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Veterinary Medicine

A prospective cohort study on seroprevalence and seroconversion of Ehrlichia canis in imported dogs from endemic regions to the Netherlands and other vector-borne coinfections

Master Research Project Veterinary Medicine

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Abstract

Canine monocytic ehrlichiosis, caused by *Ehrlichia canis*, poses a significant health concern in dogs, particularly in regions with a high prevalence of the disease, such as the Mediterranean basin. This prospective cohort study aimed to assess the seroprevalence of *Ehrlichia canis* infections in dogs imported from the Mediterranean basin to the Netherlands. A total of 250 dogs underwent serological testing using the indirect immunofluorescence antibody (IFA) test, with samples collected twice—initially within 6 weeks of importation and subsequently during a follow-up consultation conducted 7-24 months later. The findings revealed a seroprevalence of 22.4% during the initial consultation and 17.9% during the follow-up consultation. Dogs infected with *Ehrlichia canis* exhibited a higher likelihood of presenting with lymphadenomegaly, specifically in the prescapular (OR 2.6) and popliteal lymph (OR 6.75) nodes. Furthermore, Greek dogs exhibited a significantly elevated seropositivity rate compared to their counterparts from Spain and Portugal, with one in three Greek dogs testing seropositive during the initial consultation. Lastly, *Ehrlichia canis* infection was correlated with *L. infantum* infection during the follow-up consultation (p=0.040).

Keywords:
Canine-vector-borne diseases, *E. canis*, Canine monocytic ehrlichiosis, *L. infantum*, seroprevalence

Introduction

The importation of foreign rescue dogs has increased in the Netherlands from 8,000 in 2012 to 12,608 dogs per year in 2016, as reported by the latest findings from the Stray Animal Foundation Platform. This upward trend, marked by an 11.5% increase between 2015 and 2016, reflects the growing demand for dogs in the Netherlands (Radstake, 2017). This increased demand has led to the importation of dogs from regions, such as the Mediterranean basin and the Balkans, where certain canine vector-borne diseases (CVBD) are endemic. In contrast, most CVBDs are not endemic in the Netherlands. CVBDs are caused by pathogens transmitted by hematophagous arthropod vectors to canine hosts. In the Mediterranean basin, these diseases pose a significant veterinary challenge and a substantial public health concern due to their zoonotic nature, with canines potentially serving as reservoirs for human infections (Angelou et al., 2019). Among the most prevalent vector-borne-diseases in canines in the Mediterranean region include canine leishmaniosis (*Leishmania infantum*), heartworm disease (*Dirofilaria immitis*), and canine monocytic ehrlichiosis (*Ehrlichia canis*) (Miró et al., 2022). The epidemiological control and monitoring of these diseases, both in the canine population and within individual patients, present noteworthy challenges. However, the benefits to canine health achieved through these efforts are substantial. Consequently, *E. canis* and other CVBDs are of great concern.

In recent years, there has been a noticeable shift in the global distribution of parasitic arthropods and the vector-borne diseases they transmit. This shift can be attributed to a multitude of factors, including climate and environmental changes, international transportation, globalization, and human and animal population dynamics. These elements collectively influence the geographical prevalence and distribution of vector-borne diseases (Beugnet & Chalvet-Monfray, 2013). Arthropod vectors and the parasitic and viral agents they harbour are intricately linked to specific (micro)climates for survival and propagation. Environmental temperature plays a pivotal role in the life cycle of these vectors and the reproduction of the parasitic and viral agents they carry. Some CVBDs have exhibited increased prevalence in certain European regions and have seen greater circulation among countries (Beugnet & Marié, 2009; Knols & Takken, 2007). The prevalence of *E. canis* can vary significantly within endemic regions in the Mediterranean basin, as the distribution of the disease is heterogenous and not all countries and or regions have been studied (Sainz et al., 1996). The highest prevalence rates of *E. canis* are found in areas in Spain, Portugal, and Italy (Trotz-William & Trees, 2003).

The distribution of *E. canis* in Europe has expanded alongside its primary vector, *Rhipicephalus sanguineus*, as illustrated in Figure 1. While *R. sanguineus* is commonly found in the Mediterranean basin, where it is considered endemic, incidental sightings of this tick species have been reported in northern European countries, including the Netherlands (Nijhof et al., 200). Many of the ticks collected can be traced back to imported dogs or dogs that have travelled to endemic areas with their owners (Kooyman et al., 2022; Buczek & Buczek, 2020). However, reports of autochthonous cases in northern Europe have also emerged (Dongus et al., 1996). A recent study conducted in the Netherlands collected 2260 ticks from dogs and found that 0.1% of these ticks belonged to the *R. sanguineus* species (Kooyman et al., 2022). While this

percentage may appear low, it is noteworthy that these ticks can survive and complete their life cycle in temperate and colder climates, particularly when they find shelter indoors, such as in kennels (Nijhof et al., 2007; Jongejan 2001). With an increasing number of dogs accompanying their traveling owners and more dogs being imported from stray dog organizations in endemic regions, the introduction of *R. sanguineus* ticks, and consequently *E. canis*, is becoming increasingly significant.

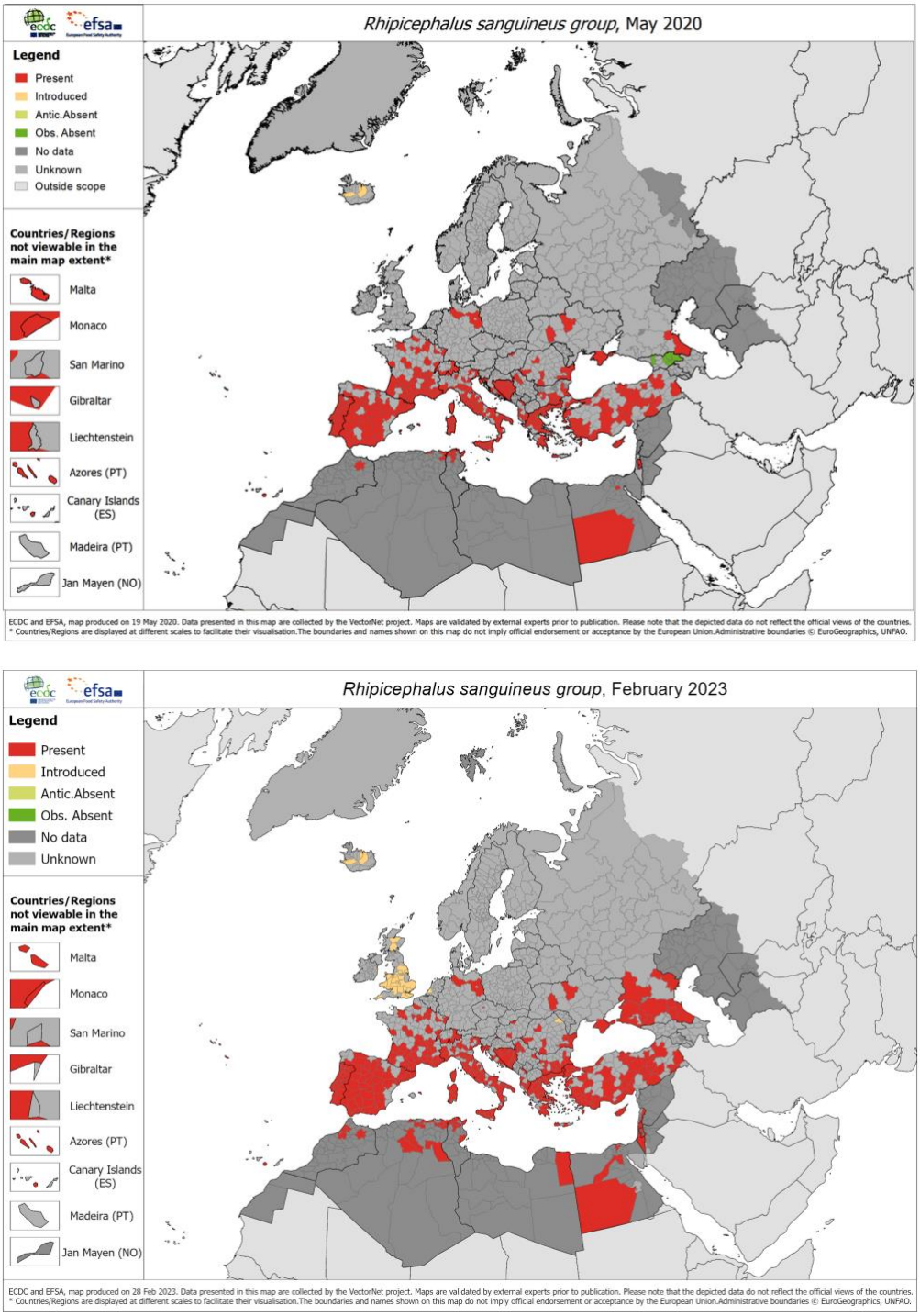

Figure 1. European Centre for Disease Prevention and Control and European Food Safety Authority. Tick maps [internet]. Stockholm: ECDC; May 2020 and February 2023. Available from: https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/tick-maps

Upon attachment of an *E. canis*-infected *R. sanguineus* tick to its canine host, transmission can occur within just a few hours (Fourie et al., 2013). This underscores the importance of preventative measures. Following transmission, an incubation period of 8-20 days ensues before IgG antibodies become detectable, often appearing as soon as 14 days after inoculation in experimentally infected dogs. Canine monocytic ehrlichiosis (CME) progresses through consecutive phases: acute, subclinical, and chronic phases (Harrus et al., 1999).

After the incubation period, the disease advances to the acute phase, typically lasting 2-4 weeks. During this phase, the pathogen multiplies within the host (Woody & Hoskins 1991; Hibler et al., 1986). *E. canis* primarily parasitizes cells of the mononuclear phagocyte system (Ebani, 2019). Clinicopathological symptoms during the acute phase may be mild or inapparent, presenting as nonspecific symptoms. Symptoms include but are not limited to fever, anorexia, lethargy, weight loss, lymphadenomegaly, splenomegaly, and oculonasal discharge. Clinicopathological findings at this stage typically reveal moderate to severe thrombocytopenia, mild anaemia, and a mild leukopenia (Harrus et al. 1997; Woody & Hoskins 1991; Pierce et al., 1977).

Following the acute phase, *E. canis* infection may persist, either following spontaneous clinical recovery or due ineffective treatment. This leads to the subclinical phase of the disease, where a significant percentage of infected dogs remain asymptomatic, exhibiting no clinical illness. Nevertheless, mild thrombocytopenia and hyperglobulinemia may persist in this phase (Harrus et al., 1999; Woody & Hoskins, 1991). In the study conducted by Codner & Farris Smith (1986) demonstrated a subclinical phase lasting 40 to 120 days. Remarkably, this subclinical phase can persist for years after the initial infection (Mylonakis et al., 2004; Codner & Farris Smith, 1986) Subsequently, a chronic phase may manifest in dogs incapable of eliminating the infection. The chronic phase is characterized by a broad spectrum of symptoms, that can vary and appear nonspecific, making it challenging to distinguish between acute and chronic phases in a patient. During CME, various clinical manifestations may occur, including fever, lethargy, weight loss, anorexia, vomiting, diarrhoea, lymphadenomegaly, splenomegaly, pale mucous membranes, and bleeding diathesis (epistaxis, petechiae, ecchymoses, haematuria, and melena). Additionally, symptoms such as uveitis, hypothermia, dyspnoea, ulcerative stomatitis, icterus, hepatomegaly, hind limb/scrotal oedema, ataxia, and convulsions can also be observed during this chronic phase (Ebani 2019; Mylonakis et al., 2004; Harrus et al., 1999; Woody & Hoskins, 1991).

Thrombocytopenia is the most common and consistent clinicopathological finding across the various disease phases. In the acute phase, in addition to thrombocytopenia, an increase in mean platelet volume is typically observed, indicating active thrombopoiesis. Conversely, in the chronic phase, thrombocytopenia persists as platelet production declines due to bone marrow hypoplasia (Woody & Hoskins, 1991). Severe chronic stages may lead to pancytopenia due to hypoplastic bone marrow (Harrus et al., 1999). Diagnosing CME presents challenges due to the different clinical phases and varied manifestations of the disease. Consequently, specific diagnostic tools and their timing present certain implications.

When addressing potential *E. canis* infections in imported rescue dogs, it becomes imperative to evaluate whether these dogs exhibit clinical symptoms and to determine the most appropriate timing for diagnostic testing. In cases where dogs display no clinical signs but yield positive

test results during screenings, it is crucial to exercise caution before considering antimicrobial treatment. This caution is especially important given the potential for encountering false-positive results within this specific group of animals, particularly when disease prevalence rates are low, which can affect the test's positive predictive value. Moreover, it is noteworthy that dogs may still be in the incubation period when undergoing testing, which can lead to false-negative test outcomes. Therefore, clinicians should consider various factors, including clinical presentation, timing of testing, test specificities, and disease prevalence within the population when interpreting test results.

Further complicating the accurate diagnosis of CME is the potential for patients to have coinfections that presents with similar clinical symptoms. An individual animal heavily infested with ticks may suffer from multiple tick-borne diseases simultaneously (Kordick et al., 1999). Coinfections can also arise when a tick serves as a vector for multiple pathogens (Schouls et al., 1999). Therefore, the absence of preventative ectoparasitic treatment can be considered a risk factor for acquiring multiple CVBDs. Coinfections with other arthropod-borne pathogens can concur with infections such as *L. infantum*, as their vectors often share the same geographical distribution (De Tommasi et al., 2013; Kordick et al., 1999). Recognizing the possibility of coinfections is essential, as they can result in more severe clinical symptoms and heightened pathogenicity (Gaunt et al., 2010).

Many dogs imported into the Netherlands have already undergone testing for *E. canis* in their country of origin. As vectors of these diseases are spreading more to the north of Europe, it becomes imperative to establish seroprevalence rates in imported dogs from endemic regions. This understanding is vital for assessing the potential spread of *E. canis* in non-endemic countries in the near future, particularly given the increasing demand for companion dogs in the Netherlands. At present, a research gap exists, as there are no existing studies presenting the prevalence of *E. canis* in imported dogs from endemic regions to the Netherlands. This may lead to delay in diagnosis and treatment for imported dogs into the Netherlands, as veterinary practitioners may place CME lower on their list of differentials when a dog presents itself with non-specific symptoms. Therefore, gathering information on the presence of *E. canis* in imported dogs from endemic regions is essential to develop diagnostic guidelines for veterinarians.

The primary aim of this research is to estimate the seroprevalence of *E. canis* in imported dogs from the Mediterranean basin, exploring potential correlations between seropositivity and clinical history or physical examination findings, while also investigating associated risk factors. The second aim of this research is to investigate the correlation between *E. canis* and *L. infantum* (and if feasible, *D. immitis* and *Hepatozoon canis*) within six weeks after importation and approximately a year later.

Material and Methods

Study population and data collection

In this prospective cohort study, a total of 250 client-owned dogs were included as part of the larger Project Leishmania study. Most of the dogs were enrolled through Dutch animal rescue foundations, as detailed in Appendix A. Additionally, a small subset of dogs was directly enrolled without the involvement of an animal rescue foundation. Enrolment of dogs into the study was contingent upon obtaining written informed consent from their owners. The consent indicated the owners' agreement to participate in the research, including the collection of information and blood samples for research purposes. The enrolment of patients in the study took place over the period from December 2018 to January 2021, at the Department of Clinical Sciences of Companion Animals (DCSCA) of the Faculty of Veterinary Medicine of Utrecht University. Inclusion criteria consisted of animals to be at least 6 months of age and imported from endemic regions for canine leishmaniasis. Dogs residing in the Netherlands for longer than 6 weeks were excluded.
The data collection process involved multiple components, including clinical history, physical examination, and blood collection. The initial consultation occurred within the first 6 weeks following importation, while the follow-up consultation after 7-24 months. To obtain comprehensive clinical history data, a standardized questionnaire (see Appendix B) was used. Concurrently, a thorough physical examination was carried out on each patient during both the initial and the follow-up consultations. Physical examinations were performed by master students and checked by internal medicine diplomates/residents. Physical examination included a dermatological and cardiovascular examination.

Diagnostic Laboratory Methods

Blood samples were collected from the animals' jugular, cephalic, or saphenous veins using EDTA and serum tubes (BD Vacutainer, BD Life Sciences). All laboratory tests were conducted at the University Veterinary Diagnostic Laboratory (UVDL), except for the *E. canis* test which was performed at the Veterinary Microbiological Diagnostic Centrum (VMDC). Complete blood counts were determined using the ADVIA 2120i with multispecies software (Siemens Healthcare, The Hague, the Netherlands) and biochemistry carried out the Olympus AU 680 (Beckman Coulter, Woerden, the Netherlands).
The Direct Agglutination Test (DAT) was used to determine the *L. infantum* antibody titers.
L. infantum titer values of < 1:1v60 are considered negative and values ≥ 1:320 positive (el Harith et al., 1989). Any values of 1:320 that were considered dubious by the UVDL were also categorized as positive for statistical analysis, following the precedent set by previous studies (Mohebali et al., 2005; Boelaert et al., 1999; el Harith et al., 1989).
For the detection of *D. immitis,* the FASTest HW antigen test (Megacor Diagnostik GmbH, Austria) was utilized along with the KNOTT test (detailed instructions in Appendix C) to detect microfilaria in the serum.

In the study, the indirect immunofluorescence antibody (IFA) test was used to detect *E. canis* antibodies. A titer of ≥1:40 was considered positive. The IFA test was performed using the MegaFLUO® *E. canis* test-kit (Megacor Diagnostik GmbH, Austria) with 1:40 and 1:80 dilutions. This method represents the standard testing procedure routinely employed at the VMDC in Utrecht.

All test kit components (apart from the conjugate) and sera had to reach room temperature before application. Dilutions were prepared by adding PBS to the serum. For the 1:40 dilution, 195 µl PBS and 5 µl of serum were added to the microtiter plate wells. To make the 1:80 dilution, 100 µl of serum from the 1:40 dilution microtiter plate wells were transferred in new wells, and 100 µl PBS was added.

Each slide well, coated with *E. canis* antigen, was initially covered with 20 µl of the negative and positive control, followed by the addition of 20 µl of the 1:40 or 1:80 dilutions to the remaining wells. Subsequently, the slides were incubated at 37°C for 30 minutes to allow possible antibody-antigen binding. To remove non-bound proteins the slides underwent two 5-minute cycles of washing with PBS on an orbital shaker. After the PBS washing, the slide wells were briefly rinsed with demineralized water, being cautious not to rinse directly onto the wells. Excess water was gently removed by tapping the slide wells onto absorbent paper. The slides were allowed to air dry briefly, ensuring that the slide wells themselves did not completely dry out. Following incubation, 20 µl of fluorescein-marked isothiocyanate (FLUO FITC) anti-dog IgG conjugate solution was added to the slide wells. This solution contains fluorescent antibodies that specifically bind to serum antibody-antigen complexes. The incubation occurred in a dark environment at 37°C and lasted for 30 minutes to protect the photosensitive conjugate. After incubation, non-bound conjugate was washed off with the same washing steps previously described.

In the final step, slide wells were covered with Mounting Medium to ensure preservation of the slides for up to 7 days at temperatures between 2-8 °C. Within a week, the slides were assessed using a fluorescence microscope equipped with a FITC filter system, with a 400x magnification. A positive test was indicated by the presence of bright, sharp, and clear yellow-green, fluorescent clusters of inclusion bodies within the cytoplasm (Figure 2). To interpret the test slide wells, the fluorescence patterns (form, density, etc.) observed in the negative and positive controls served as reference patterns. Patterns of reactivity different than that seen in the controls were considered non-specific and indicated a negative test result.

All slides were handled and interpreted by the same individuals (CM, CV). At time of interpretation, previous serological results and clinical history and findings were unknown to these individuals. To obtain final test results, another experienced laboratory technician (CV) or (AS) revaluated the samples. Tests were repeated in cases of ambiguous results.

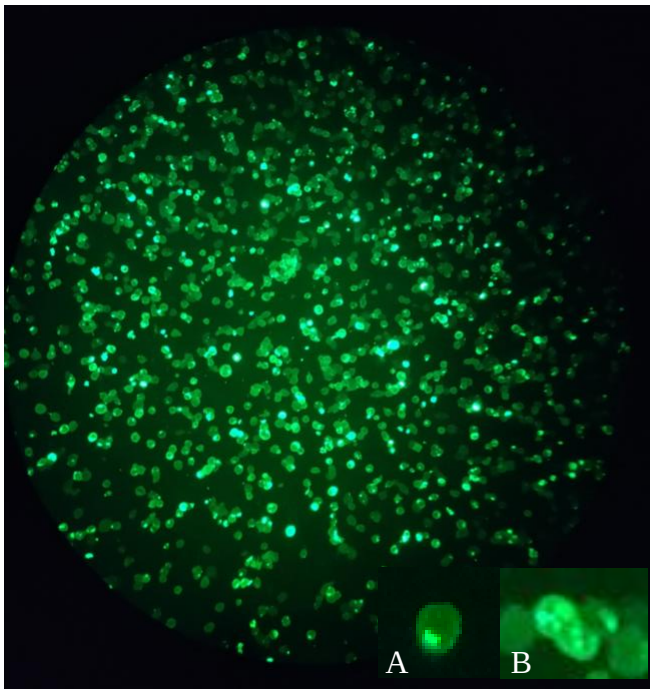

Figure 2. A positive test result indicating by a single inclusion body (A) and morulae (B) (400x magnification)

Statistical analysis

All data was organized in a database using Microsoft Office Excel for Windows 365® and subsequently exported to RStudio (Version 2023.03.1+446) for statistical analysis.
To determine the appropriate sample size, a power analysis was performed based on the number of dogs imported to the Netherlands in 2015 (n=11,300), as reported by the Stray Animal Foundation Platform (Radstake, 2017). Given the focus of the Project Leishmania study on *L. infantum*, the power analysis was specifically calculated based on *L. infantum* and not *E. canis* prevalence. The results of the power analysis indicated that a sample size of 241 dogs would be sufficient to detect a prevalence of 20% with a 95% confidence interval ranging from 15% to 25%. Nonetheless, to account for potential attrition during the study, a sample size of 250 participating dogs was selected.

Descriptive statistics regarding the breed, sex, age, weight, and country of origin were performed. Furthermore, univariate logistic regression was implemented to identify potential risk factors associated with *E. canis* seropositivity. Proportions of seropositive canines for *E. canis*, *L. infantum*, *D. immitis*, and *H. canis* were calculated for the entire study population during both initial and follow-up consultations.
All data underwent dichotomization to facilitate subsequent statistical analysis. Mcnemar's test was utilized to assess changes in seroprevalences after blood withdrawal during the follow-up consultation. For the assessment of coinfections, the proportions of *L. infantum*, *D. immitis*, and *H. canis* seropositive dogs were calculated from the total number of *E. canis* seropositive dogs. Associations between coinfections were determined through the application of either a Chi-square test or, when dictated by a low number of data entries, Fisher's exact test.

Statistical associations between *E. canis* seropositivity and clinical risk factors (variables) were evaluated in two stages. Initially, all selected variables underwent screening using Fisher's exact test. Variables with p-values <0.25 and cell values exceeding n=3 in the 2x2 contingency tables were incorporated into univariate logistic regression. Subsequently, backward multivariate logistic regression was employed to select the definitive variables for analysis. Significance levels for all tests were set at $p<0.05$.

Results

Population

A total of 250 dogs participated in the study, of which 92.4% were enrolled through animal rescue foundations (Appendix A). During the initial consultation, all 250 dogs were present. At the follow-up consultation the number reduced to 213 dogs. Six dogs were euthanized during the follow-up period, and blood collection was not possible for one dog during that consultation. Consequently, a total of 38 dogs were excluded from the statistical analysis of follow-up consultation data.

Among the participants, 54.4% of dogs were female (n=136). Out of these, 113 were castrated, and an additional seven female dogs were castrated later in the study. The population consisted of 45.6% male dogs (n=114 males), with 100 of them being castrated. The median age of the dogs at the beginning of the study was 2 years (range 6 months to 12 years). The median weight at the study's outset was 15.0 kg (range 4.3 kg to 65.8 kg), with a median BCS of 4 on a scale of 1-9. By the time of the follow-up consultation, the median weight had increased to 16.2 kg, with a median BCS of 5. The majority of dogs were crossbreeds (n=202), while the most prevalent purebred dogs included Galgo Españols (n=13), Podencos (n=9) and Mastin Españols (n=5). Other purebred dogs in the study comprised Beagles (n=3), German Shorthaired Pointers (n=3), English Setters (n=3), Yorkshire Terriers (n=3) and Epagneul Bretons (n=2). There was one of each of the following purebreds: English Pointer, Border Collie, Cirneco dell' Etna, Fox Terrier, Kokoni, Mastiff and Spanish Water Dog. Most dogs were imported from Spain, accounting for 41.6% (n=104), and Greece, constituting 36.0% (n=90) of the study population. Other countries of origin were Portugal (n=42), Italy (n=10), and Cyprus (n=4).

Risk factors of population characteristics on *E. canis* seropositivity

The variables "Age" and "Sex" did not exhibit a significant association with *E. canis* seroprevalence. Similarly, the variable "Breed," categorized as crossbreed and purebred, also did not show a significant association with *E. canis* seroprevalence. However, significant associations were identified between *E. canis* seroprevalence in dogs originating from Spain (p=0.039) and Portugal (p=0.017) compared to Greek dogs during the initial consultation. During the initial consultation, the odds of dogs from Spain being seropositive for *E. canis* were 50% lower than those of dogs from Greece (OR 0.5, CI 0.26-0.96). For dogs from Portugal, the odds were 72% lower than those of dogs from Greece (OR 0.28, CI 0.09-0.74). This suggests that dogs from Greece face a higher risk of *E. canis* seroprevalence compared to dogs from Spain or Portugal. As most dogs were imported from Spain and Greece and Greek dogs having the highest *E. canis* seroprevalence, Greek dogs were taken as the intercept in the logistic regression. Because there were only four dogs in this study from Cyprus, no odds ratio could be calculated for this country.

Below, Table 1 provides a detailed breakdown of the study population's characteristics during both the initial and follow-up consultations and the potential risk factors associated with *E. canis*.

Variable	Category	Number of dogs (%)	Positive dogs (%)	OR (CI 95%)	p-value	Number of dogs (%)	Positive dogs (%)	OR (CI 95%)	p-value
Sex	Male	114 (45.6)	25 (21.9)	0.95 (0.52-1.73)	0.870	94 (44.3)	19 (20.2)	1.32 (0.65-2.68)	0.439
	Female	136 (54.4)	31 (22.8)	-	-	118 (55.7)	19 (16.1)	-	-
Age	<1	61 (24.4)	12 (19.7)	-	-	0 (0)	0 (0)	-	-
	1-5	137 (54.8)	32 (23.4)	1.24 (0.12-0.44)	0.565	162 (76.4)	29 (17.9)	-	-
	6-10	45 (18.0)	10 (22.2)	1.17 (0.45-3.01)	0.749	44 (20.8)	9 (20.5)	1.18 (0.49 – 2.64)	0.699
	>10	7 (2.8)	2 (28.6)	1.63 (0.22-8.66)	0.584	6 (2.8)	0 (0)	0 (-)	0.988
Country of origin	Spain	104 (41.6)	20 (19.2)	0.50 (0.26-0.96)	0.039*	91 (42.9)	13 (14.3)	0.50 (0.22 -1.09)	0.083
	Greece	90 (36.0)	29 (32.2)	-	-	76 (35.8)	19 (25.0)	-	-
	Portugal	42 (16.8)	5 (11.9)	0.28 (0.09-0.74)	0.017*	33 (15.6)	5 (15.2)	0.54 (0.16-1.49)	0.259
	Italy	10 (4.0)	2 (20.0)	0.53 (0.08-2.26)	0.434	9 (4.2)	1 (11.1)	0.37 (0.02-2.24	0.370
	Cyprus	4 (1.6)	0 (0)	-	-	3 (1.4)	0 (0)	-	-
Breed	Crossbreed	202 (80.8)	47 (23.3)	-	-	174 (82.1)	35 (20.1)	-	-
	Purebred	48 (19.2)	9 (18.7)	0.76 (0.33-1.62)	0.501	38 (17.9)	3 (7.9)	0.34 (0.08-1.02)	0.087
Total		250	56 (22.4)			212	38 (17.9)		

Table 1. Seroprevalence of *E. canis* studied by sex, age, and country of origin and breed for the initial and follow-up consultation by logistic regression. Significant results ($p<0.05$) are indicated by *.

Predictive value of history, physical examination and clinicopathology on *E. canis* seropositivity

Before conducting statistical analysis, data from animals with coinfections with *L. infantum, D. immitis or H. canis* were omitted to minimize potential confounding bias. The *E. canis* status was set as the dependent variable. Out of all variables (n=76, see Appendix D), 22 variables remained after screening using Fisher's exact tests (Table 2).

Variables	Description	Coding of the variables
Weight loss	Mention of weight loss during history	Yes/No
Anorexia	Mention of anorexia during history	Yes/No
Polyuria/Polydipsia	Mention of polyuria and polydipsia during history	Yes/No
Diarrhoea	Mention of (recent) diarrheal episode (more than 1 day) during history	Yes/No
Vomiting	Mention of (recent) vomiting (more than 1 day) during history	Yes/No
Locomotion abnormalities	Mention of lameness, stiffness, or other locomotion abnormalities unrelated to orthopaedic diagnosis during history	Yes/No
Elevated temperature	Temperature above 39.0° C	Yes: >39.0° C No: ≤39.0° C
Pale mucous membranes	Pale conjunctival or buccal mucous membranes	Yes/No
Lymphadenomegaly Mandibular Prescapular Retropharyngeal Inguinal Popliteal Accessorial Axillar	(Unilateral or bilateral) enlarged lymph nodes	Yes: three or more enlarged lymph nodes No: one or two enlarges lymph nodes Yes/No
Oedema	Oedema present during physical examination	Yes/No
Splenomegaly	Enlarged spleen during palpation of the abdomen	Yes/No
Anaemia	Haematocrit below 0.420 L/L	Yes: <0.420 L/L No: ≥0.420 L/L
Leukopenia	Leukocyte count below $4.5x10^9$/L	Yes: $<4.5x10^9$/L No: $\geq 4.5x10^9$L/L
Thrombocytopenia	Thrombocyte count below $80x10^9$/L. Samples with thrombocyte aggregation were excluded.	Yes: $<80x10^9$/L No: $\geq 80x10^9$/L

Table 2. Names, descriptions, and coding of the 22 variables and their categories included in the study as potential clinical predictive factor variables for *E. canis* seropositivity.

These 22 variables were used for the univariate logistic regression. For the initial consultation only the variable "Prescapular" (p= 0.049) remained. *E. canis* seropositive dogs were more than 2.5 times (OR 2.59, CI 0.97-6.55) more likely than seronegative dogs to have enlarged prescapular lymph nodes. No other variables showed significant associations with *E. canis* seropositivity, making multivariate logistic regression unnecessary for the initial consultation. For the follow-up consultation, the variables "Elevated temperature" and "Popliteal" remained (Table 3). Since no variables from the same group (e.g. lymph nodes, pulse, respiration etc.)

remained after univariate logistic regression for the follow-up consultation, testing for collinearity between those variables (e.g. “Popliteal” and “Inguinal) was not needed.
After backward multivariate logistic regression, the final model retained only the variable “Popliteal” (p= .007) (Table 4). *E. canis* seropositive dogs were 6.75 times more likely to have enlarged popliteal lymph nodes (OR 6.75, CI 1.68-28.96).
The also significant population characteristic risk factor “Country of origin” was not used in the backward multivariate logistic regression, as it may overshadow the potential influence of biological factors in the analysis.

		Initial consultation				
Variables	**Category**	**Total**	**Positive dogs (%)**	**Negative dogs (%)**	**OR (CI 95%)**	**p-value**
Prescapular	Yes No	22 188	8 (36.4) 34 (18.1%)	14 (63.6%) 154 (81.9%)	2.59 (0.97-6.55)	0.049*
		Follow-up consultation				
Variables		**Total**	**Positive dogs (%)**	**Negative dogs (%)**	**OR (CI 95%)**	**p-value**
Elevated temperature	Yes No	76 93	15 (19.7%) 11 (11.8%)	61 (80.3%) 82 (88.2)	1.28 (0.58-2.84)	0.542
Popliteal	Yes No	10 177	3 (30.0 26 (14.7)	7 (70.0) 151 (85.3)	5.10 (1.34-19.46)	0.014*

Table 3. Predictive factor variables screened by univariate analysis with *p*-values <0.25, their *p*-values of Fisher's exact test, odds ratios (OR) and 95% confidence intervals. Significant results (p<0.05) are indicated by *.

Final model follow-up consultation n =169			
	Category	**OR (CI 95%)**	**p-value**
Elevated temperature	Yes No	1.30 (0.57 – 2.96)	0.525
Popliteal	Yes No	6.75 (1.68-28.96)	0.007*

Table 4. Final logistic regression model, after multivariate logistic regression, of the predictive factor variables associated with *E. canis* seropositivity. Significant results (p<0.05) are indicated by *.

Seroprevalence and seroconversion

During the initial consultation, 56 out of 250 dogs tested positive for *E. canis*, resulting in a seroprevalence of 22.4%. At the follow-up consultation, which involved 212 tested dogs, 38 of them tested positive for *E. canis*, resulting in a seroprevalence of 17.9% (as shown in Table 5). Among these dogs, 27 tested negative during the initial consultation, while 11 had previously tested positive for *E. canis*. Notably, during this study, six dogs tested positive for *H. canis* during the initial consultation, and one dog was found to be infected with *H. canis* during the follow-up consultation. The detection of *H. canis* in these dogs was incidental, discovered during blood smear examinations. Table 5 provides (sero)prevalence data for *E. canis*, *L. infantum*, *D. immitis*, and *H. canis*. No significant decrease or increase in (sero)prevalence rates were found between the initial and follow-up consultation for these infections. Most dogs remained within the same positive or negative *E. canis* immunostate (85.3%). During the initial consult 167 dogs were seronegative. Of these dogs, 12 seroconverted, resulting in a

seroconversion rate of 7.2%. Of the 45 dogs that were positive during the initial consult, 19 dogs seroreverted, resulting in a seroreversion rate of 42.2%.

Pathogen	Number of infected dogs initial consultation	(Sero)prevalence	Number of infected dogs follow-up consultation	(Sero)prevalence	p-value
E. canis	56/250	22.4%	38/212	17.9%	0.2812
L. infantum	30/250	12.0%	23/212	10.8%	0.6276
D. immitis	6/249	2.4%	1/212	0.5%	0.3711
H. canis	6/250	2.4%‡	1/212	0.5%‡	0.1336

Table 5. (Sero)prevalences of dogs infected with different CVBDs and their *p*-values of McNemar's test. (‡: *H. canis* prevalence was only based on incidental findings on blood smear examinations.)

Coinfections

During the initial consultation, no significant associations were found between different infections and *E. canis* seropositivity (Table 6). Notably, during the initial consultation, one dog exhibited coinfections with *E. canis*, *L. infantum* and *D. immitis*.
At the follow-up consultation, a significant association was observed between *L. infantum* and *E. canis* seropositivity (p=0.040).

Pathogen	Number of coinfected dogs of total ***E. canis*** seropositive dogs at initial consultation	Prevalence	p-value	Number of coinfected dogs of total ***E. canis*** seropositive dogs at follow-up consultation	Prevalence	p-value
E. canis + ***L. infantum***	10/56	17.9%	0.1944	8/38	21.1%	0.040*
E. canis + ***D. immitis***	2/55‡	3.6%	0.6162	0/38	0%	1.0
E. canis + ***H. canis***	2/56	3.6%	0.6186	1/38	2.6%	0.1792

Table 6. Number of coinfections among total *E. canis* seropositive dogs and prevalence of these coinfections. (‡: one dog was omitted due to the absence of a FASTest HW antigen test for *D. immitis*)

Discussion

This is the first study examining the seroprevalence of *E. canis* in imported dogs within the Netherlands. *E. canis* seroprevalences of 22.4% during the initial consultation and 17.9% at the follow-up consultation were observed among dogs imported from the Mediterranean basin. These seroprevalences did not exhibit a significant difference between the two time points. Intriguingly, the observed seroprevalences exceeded the researchers' initial expectations, based on their clinical experience.

The study by Schäfer et al., (2019) reported a seroprevalence of 16.0% (45/278 dogs) among dogs imported from Mediterranean and South-eastern European countries. This retrospective study analysed clinical records of dogs presented to the Small Animal Clinic at FU Berlin, and selected cases with at least one direct or indirect examination for vector-borne infections in their clinical record. Germany as well as the Netherlands is not endemic for *E. canis*. The selection of the clinical records based on direct or indirect examination for CVBDs may have introduced a bias toward choosing dogs with a higher likelihood of *E. canis* seropositivity.

Another study reported a seroprevalence of 10.1% (492/4681 dogs). Among the 4681 dogs in this study, 90.3% were imported from Mediterranean and South-eastern European countries to Germany, 1.8% were German dogs that travelled with their owners to other countries, and 7.9% had an of unknown country of importation. Notably, dogs living in Portugal exhibited a significantly higher seroprevalence of 24.8% (82/331 dogs). It is worth highlighting that in the study by Menn & Naucke (2010), nearly 10% of cases had an unknown country of importation or were from Germany, rendering the comparison of the reported seroprevalence more difficult with the seroprevalence found in the present study.

Both studies reported lower seroprevalences than the seroprevalence of 22.4%, during the initial consultation of the current study. While the current study focused solely on dogs from the Mediterranean basin, Schäfer et al. (2019) included dogs imported from South-eastern European countries. In their study, 16.9% of dogs fell into this category, whereas in the study by Menn & Naucke (2010), this proportion was higher at 31.2%. A possible explanation for higher seroprevalences in the current study may be based on the imported population of dogs being solely from the Mediterranean basin, where *E. canis* is more prevalent than in South-eastern European countries, with prevalence ranging from 0.16% to 11.06% (Jurković et al., 2019; Bogićević et al 2017; Farkas et al., 2014; Mircean et al., 2012). Disparity in seroprevalences can therefore be attributed to differences in population characteristics, particularly the origin of the imported dogs.

In the present study, Greek dogs exhibited a higher risk of *E. canis* seropositivity during the initial consult compared to dogs from Spain or Portugal. The lack of statistical significance for dogs from Portugal during the follow-up consultation may be attributed to a smaller sample size during the follow-up consultation.

Notably, this study found a 32.2% seroprevalence for *E. canis* in Greek dogs, indicating that one in three dogs in the study population tested seropositive. This rate is significantly higher compared to previous reports of 12.5% (Angelou et al., 2019) and 12.3% (Athanasiou et al., 2019). However, a study conducted by Chochlios et al. (2020) in Thessaloniki reported a

seroprevalence of 33.9%, aligning more closely with the findings in the present study. It is worth noting that a significant proportion of the dogs in the current study (80.0%) were imported from the North Aegean islands, including Lesvos, which differs from the region covered in Chochlios et al.'s study. A smaller study conducted in Lesvos found a notably higher seroprevalence of 58.3% (Geromichalou & Faixová, 2017).
In Spain, Italy, and Portugal, seroprevalence rates also vary between studies. In Spain seroprevalence rates of 0.9% (Díaz-Regañón et al., 2020), 4.3% (Montoya-Alonso et al., 2020), 16.7% (Solano-Gallego et al., 2006), 54.7% and 3.1% (Amusategui et al., 2008) were reported. In Portugal studies have reported seroprevalence rates of 4.1% and 16.4% (Cardoso et al., 2012) and 11.4% (da Silva, 2010). Meanwhile, in Italy seroprevalence rates have been documented at 16.2% (Ebani, 2019), 16.0% (Petrucilli et al., 2020), 28,7% Mendoza-Roldan et al., 2021), 29.6% (Migliore et al., 2020), and 46% (Pennisi et al., 2012).
Only one study was found that reported a seroprevalence in Cyprus. In this study a seroprevalence of 12% for *E. canis* in the *L. infantum* positive group and a seroprevalence of 3% in the control group was reported (Attipa et al., 2018). In the current study, the seroprevalence data from Cyprus is considered unreliable due to the inclusion of only four imported dogs from that country. Seroprevalence rates in the present study for Spain (19.2%), Portugal (11.9%) and Italy (20%) are often higher than rates mentioned in previous studies.
Lastly, a recent study from 2022 that studied the seroprevalences of major CVBDs across Europe found that, especially in countries such as Greece, Italy, Spain, Portugal, the Netherlands, Romania, Russia and Switzerland, antibody positivity rates for *E. canis* were higher (>3%) than other countries across Europe. Greece showed the highest seropositivity with 19.6% (Miró er al., 2022).
Above mentioned prevalence rates for *E. canis* show that the seroprevalences differ greatly between countries and between different regions within those countries. An explanation for these differences may be due to different temperature, humidity, and rainfall in different regions (Migliorie et al., 2020; Dantas-Torres, 2015). Because *E. canis* is a latent infection, stray dogs can be transported to different shelters in different regions, skewing results of seroprevalences between regions. Furthermore, also differences in population characteristics can affect outcomes of *E. canis* seroprevalence. Stray and shelter dogs in the above-mentioned studies often have a higher seroprevalence than client-owned dogs as have dogs which are largely housed outdoors. Lastly, dogs with clinical signs associated with a vector-borne-disease have higher seroprevalence rates in comparison with healthy dogs. Dogs in the current study were rescue dog, and many of them were likely shelter dogs or stray dogs.

Another factor that might contribute to the unexpected high seroprevalence in this study might be the test characteristics.
The IFA test is a serologic method that is considered the “golden standard” indicating exposure to *E. canis* rather than active infection. An IgG titer of $\geqslant$ 1:40 is indicative of previous exposure to *E. canis*. It must be noted that a positive IFA test titer is not necessarily related to an active state of CME, as antibodies can persist long after elimination of *E. canis*. Only increasing titers after two consecutive IFA tests 1-2 weeks apart and a 4-fold increase in antibody titers are suggestive of an active infection (Bartsch & Greene, 1996). During this study only two titer dilutions were performed, but for further research more dilutions are advisable to interpret

results more accurately. Furthermore, the IFA test is dependable on visual interpretation from the researcher and thus is subject to interobserver variability. Also, cross-reactivity with *A. phagocytophilum* may have occurred, affecting the specificity of the IFA test for the detection of *E. canis*, as *A. phagocytophilum* is found globally, including the Netherlands. (Karshima et al., 2022) Nevertheless, these cross-reactivities have been considered to occur rarely and in hyperimmunized sera only or be due to non-specific antibody bindings (Nicholson et al., 1997; Dumler et al., 1995). Although in another study cross-reaction between *E. canis* and *A. phagocytophilum* has been documented to occur over time. All dogs were seropositive for *A. phagocytophilum* 150 days post-inoculation with. *E. canis*, while also remaining seropositive for *E canis* (Waner et al., 1998). In this study it was proposed that development of these cross-reactive antibodies may be dependent on persistence of infection with *E. canis*. This study suggested that *E. canis* IgG antibodies to also bind to antigen of the *A. phagocytophilum* genus. It is unknown if the reverse is also true; if these antibodies are also produced during infection with *A. phagocytophilum* that can cross-react with *E. canis* antigen. Therefore, possible cross-reactivity with *A. phagocytophilum* infected dogs is unlikely but may not be excluded. Cross-reactivity with other Ehrlichia species of the same genus as *E. canis*, such as *E. chaffeensis*, *E. ewingii and E. equi* is possible (Beall et al., 2012; Unver et al., 1999; Murphy et al., 1998; Goldman et al., 1998; Breitschwerdt et al., 1998; Rikihisa et al., 1994). However, *E. chaffeensis, E. ewingii* and *E. equi* have not yet been isolated in dogs in Europe (Sainz et al., 2015; Shaw et al., 2001; Dumler & Bakken 1995).

Both ELISA and PCR are sensitive methods for detecting *E. canis* infection, though it is important to note that the sensitivity of ELISA may not be as high as PCR and IFA. PCR has the advantage of early detection, often within 4-10 days after inoculation. This makes PCR particularly advantageous for diagnosing *E. canis* infection in the acute phase before seroconversion occurs when antibody levels are too low to be detected by serological tests. A combination of both molecular and serological tests can increase the chances of acquiring the most reliable results in combination with the history, clinical signs and laboratory finding for an individual patient (Waner et al., 2022, Harrus & Waner, 2011; Iqbal et al., 1994). For this study only the use of a serological method was sufficient to examine seroprevalence in the examined population.

During this study serum samples were stored at a temperature of -20 ºC and undergone multiple freeze-thaw cycles, because of necessary transportation between laboratory buildings and sorting of samples. While it is common to assume extensive antibody stability, the evidence is scarce for human serum and even non-existent for the veterinary industry. Two studies done with examining the stability of human antibodies found that after multiple freeze and thaw cycles of serum samples refrigerated (2-8ºC) or freezed (-20 ºC) were still reliably analysable as fresh sample (Demir et al., 2014; Castro & Jost, 2013).

Interestingly, in the present study 7.2% (n=12) of dogs infected with *E. canis* seroconverted while 42.2% (n=19) dogs seroreverted.

These shifts in immunostatus likely stem from false-negative or false-positive outcomes. However, it is conceivable that the twelve dogs initially testing negative during the initial consult and later seroconverting were still in the incubation period, thus only developing measurable *E. canis* antibodies by the follow-up consultation. Employing a combination of

serologic tests (such as IFA or ELISA) and molecular assays (like PCR) could have aided in distinguishing between false-negative results and dogs in the incubation period when clinical signs are absent. Given the high sensitivity of the IFA test, false positives may explain why these dogs initially tested negative and then tested positive during the follow-up consultation. Additional factors contributing to false-positive results might include cross-reactivity, non-specific antibody binding, or serum contamination. Moreover, antibodies can naturally decrease over time, or dogs may have undergone treatment, although antibody levels may remain elevated for an extended period, even after treatment (Ojeda-Chi et al., 2019; Waner et al., 2001).
The primary aim of this research was to screen dogs and determine the prevalence of *E. canis* exposure among imported dogs, rather than diagnosing individual cases. Therefore, it was not necessary to establish *E. canis* as the causative agent for possible clinical signs, but only to access possible (past) exposure, especially considering that antibody levels may remain elevated for an extended duration after exposure. In a clinical context, it is crucial to retest dogs displaying clinical signs of CME or non-specific symptoms who initially tested negative using serologic methods. The recommended practice is to retest these dogs within 1-3 weeks (Dubie et al., 2014).

Infection with *E. canis* can lead to a range of clinical signs and symptoms, of which many are non-specific (Harrus et al., 1999). In this study, during the initial consultation, lymphadenomegaly of the prescapular lymph nodes, and during the second follow-up consultation, lymphadenomegaly of the popliteal lymph nodes were identified as predictive variables associated with an *E. canis* infection. Seropositive dogs during the initial consultation had a 2.59 higher likelihood of having enlarged prescapular lymph nodes, while dogs during the follow-up consultation were 5.10 times more likely to display enlarged popliteal lymph nodes compared to seronegative dogs. Both of these findings suggest a correlation between lymphadenomegaly and exposure to *E. canis*.
However, no association could be found between other variables. Thrombocytopenia for instance was not associated in the current study with *E. canis* seropositivity. It is conceivable that during this study, most seropositive dogs were in the subclinical phase of the disease, which can be lengthy. During the subclinical phase, milder cases of thrombocytopenia or even the absence of it are commonly reported (Harrus et al., 1999). Hence, it is possible that mild cases of thrombocytopenia were missed during the logistic regression as thrombocytopenia was set at a value below $80x10^9$/L, which was stricter than what the University Veterinary Diagnostic Laboratory references ($144x10^9$/L) as cut-off value. Lowering the cut-off value was done to enhance the sensitivity of this parameter in detecting *E. canis* infection (Bulla et al., 2004).
In the study of Waner et al., (1997), all Beagle dogs experimentally infected with *E. canis* exhibited decreased platelet counts, although none of the values ranged below the $130x10^9$/L. It is plausible that, as most dogs were likely in the subclinical phase in the current study, significant associations with clinical symptoms and seropositive dogs, aside from lymphadenomegaly, were not observed. The study of Rodríguez-Alarcón et al., (2020) found that during the subclinical phases, PCR testing on blood samples yielded negative results, whereas positive results were obtained when biopsies of bone marrow, liver, spleen, or lymph

nodes were tested. It may be possible that therefore the only significant clinical symptom in this study may be the lymphadenomegaly.
Coinfections with *L. infantum* and *E. canis* are reported commonly as vector activity and transmission periods are similar (Mekuzas et al., 2009). During the current study no association was found between *L. infantum* and *E. canis* at the initial consultation. At the follow-up consultation 21.1% (p=0.040) of *E. canis* seropositive dogs, tested positive for *L. infantum*. A possible reason for the difference between the two moments might be that leishmaniasis can remain undiagnosed for a long time as the incubation period and seroconversion can take a long time (Foglia Manzillo et al., 2013). The study of Mekuzas et al., (2009) endorses this finding, as *E. canis* significantly proceeded the *L. infantum* infection in 82% of the cases.
Several studies conducted in different regions and countries have found that dogs seropositive for *E. canis* were significantly more likely to be seropositive for *L. infantum* (Ramos et al., 2022; Montoya-Alonso et al., 2019; Toepp et al., 2019; Attipa et al., 2018; Mekuszas et al., 2009). Furthermore *E. canis* infected dogs are also found to be occasionally coinfected with *D. immitis* (De Tommasi et al., 2013, Ramos et al., 2022). Like *E. canis*, *H. canis* is also transmitted by *R. sanguineus* and autochthonous in southern Europe (Schäfer et al., 2019; Giannelli et al., 2013). Coinfections of *E. canis* and *H. canis* have been documented (Sukara et al., 2023; Attipa et al., 2017; Baneth et al., 2015; Mylonakis et al., 2005).
In the current study the prevalence of *D. immitis* as well as *H. canis* were very low, therefore no conclusions can be drawn for these infections on behalf of data shown in this study. Furthermore, the detection of *H. canis* was no primary goal of the present study, rather it was found as incidental findings on blood smears already conducted during the study.

This study has some limitations. Firstly, the majority of dogs (94.2%) were sourced through stray animal foundations. This could introduce selection bias, as these foundations might have predominantly attracted owners of sicker dogs or dogs that had previously tested positive for a CVBD in their country of origin. Additionally, it is possible that some dogs received treatment for *E. canis* in their country of origin, which could potentially skew the results in this study. Treatment with doxycycline, for example, can lead to a gradual decrease in IgG antibodies against *E. canis*, although this effect varies among individual dogs and decrease might be slow (Sainz et al., 2000). Furthermore, changes in the electronic health records system during the study period may have affected data quality. In the old system, fixed forms with predefined options for each clinical observation were used, while the new system required examiners to manually complete the examination form. This change may have led to inconsistencies in data documentation, with examiners potentially forgetting or omitting certain aspects of the physical exam.

Conclusion

This is the first study revealing the seroprevalence of *E. canis* in dogs imported from the Mediterranean basin to the Netherlands. The research revealed a seroprevalence of 22.4% during the initial consultation and 17.9% during the follow-up consultation. Notably, dogs with lymphadenomegaly were at a higher risk of *E. canis* infection. Additionally, Greek dogs exhibited a higher seropositivity rate compared to those from Spain and Portugal, with one in three Greek dogs testing seropositive during the initial consultation. Lastly, *E. canis* infection was correlated with *L. infantum* infection during the follow-up consultation ($p=0.040$). This study demonstrates the importance for veterinarians to include CVBDs in their differentials and to inform potential owners of imported dogs about the vector-borne diseases and risks.

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Appendix A

ACE (Animal Care España)
Adoption Dogs Portugal
Álora Dog Rescue
Animal in Need (Second Chance Foundation Nederland)
Stichting Monemvasia Dog Rescue
Stichting Hondjes Protectora Arca International
Asociacion Arca Noah
Atlas Animal Project
Dogs and Cats on the move Portugal
Dierenhulp zonder Grenzen Internationaal
Stichting Dogateers United
Stichting Filosdogrescue
Stichting GaGa Animal Care
Grenzeloze Dierenvrienden Nederland
Stichting Greyhounds Rescue Holland
Greyhounds in Nood Nederland
Stichting Hobodogs
Stichting Hond Zoekt Huis
Stichting Hondenzorg & Welzijn
Hope for Homeless Cyprus
Humans for dogs
IARA - International Animal Rescue Alliance Norge
KWSPL.
Stichting Dierensteun La Vida
Mastines en mi Salón
Stichting Dierenhulp Mirbessa
Stichting Mirtos Animal Project
Stichting Modestos
Stichting Perro&Co
ProZUS (Pro Zwerfdieren Uit Spanje)
Puntanimals
Stichting Pups voor Adoptie
Stichting Sphoek
Stichting Galgo Project
Stichting story of the strays
Stichting Straathonden van Lesbos
Kaya VagabunDOGs
Stichting We Are Here Lesvos
Stichting Dog Rescue Greece
Zante Strays
Stichting For The Strays – Paws of Greece

Appendix B

Clinical History

- Social attitude towards humans and other dogs?
- Information about food intake (e.g. anorexia/hyporexia, type of food)
- Information about water intake
- Information about urination (e.g. polyuria, stranguria, pollakisuria etc.)
- Information about defecation (e.g. diarrhoea, straining etc.)
- Information about possible vomiting (e.g. frequency)
- Information about possible dyspnoea and/or coughing
- Information about possible pruritus
- Information about locomotion abnormalities (e.g. lameness, stiffness, frequency)
- Information about stamina
- Medical history of the dog (previous or other present diseases, medication, and vaccination)

Appendix C

1. Mix 1 ml of EDTA blood with 9 ml of 2% formaldehyde by swirling the tube five times (with the cap on or using a gloved finger). If the hematocrit is greater than 0.45, let the formaldehyde solution sit for an additional 2 minutes*.
2. Centrifuge at room temperature (21 °Celsius) for 5 minutes at 1000 - 1500 rpm.
3. Pipette off the liquid until you have exactly 1 ml.
4. Apply 1 drop of the liquid onto a microscope slide and cover it with a cover slip.
5. Mix the remaining ml of liquid with approximately 8 drops of methylene blue.
6. Apply 1 drop of the mixed liquid onto a microscope slide and cover it with a cover slip.
7. Examine under a microscope at 10x and/or 40x magnification.

(* In cases where the hematocrit is 0.45 or higher, not all erythrocytes may be lysed by the formaldehyde initially. Allowing the formaldehyde to work for an additional 2 minutes ensures proper lysis. If, after this, the erythrocytes are still not sufficiently lysed under the microscope, you may observe large clumps of different layers of intact erythrocytes, making the interpretation of the image less reliable. In cases where the hematocrit is within the normal range, you can proceed with centrifugation directly.)

Appendix D

History of (during initial and follow-up consultation):

- Weight loss
- Anorexia
- Locomotion abnormalities
- Polyuria/polydipsia
- Diarrhoea
- Pruritus
- Ocular problems
- Nail abnormalities
- Vomiting
- Coughing

Physical examination (during initial and follow-up consultation):

- Respiration
    - Frequency
    - Depth
    - Type
- Pulse
    - Amplitude
    - Equality
    - Shape and symmetry
    - Regularity and rhythm
    - Pulse deficit
- Temperature
- Mucous membranes
    - Colour
    - Moisture
    - Bleeding and ulceration
- Lymph nodes
    - Mandibular
    - Prescapular
    - Accessorial
    - Axillar
    - Retropharyngeal
    - Inguinal
    - Popliteal
- Dermatology
    - Coat
        - Alopecia
        - Dullness
        - Hypotrichosis
        - Ectoparasites
        - Change in colour
        - Loose hair
    - Skin
        - Morphology

                - Squamae
                - Pustulae
                - Papulae
                - Crustae
                - Hyperkeratosis
                - Lichenfication
                - Collarettes
                - Excoriations
                - Ulceration
                - Noduli
                - Comodones
                - Acanthosis
                - Cysts
                - Tumour
                - Thickening of the ear margins
                - Vesiculae or bullae
                - Maculae
            - Skin colour
                - Erythematous
                - Hyperpigmentation
                - Depigmentation
            - Skin scent
            - Turgor
            - Elasticity
            - Skin thickness
            - Skin temperature
            - Skin sensibility
        - Nail abnormalities
- Circulation
    - Capillary system
        - Temperature of the extremities
        - Capillary refill time
    - Venous system
        - Episcleral veins
        - Jugular venous pressure and pulsation
        - Oedema
        - Abdominal circumference
        - Liver enlargement
        - Splenomegaly
    - Heart
        - Palpation of ictus cordis
        - Fremitus
        - Heart murmur

<u>Clinicopathological examination (during initial and follow-up consultation):</u>

- Anaemia
- Leukopenia
- Leucocytosis
- Thrombocytopenia