–4.5 (0–28.0)	2.0, 1.0–2.5 (0–22.0)	0.109
Abnormal spermatozoa [%]	8.0, 5.5–11.5 (3.0–40.0)	8.0, 4.5–11.0 (2.5–49.0)	0.519
Total motility [%]	96.3, 93.3–98.9 (65.4–99.8)	97.0, 93.7–99.2 (87.5–99.8)	0.553
Progressive motility [%]	36.8, 22.1–46.0 (3.0–69.3)	39.5, 30.2–56.5 (19.7–69.3)	0.380
Medium-progressive motility [%]	25.5, 16.6–32.8 (5.1–63.1)	25.9, 13.8–34.9 (12.9–60.5)	0.988
Non-progressive motility [%]	30.6, 20.9–43.2 (11.0–62.7)	23.3, 17.7–34.6 (14.0–48.2)	0.341
Spherical tracks [%]	38.4, 23.5–49.9 (7.5–91.2)	35.4, 21.3–46.7 (15.1–80.4)	0.598
Rapid motility [%]	52.9, 29.3–67.9 (3.8–82.4)	60.5, 42.8–74.4 (28.8–82.6)	0.272
Medium motility [%]	16.8, 10.5–24.1 (4.1–54.0)	12.0, 7.7–26.5 (3.2–34.4)	0.341
Slow motility [%]	22.9, 14.7–37.6 (2.5–57.4)	17.4, 14.1–25.7 (9.3–46.6)	0.412
Mucus penetration [%]	49.8, 39.9–63.0 (0–76.3)	53.7, 42.9–68.4 (21.3–76.6)	0.529
Viability [%]	90.0, 86.0–93.0 (60.0–98.0)	88.0, 80.0–91.5 (76.5–94.0)	0.253

[a] Presented as the median, interquartile range (IQR), and range unless otherwise stated.
[b] Presented as the number and percentage of dogs in a group.

all 15 described canine *Mycoplasma* species in the male reproductive tract of clinically healthy dogs. Studies of *Mycoplasma* species in the reproductive system are limited and yield various results, with different methods employed. Maksimovic et al. [14] identified *Mycoplasma* spp. species in 60 bitches and detected only 3 species: *M. canis, M. spumans* and *M. edwardi*[14]. Lechner et al., detected *M. arginini, M. canis, M. cynos, M. edwardii* and *M. spumans* in canine ejaculates [36], but PCR was not utilized in this study. Currently, molecular methods are considered the gold standard in mycoplasmology. To the best of our knowledge, the occurrence of *M. molare, M. HRC 689 & VJC 358* and *M. feliminutum* has not been described in the canine reproductive tract thus far. Alves et al., evaluated 294 samples and did not found *M. cynos* [37].

Our results indicate that the occurrence of *M. canis* was not correlated with abnormalities in spermatozoa's morphology. Previous studies have shown that following *M. canis* infection, the number of normal spermatozoa decreases [15]. However, there are methodological differences between our study and the much older one. We examined the preputial microbiota of male dogs, whereas Laber & Holzmann [15] inoculated dogs with *M. canis* via vasostomy [15]. Manipulations in the reproductive tract could have caused regional inflammation, resulting in a decrease in semen quality. Additionally the group size in the study by Laber & Holzmann (1977 was much smaller (4 dogs) than ours (18 healthy dogs with *M. canis*), suggesting that this strain may not be as pathogenic as previously suggested.

The occurrence of *M. maculosum* was not associated with any disorders in our study. We did not find *M. spumans* in our samples, and this *Mycoplasma* species may be suspected as particularly important due to its association with subfertility episodes in a Bernese Mountain Dog kennel [38]. A male dog introduced to this kennel had low sperm counts, lack of motility, and high rate of abnormalities, but these largely improved after doxycycline therapy administered to both females and this male following the detection of Mycoplasmas [38]. We found 14 dogs with this strain of *Mycoplasma* spp. and we did not observe any negative influence on their semen quality and clinical appearance. Again, there is a difference in the methodology between our and Tamiozzo [38,38], which employed gene sequencing and curated product using the BioEdit software, aligning them against the database using nucleotide BLAST (http://www.ncbi.nlm.nih.gov/blast). In our opinion, our methodology based on specific primers yields more reliable results. The methodology proposed by Tamiozzo [38] could be applied in further research aiming to discover new species or subspecies of this bacteria[38].

It still remains unanswered how the local and general immune system behave in a *Mycoplasma* spp. -positive dogs. We evaluated the concentration of round cells in seminal fluid, a parameter known to increase during infection [39]. However, our results did not indicate any influence of type of *Mycoplasma* spp. on the number of round cells in the ejaculate, suggesting that bacteria in the prepuce do not induce active inflammation in the upper parts of the reproductive system.

Kustritz et al. [40] have analysed the cytology results of seminal fluid and found both inflammatory and non-inflammatory results (with a predominance of non-inflammatory) in *Mycoplasma* spp. positive semen [40]. This comparison revealed that our study deviates from the cited study, although the overall trends remained the same. This supports the importance of presented results in guiding further research. It is worth considering expanding the number of carriers of particular *Mycoplasma* species and evaluating the functions of their immune system.

There are numerous reports on the pathogenicity of specific species of *Mycoplasma* spp. Mycoplasmas of the following species were isolated from the lungs of dog with distemper: *Mycoplasma cynos, Mycoplasma bovigenitalium, Mycoplasma canis, Mycoplasma spumans,* and *Mycoplasma gateae* [8]. In addition, M. *canis* has been detected in dog with haematuria [18,41]. In our study, all species listed above were identified except for *M. bovigenitalium, M. haemocanis, M. gateae and M.sputum.* None of the detected species were related to decreased semen quality

parameters or any clinical findings in male dogs. These results may suggest that the bacteria may be pathogenic only to certain organs or systems. Another possibility could be that the development of clinical symptoms depends on the colony count of this bacteria. The effectiveness of bacterial multiplication depends on many factors, such as the pH of the environment. The best pH value for *Mycoplasma* growth is 7.8–8 [42] In canine prepuce pH amounts to 6.3–6.7[43]. For this reason, Mycoplasma spp. colonizing mucous membranes may not be numerous enough to cause clinical symptoms. Unfortunately, our study was conducted using qualitative, not quantitative methods, and there are insufficient data to confirm this thesis. Another aspect concerning the impact of pathogens on clinical symptoms may be the duration of infection. Our results are not adequate to reach conclusions in this field, as we do not know how long the dogs we studied have been carriers of *Mycoplasma* spp. Further studies are needed to draw long-term conclusions.

Our study provides a new perspective on *Mycoplasma* spp. -positive dogs. Until now, it has been common practice to administer antibiotic therapy to the dogs with *Mycoplasma* spp. positive PCR results [44]. The results of our study indicate that carriers may still have physiological parameters of semen quality and no clinical signs. Other authors have found this bacteria in dogs with orchitis, epididymitis, benign prostate hyperplasia and prostatitis [13,17]. In our opinion, this bacteria, although detected in clinical cases, may not be an etiological factor of those diseases but rather a commensal of the prepuce in dogs that become ill from other reasons.

Even the presence of many species of *Mycoplasma* on the prepuce did not negatively affect the semen quality. This raises the question of whether it is reasonable to treat a fertile and clinically healthy carrier of *Mycoplasma* spp. From the clinical point of view, long-term use of antibiotics can result in a number of adverse effects [45], suggesting that the best choice would be not to treat a healthy *Mycoplasma* spp. carrier. On the other hand, it is unclear if using the carrier to mate a *Mycoplasma* spp.-negative bitch is safe. Watts et al. [46] suggested that *Mycoplasma* spp. should not be considered as normal microbiota of canine uterus [46], so it is very likely to infect the bitch during mating [47]. More research in this field is needed.

Studies have revealed that bacteria can communicate among each other to carry out a wide range of complex social behaviors, including cooperation [48]. Such social behaviors are widespread, and it is now clear that have important consequences in shaping structure of polymicrobial communities. In bacteria, many secreted or excreted goods, like proteases can be shared by the entire group and are of importance for cooperation. Some of the cooperative activities, such as secretion of toxins, might also be important for competition with other strains or species [48]. Recent scientific advances have added much to our understanding of biofilms, polymicrobial communities, which are commonly associated with chronic infection[49]. Our study have shown that in the majority of *Mycoplasma* spp.- positive dogs, over 72% of cases, more than one species of *Mycoplasma* were detected. This result may suggest a kind of cooperation among various species of this bacteria. To our best knowledge this is first report that presented the multi-species nature of *Mycoplasma* population in canine reproductive tract. Other authors found only single species [14,17].

The main limitation of our study is the size of the groups. Some species of *Mycoplasma* did not occur in any of the dogs or were too rare to draw objective conclusions. A second weakness of our study was the examination of the occurrence of *Mycoplasmas* only on the mucosal surface of the prepuce, as is done in routine clinical practice. Semen during ejaculation has brief contact with the mucous membranes of the prepuce and penis, and thus the presence of bacteria in this area may not affect semen quality. The ideal solution would be to test along the entire length of the reproductive tract for the presence of *Mycoplasmas*. Unfortunately, this type of testing is not possible, as it would involve a biopsy of the testicles and prostate, which is likely to affect the future fertility of the sire and the possibility of using him for reproduction shortly after the test.

5. Conclusions

In conclusion, Mycoplasma spp. are common in the canine prepuce, even in dogs that have not been used for reproduction. Semen quality parameters are not related neither to the general presence of *Mycoplasma* nor to the species found on the prepuce. Over 70% *Mycoplasma* spp.- carriers have more than one species of this bacteria in the reproductive tract. Despite the finding of *Mycoplasma* spp. in prepuce of azoospermic dogs, we suggest that they were not the cause of low semen quality.

CRediT authorship contribution statement

K. Domrazek: Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Conceptualization. **P. Konieczny:** Writing – review & editing, Methodology, Investigation. **M. Majka:** Writing – review & editing, Supervision, Methodology, Conceptualization. **M. Czopowicz:** Writing – review & editing, Formal analysis, Data curation. **A. Cywińska:** Writing – review & editing, Validation, Methodology. **P. Jurka:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

We would like to extend our sincere gratitude to Filip Tobolewski for his invaluable linguistic corrections of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2024.02.018.

List of abbreviations:

M	*Mycoplasma*
spp.	species
PCR	polymerase chain reaction
ELPHA	final fluorescent detection
T	testosterone
E2	oestradiol
T4	thyroxin
CASA	Computer-Assisted Sperm Analysis
IQR	interquartile range

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Table S1. The correlations between semen characteristics and the number of *Mycoplasma* species detected in a dog

Semen characteristics	Spearman's rank correlation coefficient (R_s)	p-value
Semen volume [ml]	-0.02	0.877
Sperm concentration [$\times 10^6$/ml]	0.13	0.325
Total sperm number [$\times 10^6$]	0.07	0.618
Number of oval cells [$\times 10^6$/ml]	0.02	0.891
Normal spermatozoa [%]	0.03	0.821
Head abnormalities [%]	-0.22	0.097
Midpiece abnormalities [%]	0.11	0.408
Tail abnormalities [%]	-0.13	0.315
Abnormal spermatozoa [%]	-0.02	0.859
Total motility [%]	-0.05	0.719
Progressive motility [%]	-0.13	0.343
Medium-progressive motility [%]	0.11	0.422
Non-progressive motility [%]	0.13	0.327
Spherical tracks [%]	0.11	0.406
Rapid motility [%]	-0.09	0.490
Medium motility [%]	0.10	0.462
Slow motility [%]	0.12	0.374
Mucus penetration [%]	-0.01	0.948
Viability [%]	0.14	0.283

Table S2. The influence of infection with a particular *Mycoplasma* species on semen characteristics

Semen characteristics	Uninfected dogs (n=13)	Dogs infected with											
		M. cynos (n=37)		HRC 689 (n=32)		M. canis (n=17)		VJC 358 (n=12)		M. molare (n=12)		M. maculosum (n=11)	
	Median, IQR (range)	Median, IQR (range)	p-value	Median, IQR (range)	p-value	Median, IQR (range)	p-value	Median, IQR (range)	p-value	Median, IQR (range)	p-value	Median, IQR (range)	p-value
Semen volume [ml]	3.5, 1.5 – 5.0 (0.8 – 7.0)	2.0, 1.5 – 3.6 (0.3 – 6.0)	0.165	2.0, 1.5 – 3.7 (0.4 – 7.0)	0.191	2, 1.7 – 3.9 (0.4 – 5.0)	0.193	2.4, 0.4 – 3.7 (0.3 – 5.0)	0.113	1.8, 0.7 – 2.9 (0.3 – 5.0)	0.056	2.0, 0.4 – 4 (0.3 – 5)	0.163
Sperm concentration [$\times 10^6$/ml]	96.5, 48.7 – 239.9 (29.7 – 536.7)	186.9, 123.8 – 351.4 (18.6 – 976.8)	0.106	204.4, 119.1 – 390.2 (11 – 976.8)	0.101	303.6, 132.7 – 404.4 (25.1 – 976.8)	0.060	202.2, 83.8 – 463.9 (9.4 – 976.8)	0.314	309.7, 192.2 – 386 (34.5 – 976.8)	0.047	157.9, 62.6 – 245.4 (25.1 – 976.8)	0.862
Total sperm number [×106]	322.8, 208.2 – 482.6 (153.8 – 839.5)	390.7, 256.8 – 742 (4.7 – 1398.6)	0.164	507.9, 252.4 – 738.8 (44.0 – 2197.4)	0.189	457.9, 333.2 – 752 (98.6 – 1031.4)	0.112	368, 114.4 – 835.3 (10.3 – 1398.6)	0.724	471.1, 288.8 – 745.1 (10.3 – 1398.6)	0.165	256.8, 156.5 – 390.7 (10.3 – 1017.4)	0.451
Number of oval cells [$\times 10^6$/ml]	1.6, 0.8 – 3.8 (0.1 – 6.4)	2.8, 1.0 – 4.5 (0 – 56)	0.325	1.3, 0.5 – 4.3 (0 – 19.7)	0.754	2.5, 0.7 – 4.0 (0 – 9.2)	0.883	3.6, 0.9 – 4.4 (0.3 – 9.2)	0.314	3.1, 1.4 – 4.6 (0 – 8.7)	0.265	1.8, 0.7 – 4.4 (0 – 9.2)	0.772
Normal spermatozoa [%]	92.0, 89.0 – 95.5 (51.0 – 97.5)	91.5, 89.5 – 94.5 (60.0 – 97.0)	0.521	92.5, 89.0 – 94.5 (78.0 – 97.0)	0.564	93.0, 86.0 – 95.0 (71.5 – 97.0)	0.917	92.3, 90.3 – 95.3 (66.5 – 97.0)	0.870	92.3, 90.8 – 93.8 (88.5 – 97.0)	0.999	89.5, 85.0 – 95.0 (71.5 – 97.0)	0.385
Head abnormalities [%]	1.5, 1.0 – 2.0 (0 – 6.5)	1.5, 1.0 – 2.5 (0 – 9.0)	0.876	1.8, 1.3 – 3.0 (0 – 9.0)	0.630	1.5, 0.5 – 2.5 (0 – 8.5)	0.882	1.8, 0.8 – 3.0 (0 – 10.0)	0.848	1.0, 0.3 – 2.8 (0 – 9.0)	0.742	2.0, 0.5 – 4.5 (0 – 9.0)	0.683
Midpiece abnormalities [%]	2.0, 1.5 – 4.0 (0.5 – 22)	2.5, 1.5 – 4.5 (0 – 23)	0.999	2.3, 1.5 – 3.5 (1.0 – 14.0)	0.705	2.5, 1.5 – 3.5 (1.0 – 23.0)	0.768	3.5, 1.5 – 4.0 (1.0 – 23)	0.913	3.3, 1.5 – 4.0 (1.0 – 4.5)	0.826	3.0, 2.0 – 4.0 (1.0 – 23.0)	0.682
Tail abnormalities [%]	2.0, 1.0 – 2.5 (0 – 22)	2.5, 2 – 4.5 (0.5 – 28)	0.070	2.3, 1.5 – 4.0 (0 – 10)	0.284	2.5, 1.0 – 5.0 (1.0 – 11.5)	0.289	2.5, 1.0 – 4.0 (0.5 – 14.0)	0.426	2.5, 2.0 – 3.3 (0.5 – 7.0)	0.306	3.5, 1.0 – 5.0 (0.5 – 10.0)	0.243
Abnormal spermatozoa [%]	8.0, 4.5 – 11.0 (2.5 – 49.0)	8.5, 5.5 – 10.5 (3.0 – 40.0)	0.514	7.5, 5.5 – 11.0 (3.0 – 22.0)	0.539	7.0, 5.0 – 14.0 (3.0 – 28.5)	0.900	7.8, 4.8 – 9.8 (3.0 – 33.5)	0.849	7.8, 6.3 – 9.3 (3.0 – 11.5)	0.978	10.5, 5.0 – 15.0 (3.0 – 28.5)	0.385
Total motility [%]	97.0, 93.7 – 99.2 (87.5 – 99.8)	95.7, 90.8 – 98.3 (70.1 – 99.8)	0.347	96.9, 95.0 – 99.1 (79.4 – 99.8)	0.999	98.1, 95.0 – 98.9 (70.1 – 99.3)	0.834	94.9, 88.9 – 96.0 (65.4 – 99.8)	0.165	95.7, 93.9 – 97.2 (88.2 – 98.9)	0.463	94.8, 93.8 – 95.8 (70.1 – 99.2)	0.297
Progressive motility [%]	39.5, 30.2 – 56.5 (19.7 – 69.3)	35.7, 21.5 – 46 (3.0 – 64.2)	0.401	36.6, 24.8 – 45.6 (10.0 – 69.3)	0.573	35.7, 21.5 – 45 (10 – 61.6)	0.295	30.5, 17.4 – 35.1 (7.6 – 61.6)	0.087	44.4, 24.1 – 47.6 (9.9 – 55.3)	0.644	37.5, 31.1 – 45.2 (9.9 – 61.6)	0.643
Medium-progressive motility [%]	25.9, 13.8 – 34.9 (12.9 – 60.5)	25.2, 15.7 – 33.8 (5.1 – 63.1)	0.894	25.4, 16.1 – 34.8 (9.6 – 63.1)	0.910	29.1, 22.3 – 32.6 (10.3 – 43.7)	0.477	27.8, 17.8 – 35.7 (10.3 – 49.4)	0.605	26.4, 22.8 – 29.8 (18.3 – 41.5)	0.605	19.4, 15.2 – 28.9 (9.6 – 32.0)	0.451
Non-progressive motility [%]	23.3, 17.7 – 34.6 (14.0 – 48.2)	30.6, 22.2 – 42.4 (11.2 – 60.8)	0.330	28.2, 21.6 – 43.3 (11.0 – 62.7)	0.335	33.1, 21 – 42.3 (12.0 – 62.7)	0.379	33.1, 25.4 – 42.8 (11.7 – 57.6)	0.314	26.0, 22.9 – 39.2 (18.2 – 57.6)	0.314	37.2, 26.9 – 42.4 (15.9 – 57.6)	0.164
Spherical tracks [%]	35.4, 21.3 – 46.7 (15.1 – 80.4)	34.7, 26.4 – 50.9 (7.5 – 91.2)	0.674	35.4, 22.4 – 49.7 (7.5 – 91.2)	0.679	40.8, 27.0 – 43.3 (18.4 – 66.3)	0.616	43.6, 26.5 – 53.8 (20.2 – 65.7)	0.314	33.2, 27.7 – 38.5 (20.2 – 65.7)	0.978	38.6, 23.5 – 45.0 (19.9 – 65.7)	0.728
Rapid motility [%]	60.5, 42.8 – 74.4 (28.8 – 82.6)	52.9, 29.4 – 66.4 (3.8 – 82.4)	0.259	57.8, 37.5 – 68.3 (12.7 – 82.4)	0.475	47.0, 27.5 – 67.9 (12.7 – 81.0)	0.155	42.6, 24.0 – 60.6 (8.6 – 78.6)	0.097	58.3, 28.2 – 66.5 (16.5 – 69.3)	0.430	47.4, 44.8 – 51.7 (16.5 – 74.9)	0.164
Medium motility [%]	12.0, 7.7 – 26.5 (3.2 – 34.4)	16.8, 10.5 – 22.9 (4.1 – 54.0)	0.432	16.8, 10.0 – 23.5 (4.7 – 54.0)	0.416	18.4, 12.0 – 25.8 (5.9 – 54.0)	0.202	18.5, 13.1 – 28.0 (5.9 – 39.7)	0.201	18.9, 12.1 – 31.1 (8.3 – 47.0)	0.174	12.5, 8.3 – 25.7 (5.9 – 38.1)	0.954
Slow motility [%]	17.4, 14.1 – 25.7 (9.3 – 46.6)	22.9, 14.7 – 35.8 (4.8 – 57.4)	0.365	22.1, 14.4 – 34.2 (2.5 – 50.5)	0.590	27.0, 14.9 – 35.8 (6.0 – 41.7)	0.241	26.0, 18.8 – 38.8 (2.5 – 42.9)	0.183	22.1, 16.8 – 29.7 (4.8 – 39.4)	0.568	29.9, 21.8 – 39.4 (14.0 – 41.7)	0.064
Mucus penetration [%]	53.7, 42.9 – 68.4 (21.3 – 76.6)	52.1, 38.7 – 64.6 (0 – 74.2)	0.521	51.2, 36.8 – 63.1 (0 – 76.3)	0.507	49.8, 46.1 – 61.0 (23.2 – 74.2)	0.834	45.4, 38 – 51.2 (29.5 – 68.3)	0.314	58.7, 54 – 63.6 (29.5 – 70.8)	0.765	49.8, 45.6 – 62.6 (29.5 – 68.3)	0.772
Viability [%]	88.0, 80.0 – 91.5 (76.5 – 94.0)	90.0, 88.0 – 92.0 (60.0 – 98.0)	0.293	90.5, 86.5 – 93.5 (72.0 – 98.0)	0.229	89.5, 88.0 – 93.0 (72.0 – 98.0)	0.402	89.3, 88.3 – 91.3 (65.5 – 94.0)	0.585	90.5, 89.0 – 94.0 (88.0 – 96.0)	0.113	91.5, 89.0 – 94.0 (87.0 – 95.0)	0.067

Azoospermia [a]	0 / 13 (0%)	3 / 40 (7.5%)	0.567	1 / 33 (3.0%)	0.999	1 / 18 (5.6%)	0.999	4 / 16 (25.0%)	0.107	0 / 12 (0%)	–	3 / 14 (21.4%)	0.222

[a] presented as the number and percentage of dogs in a group

Załącznik nr 2 – Wzór oświadczenia o współautorstwie

Warszawa, 17.06.2024

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Rada Dyscypliny Weterynaria

Szkoły Głównej Gospodarstwa Wiejskiego w Warszawie

Oświadczenie o współautorstwie

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Warszawa, 13.06.2024

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Article

The Impact of Microorganisms on Canine Semen Quality

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Simple Summary: Various microorganisms, including *Mycoplasma* spp., have been reported in canine ejaculate. The impact of these microorganisms on semen quality remains unclear. The aim of this study was to evaluate the prevalence of bacteria and *Mycoplasma* spp. (and various species) in canine semen. Interestingly, 36.5% of the examined dogs tested negative for both aerobic bacteria and mycoplasmas, while 12.7% tested positive for bacterial presence. Additionally, 60.3% of the dogs tested positive for *Mycoplasma* spp. using PCR, with most carrying 1–2 *Mycoplasma* species. We found no significant difference in semen characteristics between *Mycoplasma*-positive and -negative dogs. The detection of *Mycoplasma* was not significantly linked to the presence of bacteria in semen. All the microorganisms identified were classified as saprophytic flora. Some canine ejaculate is sterile. Our findings suggest the existence of undescribed species of canine mycoplasmas, necessitating advanced diagnostic techniques like NGS for their identification.

Abstract: Various microorganisms, including *Mycoplasma* spp., have been reported in canine ejaculate. The impact of these microorganisms on semen quality remains unclear. This study included 63 male intact healthy dogs aged 1–8 years. One dog exhibited azoospermia, indicating a relatively low incidence of this condition. Interestingly, 36.5% of the examined dogs tested negative for both aerobic bacteria and mycoplasmas, while 12.7% tested positive for bacterial presence. Additionally, 60.3% of the dogs tested positive for *Mycoplasma* spp. using PCR, with most carrying 1–2 *Mycoplasma* species. We found no significant difference in semen characteristics between *Mycoplasma*-positive and -negative dogs. The detection of *Mycoplasma* was not significantly linked to the presence of bacteria in semen. All the microorganisms identified were classified as saprophytic flora. Our findings indicate that *Mycoplasma* spp. is common in canine ejaculate. Semen quality parameters were not correlated with the presence of *Mycoplasma* spp. in semen. *Mycoplasma* HRC689 was the most common species. Some dogs exhibited no presence of aerobic bacteria or mycoplasmas in their semen. Our study highlights the common presence of *Mycoplasma* spp. in canine ejaculate. Semen quality shows no correlation with *Mycoplasma* presence. Some canine ejaculate is sterile. Our findings suggest the existence of undescribed species of canine mycoplasmas, necessitating advanced diagnostic techniques like NGS for their identification.

Keywords: mycoplasma; semen quality; CASA-system

Citation: Domrazek, K.; Konieczny, P.; Majka, M.; Czopowicz, M.; Jurka, P. The Impact of Microorganisms on Canine Semen Quality. *Animals* **2024**, *14*, 1267. https://doi.org/10.3390/ani14091267

Academic Editor: Anna Wysokińska

Received: 4 April 2024
Revised: 18 April 2024
Accepted: 20 April 2024
Published: 23 April 2024

1. Introduction

The importance of semen quality in canine reproduction cannot be overstated, as it directly influences the success of breeding programs and the health of the offspring [1]. The evaluation of semen quality encompasses various parameters, including sperm count,

motility, morphology, and viability, all of which directly influence the likelihood of successful conception [1]. The contribution of the stud dog constitutes half of the factors that are to be considered when assessing the potential causes of infertility in canine breeding [2]. Due to this fact, the semen quality of the stud dog should be routinely evaluated before mating. Being a carrier of various pathogens is another major factor that needs to be controlled in a dog used for reproduction, as some pathogens may be transmitted via the sexual route to the bitch and lead to reproductive failure [3]. The presence of bacteria in canine ejaculate is a problematic issue in veterinary medicine. Many studies have shown that canine ejaculate is not sterile [4]. It is difficult, however, to distinguish between contamination from the urethra or foreskin and a primary infection of the urogenital tract [4]. Organisms commonly cultured from the semen of healthy male dogs include *Escherichia coli*, *Pasteurella multocida*, beta-hemolytic *Streptococcus*, coagulase-negative *Staphylococcus*, *Staphylococcus pseudintermedius*, *Canicola haemoglobinophilus*, *Klebsiella* spp., and *Pseudomonas* spp. [5–7]. Bacterial infections of the urogenital tract can have detrimental effects on canine semen quality, potentially leading to reproductive failures. Some studies describe a negative influence of bacteria belonging to the natural urogenital microbiome, e.g., *E. coli* on fertility [8]. However, except for *Brucella canis*, bacteria appear to be an uncommon cause of compromised fertility in dogs [9]. A negative impact on the seminal quality parameters is likely associated with an increasing number of bacterial species in canine sperm [7]. On the other hand, the bacteria commonly found in semen may play a protective role by inhibiting the growth of pathogenic microorganisms.

The negative influence of bacteria on sperm results from various mechanisms, including direct contact, competition for nutrients, and detritus production [10,11]. Bacterial contamination of ejaculate can lead to decreased spermatozoa motility, increased percentage of dead spermatozoa, and changes in morphology [8,12]. Moreover, after artificial insemination or natural mating, bacteria from ejaculate may induce uterine infections, fertilization failure, embryonic and fetal resorption, abortions, or stillbirths, contributing to decreased litter size and even leading to septicemia in the bitch [13]. One group of bacteria with potential negative impact on semen quality is mycoplasmas.

The data about the occurrence and role of *Mycoplasma* spp. in canine semen are contradictory. Some authors suggest that they have a negative influence on canine fertility [14] and can cause orchitis, epididymitis, and prostatitis [15]. In vitro studies have shown that *Mycoplasma* spp. can be attached to the spermatozoa by interlacing fibrils of variable diameter, which may reduce its motility [16]. Furthermore, Laber and Holtzmann [14] reported a significant increase in the percentage of abnormal spermatozoa and decrease in their motility caused by *M. canis*. *M. maculosum*, and *M. spumans* were described as a cause of 100% of dead forms and 70% of abnormalities in the head, midpiece, and tail of spermatozoa in Bernese Mountain Dogs [12].

Our study aimed to determine the prevalence of aerobic bacteria and mycoplasmas in Polish male dogs and the impact of these microorganism on semen quality.

2. Materials and Methods

2.1. Study Population and Sampling

This study enrolled adult male intact dogs between 1 and 8 years of age to avoid the potential influence of extreme age on their fertility. These dogs were sourced from kennels affiliated with the Polish Kennel Club (ZKwP, Poland), as well as from shelters for homeless animals. Subsequently, each dog underwent a routine clinical examination to ensure they were free of systemic diseases, and serum testosterone, estradiol, and total thyroxin concentrations were measured to eliminate the potential influence of endocrine disorders on semen quality.

All medical procedures were performed as a part of routine veterinary examination on the owners' request and thus, according to the European directive EU/2010/63 and Polish legal regulations, the approval of Ethical Committee for the described procedures was not required, as they could be qualified as nonexperimental clinical veterinary practices

excluded from the directive (Act of 15 January 2015 on the protection of animals used for scientific or educational purposes).

Eventually, 63 clinically healthy male dogs with the aforementioned hormones within the reference intervals were enrolled in study. Semen was collected in the sterile containers by digital manipulation, and the sperm-rich fraction of the ejaculate was analyzed according to standards [1]. From each semen sample, the swab was collected and sent to the commercial laboratory (Vetlab, Warsaw, Poland) for the routine bacteriological examination. Additionally, three cotton swabs were taken from each semen sample and air-dried. One of these swabs was sent to the same commercial veterinary laboratory (Vetlab, Poland) for PCR for *Mycoplasma* spp., canine herpesvirus type 1 (CHV-1), and *Chlamydia* spp., while the remaining two were kept at −80 °C until the results of PCR had been obtained. Based on the PCR results, dogs were categorized into the *Mycoplasma*-positive or *Mycoplasma*-negative group. The samples from *Mycoplasma* spp.-positive dogs were further analyzed to identify the exact *Mycoplasma* species. No samples were positive for CHV-1 or *Chlamydia* spp., as described elsewhere [17].

2.2. Hormone Measurements

After clotting, blood samples were centrifuged at 2057× *g* for 5 min, and serum was harvested. Hormones were quantified using the competitive enzyme immunoassay competition method with final fluorescent detection (ELFA) (MINI, VIDAS, bioMérieux, Marcy l'Etoile, France) in accordance with the manufacturers' manuals. Reference intervals were defined as follows: testosterone $\geq$ 1 ng/mL [18], estradiol < 115 pg/mL [19], and total thyroxin within the range of 10–50 nmol/L [20].

2.3. PCR Analyses

To increase the efficiency of the reactions, two swabs were used to carry out the PCR reaction. DNA isolation was performed using the Swab-Extract DNA Purification Kit (Eurx, Gdańsk, Poland), following the manufacturer's guidelines. PCR reactions were performed using recently published primers specific to various *Mycoplasma* species [21], along with Taq PCR Master Mix (2x) (Eurx, Gdańsk, Poland). Protocols for PCR were adapted from standard procedures described previously [22,23]. Subsequently, the PCR products were analyzed via electrophoresis in a 2% agarose gel, and the approximate lengths of the amplicons were determined using a molecular-weight size marker (100 bp DNA ladder) as a reference.

2.4. Bacteriological Examination

The semen samples were collected for bacteriological tests using transport agar medium swabs and promptly send to the commercial laboratory (Vetlab, Poland). The samples were cultured on the following microbiological media: Columbia agar with 5% ovine blood, MacConkey agar, Columbia CNA agar with 5% ovine blood, and chocolate agar. Incubation conditions included maintaining a temperature of 35–37 °C for 48 h in an oxygen atmosphere (Columbia agar with 5% ovine blood, MacConkey agar, and Columbia CNA agar with 5% ovine blood) or an atmosphere with an elevated concentration of CO_2 (Chocolate Agar), facilitated by a CO_2 atmosphere generator (Gen Compact, bioMérieux, Marcy l'Etoile, France). The bacterial growth was reviewed 24 and 48 h post-incubation. Subsequently, the obtained bacterial colonies underwent analysis in the MALDI TOF Biotyper Sirius IV (Billerica, MA, USA).

2.5. Semen Quality Evaluation

2.5.1. Macroscopic Evaluation

The volume of the sperm-rich fraction was measured by using calibrated pipettes, and the color of the semen was visually assessed. Cloudy or milky opacity were considered normal, following guidelines outlined by Root Kustritz [24]. The pH value was determined in each semen sample by dipping litmus strips.

2.5.2. Morphology Evaluation

The morphology of spermatozoa was evaluated by preparing smears from the second fraction, which were then air-dried and immersed in the sperm stain (Microptic, Barcelona, Spain) for 5 min. Then, the samples were examined in the light microscope (ECLIPSE E 200, Nikon, Tokyo, Japan) at 100-fold magnification. At least 200 spermatozoa were reviewed and categorized according to the criteria established by Freshman [1] into normal spermatozoa and spermatozoa exhibiting defects of the head, midpiece, or tail. Semen samples with more than 70% of spermatozoa of normal morphology were classified as physiological [1].

2.5.3. Viability Evaluation

The conventional microscopic assessment of the proportion of viable (with intact cell membrane) and dead (with compromised cell membrane) spermatozoa was conducted using nigrosine–eosin stain according to established protocols [25]. A warm mixture of the stain and semen (comprising 3 µL of eosin, 3 µL of nigrosine, and 3 µL of semen) was smeared on a heated glass slide [26] and air-dried. Then, the samples were examined under the light microscope (ECLIPSE E 200, Nikon, Tokyo, Japan) at 100-fold magnification. Each assessment involved the evaluation of at least 200 cells, with the results presented as the percentage of viable and dead spermatozoa.

2.6. Computer-Assisted Sperm Analysis (CASA)

The computer-assisted sperm analysis (CASA) was conducted using the sperm class analyzer (SCA version 6.5.0.67, Microptic, Barcelona, Spain) in conjunction with the light microscope (ECLIPSE E 200, Nikon, Tokyo, Japan) and camera (Basler, Ahrensburg, Germany). The thermostable table of the analyzer was heated to a temperature of 37 °C according to the established protocols [27]. The sperm-rich semen fraction was diluted in the proportion 1:1–1:5 with phosphate-buffered saline (PBS, Sigma Aldrich, Saint Louis, MI, USA) and incubated for 5 min at 37 °C prior to evaluation. Analysis was performed using a 20-micron GoldCyto 4-chamber slide (Goldcyto Biotech corp., Shanghai, China), using the manufacturer settings for dogs, as follows: VLC Rapid 165 µm/s, Lin Rapid 55%, and the average head area 20 μm^2.

In each analysis, a minimum of 500 spermatozoa were counted and examined for the following characteristics: concentration, motility, mucus penetration, and round cell count. Additionally, spermatozoa were categorized into subpopulations based on their movement characteristics, including velocity (fast (RAPID), moderate (MEDIUM), slow (SLOW)), direction (progressive, moderately progressive, nonprogressive), and the percentage of spherical tracks. A total spermatozoa count exceeding 200×10^6 and the percentage of motile spermatozoa exceeding 70% was considered normal [24]. To ensure the reliability of the results, all semen samples were microscopically evaluated by the same highly qualified staff member.

2.7. Statistical Methods

Categorical variables were presented as counts of groups and percentages from this study population and compared between groups using the likelihood ratio G test or Fisher exact test (if any expected cell count in the contingency table was <5). The 95% confidence interval (CI 95%) for proportions was calculated using the Wilson score method [28]. Numerical variables were tested for normality of distribution through the inspection of normal probability Q-Q plots and using the Shapiro–Wilk W test. As normality assumption was violated in most cases, the numerical variables were expressed as the median, interquartile range (IQR), and range and compared between groups using the Mann–Whitney U test. Their correlations were tested using Spearman's rank correlation coefficient (R_s). All statistical tests were 2-tailed, and the significance level (α) was set at 0.05. Statistical analysis was performed using TIBCO Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA).

3. Results

3.1. Study Population

This study included sixty-three male, intact, clinically healthy dogs aged from 1 to 8 years with a median (IQR) of 3.0 (1.5–4.5) years; twenty-three dogs (36.5%) were 1 year old, eight dogs (12.7%) were 2 years old, nine dogs (14.3%) were 3 years old, nine dogs (14.3%) were 4 years old, five dogs (7.9%) were 5 years old, two dogs (3.2%) were 6 years old, three dogs (4.8%) were 7 years old, and four dogs (6.3%) were 8 years old. Three dogs were crossbreeds (4.8%), and the remaining sixty dogs belonged to forty-seven breeds, among which Border Collie was represented by six dogs and springer spaniel and English Mastiff were represented by three dogs (Table S1). Body weight ranged from 3 to 120 kg, with a median (IQR) of 24 (15–31) kg.

Testosterone and estradiol concentrations were within the reference interval in all dogs. Total thyroxin concentration was lowered in twelve dogs (only in two dogs had <10 nmol/L) and slightly elevated in one dog (56.5 nmol/L).

3.2. Semen Characteristics

Only 1/63 dogs (1.6%; CI 95%: 0.3–8.5%) had azoospermia, and 5/63 dogs (7.9%; CI 95%: 3.4–17.3%) had oligospermia (<200 $\times$ 10^6 sperms). Abnormal spermatozoa morphology ($\leq$70% of normal spermatozoa in semen) was found in 3/62 dogs (4.8%, CI 95%: 1.7–13.3%). Details of semen characteristics are presented in Table 1. Semen volume was significantly positively correlated with the body weight of dogs (R_s = 0.37, p = 0.003), while round cell count and the proportion of normal spermatozoa was significantly correlated with the age of dogs, the former positively (R_s = 0.34, p = 0.006) and the latter negatively (R_s = $-$0.35, p = 0.005)

Table 1. General characteristics of the semen of study dogs.

Semen Characteristics [a]	Median	Interquartile Range (Range)
General semen characteristic		
Semen volume [mL]	2.5	1.5–3.5 (0.4–5.0)
pH	6.0	6.0–6.5 (3.0–7.5)
Sperm concentration [$\times 10^6/^{mL}$]	365.7	204.3–596.6 (42.2–1649.3)
Total sperm number [$\times 10^6$]	671.2	398.9–1401.7 (113.4–3298.7)
Oval cell count [$\times 10^6/^{mL}$]	1.0	0.4–3.0 (0–17.7)
Spermatozoa morphology		
Normal spermatozoa [%]	92.5	88.0–95.0 (58.5–99.0)
Head abnormalities [%]	1.8	1.0–4.0 (0–18.0)
Midpiece abnormalities [%]	2.0	1.5–3.5 (0–20.0)
Tail abnormalities [%]	2.8	1.0–5.0 (0–29.5)
Abnormal spermatozoa [%]	7.5	5.0–12.0 (1.0–41.5)
Spermatozoa motility		
Total motility [%]	93.4	87.7–96.4 (34.4–99.8)
Progressive motility [%]	31.7	21.2–39.4 (0.7–55.9)
Medium-progressive motility [%]	32.1	26.5–45.9 (4.6–78.7)
Non-progressive motility [%]	23.6	17.8–29.5 (5.1–49.5)
Spherical tracks [%]	38.3	31.4–49.7 (1.2–83.7)
Rapid motility [%]	57.4	45.6–68.4 (3.7–94.2)
Medium motility [%]	24.7	17.6–31.5 (5.1–53.4)
Slow motility [%]	7.0	4.4–10.9 (0.5–38.8)

Table 1. *Cont.*

Semen Characteristics [a]	Median	Interquartile Range (Range)
Mucus penetration [%]	31.2	20.7–41.1 (2.4–64.9)
Viability [%]	91.8	87.0–95.0 (40.0–98.5)

[a] spermatozoa characteristics for 62 dogs that had spermatozoa in semen.

3.3. Bacteriological and PCR Findings

In 8/63 dogs (12.7%, CI 95%: 6.6–23.1%), the following aerobic bacteria were cultured from the semen: *Staphylococcus pseudintermedius* in 3 dogs, *Streptococcus canis* in 2 dogs, followed by *Staphylococcus vitulinus*, *E. coli*, and *Pseudomonas* sp. in 1 dog each. *Mycoplasma* spp. was detected using PCR in 38/63 dogs (60.3%; CI 95%: 48.0–71.5%). In 10/38 *Mycoplasma*-positive dogs (26.3%), the *Mycoplasma* species could not be determined using routine PCR primers. In the remaining 28 dogs, 54 *Mycoplasma* strains belonging to twelve species were identified (Table 2)—one species in 11/28 dogs (39.3%), two species in 10 dogs (35.7%), three species in 5 dogs (17.9%), and four species in 2 dogs (7.1%). Except for three dogs with *M. canis*, two dogs with *M. haemocanis*, and two dogs with M. HRC689, all other *Mycoplasma*-positive dogs had unique combinations of various *Mycoplasma* species (Table 3). The detection of *Mycoplasma* was not significantly associated with the presence of bacteria in the semen ($p = 0.461$).

Table 2. Species of *Mycoplasma* spp. detected in canine semen.

Mycoplasma Species	Number of Dogs	Prevalence (CI 95%) [%]
M. HRC689	13/38	34.2 (21.2–50.1)
M. canis	7/38	18.4 (9.2–33.4)
M. haemocanis	6/38	15.8 (7.4–30.4)
M. arginini	5/38	13.2 (5.8–27.3)
M. VJC365	4/38	10.5 (4.2–24.1)
M. molare	3/38	7.9 (2.7–20.8)
M. maculosum	3/38	7.9 (2.7–20.8)
M. feliminutum	3/38	7.9 (2.7–20.8)
M. edwardii	3/38	7.9 (2.7–20.8)
M. opalescens	3/38	7.9 (2.7–20.8)
M. cynos	3/38	5.3 (1.5–17.3)
M. bovigenitalium	3/38	5.3 (1.5–17.3)
Unidentified	10/38	26.3 (15.0–42.0)

Table 3. The combinations of *Mycoplasma* species detected in canine semen.

Mycoplasma spp.	Number of Dogs
1 *Mycoplasma* species	
M. canis	3
M. haemocanis	2
M. HRC689	2
M. arginini	1
M. edwardii	1
M. molare	1
M. VJC 358	1

Table 3. *Cont.*

Mycoplasma spp.	**Number of Dogs**
10 Combinations of 2 *Mycoplasma* species	
M. HRC689 and *M. canis*	1
M. HRC689 and *M. cynos*	1
M. HRC689 and *M. edwardii*	1
M. HRC689 and *M. arginini*	1
M. HRC689 and *M. feliminutum*	1
M. HRC689 and *M. bovigenitalium*	1
M. HRC689 and *M. haemocanis*	1
M. VJC358 and *M. haemocanis*	1
M. VJC358 and *M. feliminutum*	1
M. arginini and *M. molare*	1
5 Combinations of 3 *Mycoplasma* species	
M. HRC689 and *M. cynos* and *M. arginini*	1
M. HRC689 and *M. maculosum*	1
M. HRC689 and *M. canis* and *M. molare*	1
M. VJC358 and *M. feliminutum* and *M. opalescens*	1
M. haemocanis and *M. maculosum* and *M. opalescens*	1
2 Combinations of 4 *Mycoplasma* species	
M. HRC689 and *M. canis* and *M. maculosum* and *M. bovigenitalium*	1
M. canis and *M. edwardii* and *M. opalescens* and *M. haemocanis*	1

3.4. Relationship between the Presence of Mycoplasma and Semen Characteristics

There was no significant difference in demographic and hormonal characteristics between *Mycoplasma*-positive and *Mycoplasma*-negative dogs (Table S2). The presence of *Mycoplasma* in the semen did not prove to be associated with any significant changes in the semen characteristics (Table S3).

4. Discussion

To the best of our knowledge, this is the first study in which PCR testing for all known canine mycoplasmas has been performed on canine semen material. Lechner et al. tried to detect only six of them [6]. Schafer-Somi et al. evaluated nine species of canine *Mycoplasma* in semen by culturing them [29]. Tamiozzo performed gene sequencing and detected only two species of these bacteria [12]. Currently, in routine veterinary practice, the gold standard for mycoplasma diagnosis is PCR testing, so our study focused on this method. Commercially available laboratories detect only *Mycoplasma* spp. without species identification of this bacteria. This leads to a lack of available statistics on the prevalence of exact species. Moreover, the knowledge regarding which species are pathogenic and which are not makes the obtained results difficult to interpret.

Studies suggest that *Mycoplasma* spp. may be present in the reproductive tract of dogs at varying rates, with estimates ranging from 30% to 89% [15,30]. In our study, the prevalence of *Mycoplasma* spp. in canine ejaculate was 60%. Schafer-Somi et al. detected these bacteria in 55% of samples, including 35% of samples of good-quality ones [29]. The prevalence seems to be similar, but the methodology is significantly different. Interestingly, the most frequently detected species of *Mycoplasma* was in our study—*Mycoplasma* HRC689. The presence of this *Mycoplasma* species in canine semen has not been investigated so far. *M. cynos* [6] or *M. canis* [29] appear to be the most common *Mycoplasma* species in ejaculate.

In our study, these two species were detected in 5.3% and 18.4% of tested dogs, respectively. Our data did not show any significant correlation between various species of this bacteria and semen quality, while Tamiozzo suggested that *M. spumans* and *M. maculosum* negatively affected male dogs' fertility [12]. Also, in another study, *Mycoplasma* was detected in a significantly higher percentage of poor-quality ejaculate samples compared to ejaculate samples of good quality [29].

Our results show that the detection of *Mycoplasma* spp. was not significantly associated with the presence of bacteria in the semen. This phenomenon could be caused by several factors. First, too small of a sample size could have undermined the statistical power required to detect meaningful differences. Consequently, even if a genuine association had existed, it may have remained undetected. Secondly, high variability in the methodologies employed for *Mycoplasma* and bacterial detection, encompassing diverse approaches, such as culture-based methods and molecular assays like polymerase chain reaction (PCR), could introduce disparities in their diagnostic sensitivity or specificity. In our opinion, using NGS technology could shed more light on these aspects. More research in this field is needed. The last explanation of this phenomenon could be the coincidental presence of those microorganisms. The co-occurrence of *Mycoplasma* and bacterial species in semen may be incidental rather than reflective of a direct causal relationship. Shared transmission routes, such as sexual activity, or similar ecological niches within the reproductive tract, could facilitate coincidental cohabitation without necessitating an intrinsic association.

Among the aerobic bacteria isolated from the semen samples in our study, various species were identified, including *Staphylococcus* spp., *Streptococcus* spp., and *E. coli.* These results are consistent with data reported in the available literature [6]. Our study also showed that not all ejaculate contained aerobic bacteria. In only 12.7% of samples, aerobic bacteria were cultured. This result is contradictory to other studies, which suggested that canine semen is not sterile [5–7]. On the other hand, data evaluated by another author suggested that the source of bacteria could be an environment, bacteria on the urethra [31], or a lack of proper hygiene of the person who collect the samples. Regardless of the quality of semen, bacterial growth is observed in various fractions of dog semen. However, higher concentrations are typically found in the first fraction, which is primarily attributable to the presence of bacteria originating from the urethra [4]. Dogs included in our study did not show any signs of urinary tract infection. The samples were collected with clean gloves in sterile containers. The samples for bacteriology were collected according to rules that are used, e.g., during urine collection, which means that the middle stream of semen was collected for bacteriology [32]. On the one hand, there are studies that describe the presence of bacteria as physiological [33], and on the other hand, some others consider bacteriospermia as pathology [7,8]. The number of bacteria and the immune status of the organism matter. Typically, the detection of over 10,000 colony-forming units of aerobic bacteria per milliliter of semen indicates an infection of the genital tract [34]. The infection is generally correlated with presence of inflammatory cells [35]. In our study there were no significant differences in round cell concentration in semen and bacterial or mycoplasmal contamination. Similar results have been obtained in the analysis of the cytology of seminal fluids performed by Kustritz et al. [36].

The predominant components of the physiological microflora in female dogs typically comprise β-hemolytic *Streptococcus* spp., *Staphylococcus* spp., *E. coli*, *Enterococcus faecalis*, *Pasteurella multocida*, *Proteus* spp., *Bacillus* spp., *Corynebacterium* spp., *Klebsiella pneumoniae*, *Actinomyces* spp., and *Neisseria* spp. Additionally, certain authors propose the presence of *Lactobacillus* spp., *Mycoplasma* spp., and *Ureaplasma* spp. [37,38]. Our bacteriology results obtained from fertile dogs indicate that the saprophytic flora of the male reproductive tract is similar. This suggests that prophylactic antibiotic therapy after positive bacteriology results in dogs with normal parameters describing semen is not justified because similar microorganisms inhabit the body of the bitch, and there is no risk of infecting her.

The findings of this study have practical implications for veterinary practice and breeding programs. By identifying the microbial flora present in canine ejaculate and its

influence on fertility parameters, this study contributes to the development of targeted screening and management protocols to improve breeding success rates and reproductive outcomes in dogs. In the current veterinary practice, the carriers of *Mycoplasma* spp. are mainly treated using doxycycline [39]. Our findings show that not every carrier of this bacteria should be treated. This result appears to be extremely important, as it will help reduce the use of antibiotic therapy in veterinary medicine. The overuse and misuse of antibiotics contribute to the development of antibiotic-resistant bacteria [40]. When antibiotics are used too frequently or inappropriately, bacteria can evolve and become resistant, making infections more difficult to treat [40]. In addition, antibiotics not only target harmful bacteria but can also affect the beneficial bacteria in the body, disrupting the natural balance of the microbiome. This disruption can lead to various health issues, including digestive problems and increased susceptibility to infections [41]. Due to this fact, it is highly recommended to evaluate semen quality, including the presence of inflammatory cells after obtaining bacteriology or PCR results from ejaculate. The decision on treatment should be made after careful consideration of all the factors.

Our study also has some limitations. While this study provides valuable insights into the prevalence and impact of aerobic bacteria and mycoplasmas in Polish male dogs, the findings may not be directly generalizable to other canine populations in different geographic regions. Factors such as breed diversity, environmental conditions, and management practices could influence the microbial composition of semen. This study focuses specifically on male dogs from Poland, which may limit the applicability of the findings to dogs from other countries or regions with different environmental conditions and management practices. Including participants from multiple geographic locations could enhance the external validity of this study. This study provides a cross-sectional snapshot of semen quality and microbial presence in male dogs at a specific point in time. Longitudinal data tracking changes in semen quality and microbial composition over time could provide deeper insights into the dynamic nature of these factors. The final limitation of our study is that the diagnostic methods we used are qualitative, not quantitative. It is possible that the quantity of bacteria has a greater impact on semen quality than the species themselves. More research in this area is needed. Our methodology, which contains PCR reactions and bacteriology culturing, is not cutting-edge technology, but it is available to both scientists and veterinarians. In the future, we want to expand our research to include the use of technology next-generation sequencing (NGS) [42]. Another limitation correlated with methodology is using basic diagnostic tools like CASA-system and microscopy evaluation of the morphology and viability of sperms. Our methodology, which includes diagnostic tools such as the CASA system, microscopic evaluation of sperm morphology and viability, PCR reactions, and bacteriological culture, are widely available to both researchers and clinical veterinarians. The use of these testing methods provides reproductive veterinarians, including practitioners and clinicians, with accessible tools for diagnosing infertility in their routine practice. Therefore, we also chose to use a simple eosin–nigrosine test as a surrogate for assessing functional membrane integrity. This test distinguishes between damaged and intact cell membranes, with damaged membranes staining pink while intact membranes remain unstained. While more sophisticated techniques, such as the hypoosmotic edema test (HOS), are typically available primarily in research settings, we anticipate incorporating them into our future research, similarly to next-generation sequencing (NGS) technology. Using these basic but effective tests, we aim to improve the diagnostic process for reproductive veterinarians and facilitate the timely and accurate identification of semen quality problems in dogs.

This study encountered challenges in identifying specific *Mycoplasma* species in some cases, with 10 out of 38 *Mycoplasma*-positive dogs having unidentified species. This limitation could affect the accuracy of the associations between *Mycoplasma* species and semen quality parameters. The presence of a positive result for *Mycoplasma* spp. and the absence of a positive result in PCR reactions for known species may suggest that these dogs were

carriers of another species. The ideal solution to this situation would be to sequence genes from samples obtained from these dogs. This will be the direction of our further research.

The significance of this study lies in its contribution to understanding the factors affecting reproductive health in dogs. This research addresses a critical gap in current knowledge by investigating the prevalence and impact of aerobic bacteria and mycoplasmas on semen quality, which is a crucial aspect of canine fertility and breeding success. By identifying and characterizing the microbial flora present in canine ejaculate, this study sheds light on potential sources of contamination and infection that may compromise semen quality. Understanding the microbial composition of semen and its influence on fertility parameters is essential for developing effective strategies to optimize reproductive outcomes in dogs. Furthermore, the study findings may have practical implications for veterinary practice and breeding programs. By elucidating the role of aerobic bacteria and mycoplasmas in semen quality, veterinarians and breeders can implement targeted screening and management protocols to minimize the risk of reproductive tract infections and improve breeding success rates.

5. Conclusions

In conclusion, in canine ejaculate, *Mycoplasma* spp. is common in dogs that have not been used for reproduction. The semen quality parameters are not related to the general presence of *Mycoplasma* spp. The most common species is *Mycoplasma* HRC689. There are dogs in whose semen neither aerobic bacteria nor mycoplasmas are present, which indicates that in some cases, the semen could be sterile. It is likely, however, that there are yet undescribed species of canine mycoplasmas that cannot be detected using conventional diagnostic tools. Therefore, further investigations employing advanced techniques, such as NGS, are imperative to unveil these elusive pathogens.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani14091267/s1, Table S1. Breeds of dogs of the study population; Table S2. Demographic and hormonal characteristics of dogs from Mycoplasma-positive and Mycoplasma-negative group; Table S3. Influence of the presence of *Mycoplasma* in the semen on semen characteristics.

Author Contributions: Conceptualization, K.D. and P.J.; methodology, P.K., M.M. and K.D.; formal analysis, K.D.; investigation, K.D.; writing—original draft preparation, K.D.; writing—review and editing, P.J., M.C. and P.K.; statistical analysis M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: All medical procedures were performed as a part of routine veterinary examination, on the owners' request and thus, according to the European directive EU/2010/63 and Polish legal regulations the approval of Ethical Committee for the described procedures was not required as they could be qualified as non-experimental clinical veterinary practices, excluded from the directive (Act of 15 January 2015 on the protection of animals used for scientific or educational purposes).

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors have read the journal's guideline and have the following competing interests: the co-authors (Konieczny P., Majka M.) are employees of Vet Cell Tech Sp. z o.o. company, which partially financed the project and provided the PCR analysis. The other authors have no competing interests.

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Załącznik nr 2 – Wzór oświadczenia o współautorstwie

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