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Molecular detection of Spirochetes and *Borrelia burgdorferi* in stray dogs of Nineveh province, Iraq

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Abstract

Background: *Borrelia burgdorferi* is a Gram-negative bacterium that causes Lyme disease or borreliosis in domestic and wild animals, including dogs, with the possible transmission to humans.

Aim: This study was conducted to investigate the infection rate of Spirochetes and B. burgdorferi in stray dogs in Nineveh province, Iraq.

Methods: During the period from May to October (2022), a total of 55 stray dogs were selected randomly from different areas in Nineveh province, Iraq. Blood samples were collected from cephalic venous and tested molecularly using the conventional polymerase chain reaction technique.

Results: The present study revealed that the total infection rates of Spirochetes and *B. burgdorferi* were 41.82% and 27.27%, respectively. Concerning age, values of infection rate, odds ratio, and relative risk of *B. burgdorferi* were increased significantly in dogs aged ? 4 months (42.86%, 3.505%, and 2.438%, respectively), while decreased in dogs of ? 1–3 (12.5%, 0.337% and 0.42%, respectively) and ? 3 (13.33%, 0.32% and 0.409%) years old when compared to dogs aged 5–12 months (27.27%, 1% and 1%, respectively). While concerning dogs sex, a significantly higher infection rate, odds ratio, and relative risk of *B. burgdorferi* were shown in females (32.56%, 5.495% and 6.792%, respectively) compared to males (8.33%, 0.182% and 0.147%, respectively).

Conclusion: To the best of our knowledge, this represents the first Iraqi study on the prevalence of spirochetes, in particular *B. burgdorferi*, in stray dogs in Nineveh province (Iraq). However, additional studies of *B. burgdorferi* infection in other animals as well as vectors such as ticks in different geographic areas, appear necessary to detect variation in the distribution patterns of infection. In addition, owners and veterinarians should be aware of zoonotic diseases transmitted from wild and domestic animals, in particular those with tick-bite histories.

Keywords: Spirochetes, Lyme disease, *Borrelia burgdorferi*, Tick-transmitted diseases, Iraq.

Introduction

Spirochetes are the etiological agents of several important animal and human diseases such as Leptospirosis (*Leptospira* spp.), Lyme disease (LM) (*Borrelia burgdorferi*), and syphilis (*Treponema pallidium*), which exhibit unique morphological and physiological features (San Martin *et al.*, 2022). *Borrelia burgdorferi*, a Gram-negative bacterium belonging to the Borreliaceae Family in the Spirochaetia Class, is a tick-borne multi-systemic zoonotic pathogen that causes (LM or Lyme Borreliosis (Borreliosis) in many countries, worldwide (Mafra and Montandon, 2017). Horizontal transmission is the main method of *B. burgdorferi* transmission within wild animals and between ticks (Milkovičová *et al.*, 2023). In most cases, *B. burgdorferi* invades salivary glands as well as the midgut of ticks (*Ixodes pacificus, Ixodes scapularis, Ixodes ricinus, and Ixodes persulcatus* called (deer tick, or black-legged tick), and delivered routinely through the transstadial the transstadial transmission (Başbulut et al., 2012; Divers *et al*., 2018).

In dogs of all ages, early infection is characterized by high temperature, loss of appetite, dullness, lymphadenopathy, and acute onset of pain or stiffness, while dogs that are infected acutely generally do not exhibit swollen joints and are usually difficult to estimate the origin of pain (Ebani *et al.*, 2014; Bajer *et al.*, 2022). In addition, lameness might be intermittent and shift from one leg to another (Divers *et al.*, 2018). At the same time, advanced manifestations of Lyme borreliosis are recurrent and non-erosive arthritis, recurrent episodes of lameness with involvement of multiple joints, particularly the carpus joint (Verbsky, 1997; Clausen *et al.*, 2020). The association between

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these lesions and erythema chronicum migrans has not been established. In addition, dogs living in endemic areas have displayed increasing erythematous rashes on the abdomen or other sparsely hairy areas. These migrans are distinct from a limited response to a tick bite due to their spreading nature (O'Connell, 2014; Quarsten *et al.*, 2017). Myocardial dysfunctions, including atrio-ventricular heart block, myocardial necrosis, and vegetative endocarditis (Saunders *et al.*, 2013; Adaszek *et al.*, 2020), as well as renal (Rolla *et al.*, 2014) and neurologic (Han *et al.*, 2015; Borys *et al.*, 2019) diseases, have been noted in some experimentally and naturally infected dogs for *B. burgdorferi*.

In stray dogs, the problems with the interpretation of different diseases have stemmed from the lack of data concerning the clinical symptoms after being naturally infected. Without a pathognomonic marker for canine *B. burgdorferi* infections, definitive advanced tests such as serological and molecular methods have been proposed to investigate and confirm active and subclinical infections, but with variable findings (Gettings *et al.*, 2019; Bajer *et al.*, 2022). However, valuable sensitivity and specificity of molecular diagnostic techniques such as polymerase chain reaction (PCR) have proven useful in the identification and differentiation of *Borrelia* species over the serological assays (Barth *et al*., 2012; Maggi *et al*., 2014; Koetsveld *et al.*, 2016; Das *et al.*, 2020). In Iraq, few studies were conducted to detect the prevalence rate of *B. burgdorferi* in humans (Ameen *et al.*, 2013; Madhi *et al.*, 2019), but not in dogs. Hence, this study aimed to investigate Spirochetes and *B. burgdorferi* in stray dogs in Iraq.

Materials and Methods

Animals and samples collections

The current study involved 55 stray dogs selected randomly from various regions in Nineveh province, Iraq, during the period between May and October (2022). Under the sedative effect of xylazine (1 mg/Kg BW), 2.5 ml of cephalic venous blood was sampled from each dog using a disposable syringe, then the blood was transferred into an EDTA tube and kept frozen at 4°C until tested.

Data regarding the sex of the study animal were determined based on observations of external sex organs and physical characteristics of the abdomen, while age was evaluated according to the dental formula for deciduous and permanent teeth of dogs (Gracis, 2018).

Molecular diagnosis

Blood samples were prepared and processed following the blood protocol procedure of gSYNC™ DNA Extraction Kit (Geneaid Biotech, Korea). The concentration (ng/ml) and purity of obtained DNAs were checked using the NanoDrop spectrophotometer (Thermo Scientific, UK) at an absorbance of A260/280. At a final volume of 20 ml, the MasterMix tubes were prepared following the manufacturer's instructions of the AccuPower PCR PreMix Kit (Bioneer, Korea) targeting the *16S rRNA* gene detect the Spirochetes [SPIRO: F (5′-GGC GGC GCT ATT AAG-3′) and SPIRO: R (5′-TAC CTT GTT ACG ACT TCA-3′)] (Campbell and Cary, 2001), and *B. burgdorferi* [BOR: F (5′-GCT GTC AGT GCG TCT TAA G-3′) and BOR: R (5′-CTT AGC TGC TGC CTC CGT A-3′)] (Skotarczak *et al.*, 2005). For DNAs amplification, conventional PCR-reaction was carried out in the Thermocycler System (BIO-RAD, USA) for both Spirochetes and *B. burgdorferi,* respectively, as 1 cycle for initial denaturation (95°C/5 minutes), 30 cycles for denaturation (95°C/20 seconds), annealing (51°C and 56°C/1 minute), and extension (72°C/1 minute); and 1 cycle for final extension (72°C/10 minute). Electrophoresis of 1.5% agarose gel stained with SYBR Safe DNA Gel Stain (ThermoFisher Scientific, USA) at 100 V, 80 Am for 1 hour was performed to analyze the PCR products. Finally, the product sizes of amplified DNAs were visualized using the UV illuminator (Clinx Science, China) and photographed by a digital camera (Nikon, Japan). The positive samples for Spirochetes were identified at product 650 bp while, for *B. burgdorferi* of 230 bp.

Statistical analysis

Data risk was analyzed using the *chi-square* test and odds ratio in the GraphPad Prism Software (GraphPad Software Inc., USA) to determine variation between specific groups for each age, and sex factors were considered significant at $p < 0.05$ (Gharban, 2023).

Ethical approval

The current study was authorized and conducted by the license of the Scientific Committee of the College of Veterinary Medicine, University of Mosul, Nineveh province, Iraq. (UM.VET.2022.064) on 17th of April 2022.

Results

Among a total of 55 stray dogs tested for detection of Spirochetes and *B. burgdorferi* by PCR assay, the total positive rate was 23 (41.82%) and 15 (27.27%), respectively (Figs. 1 and 2).

Concerning age factor, *B. burgdorferi* increased significantly ($p < 0.0487$) in dogs aged £4 months [9/21 (42.86%)] and decreased significantly in dogs aged ^{3}1–3 years [1/8 (12.5%)] and ^{3}3 years [2/15 (13.33%)] when compared to dogs aged 5–12 months [3/11 (27.27%)]. Higher odds ratio and relative risk (0.0002 and 0.0003, respectively) values were seen in dogs aged £4 months (3.505 and 2.438, respectively) than 5–12 months old (1.523 and 1.333, respectively), ^{3} 1–3 years old (0.305 and 0.392, respectively), and ^{3}3 years old (0.286 and 0.381, respectively), (Figs. 3 and 4).

Moreover, the association of sex to *B. burgdorferi* showed a significant elevation ($p < 0.0354$) in positive females [14/43 (32.56%)] in comparison with males [1/12 (8.33%)]. In addition, values of odds ratio and relative risk were significantly higher in females (5.495

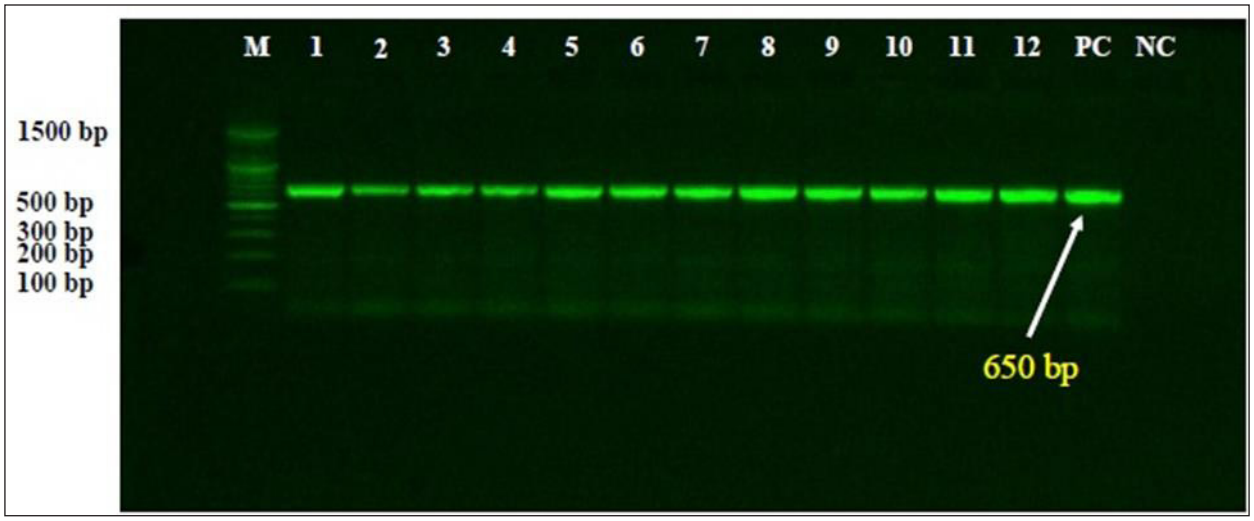

Fig. 1. Representative image for electrophoresis of PCR products in 1.5% agarose gel to detect amplified DNAs of Spirochetes targeting 16S rRNA gene. While, Lane M: Ladder marker (100–1,500 bp); Lane PC: Positive control, Lane NC: Negative control, Lanes 1–12: Positive samples of Spirochetes at 650 bp.

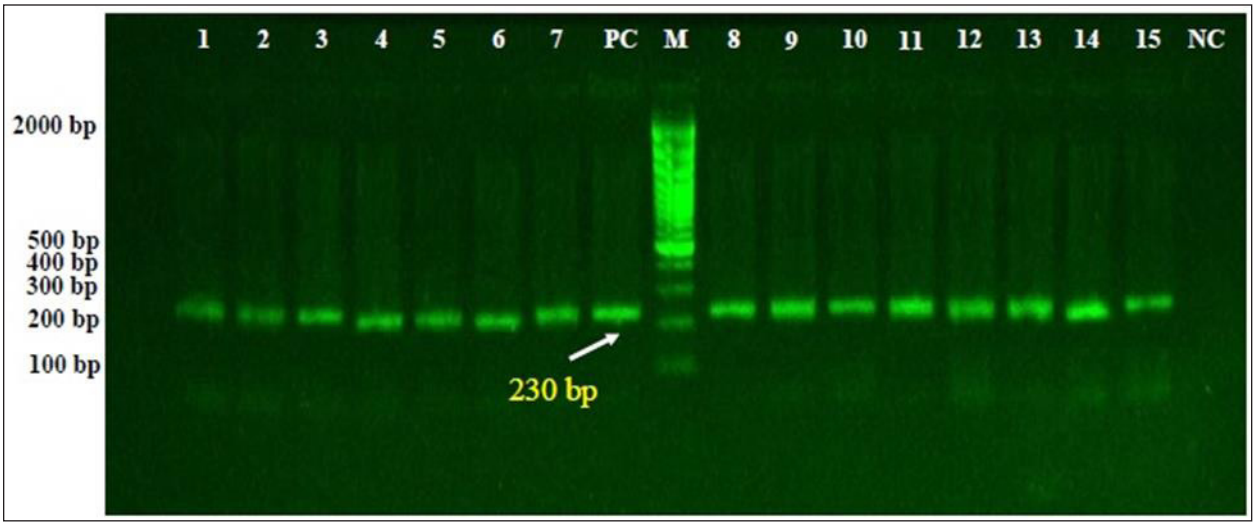

Fig. 2. Electrophoresis of PCR products in 1.5% agarose gel to detect amplified DNAs of B. *burgdorferi* targeting 16S rRNA gene. While Lane M: Ladder marker (100–2,000 bp); Lane PC: Positive control, Lane NC: Negative control, Lanes 1–12: Positive samples of B. *burgdorferi* at 230 bp.

and 6.792, respectively) than in males (0.182 and 0.147, respectively) (Fig. 5).

Discussion

Several worldwide studies attempted to identify or isolate Spirochetes and *B. burgdorferi* from different samples (blood or joint and cerebrospinal fluids) of patients, which often failed due to the low number of organisms found in tested samples, lack of ideal culture conditions, long time consumed, and excessive laboratory steps in addition to the risk of infection of transmission for workers. In the last decades, molecular techniques have been provided highly sensitive, specific, and safe alternative tools for the diagnosis of agents such as Spirochetes and *B. burgdorferi* in humans and animals (Straubinger *et al.*, 1997; Straubinger, 2000; Coulter *et al.*, 2005; Anthony *et al.*, 2013; Bil-Lula *et al.*, 2015). Duhamel *et al.* (1998) confirmed that the large intestines of dogs, like those of humans and other animals, including birds, are colonized by more than one species of spirochete. The present study reported that the prevalence rate of *B. burgdorferi* in dogs by PCR assay was 29.09%. In other studies, there were 0.1%–17.3% in Mexico (Solís-Hernández *et al.*, 2018; Bedoya *et al.*, 2023), 0.4%–

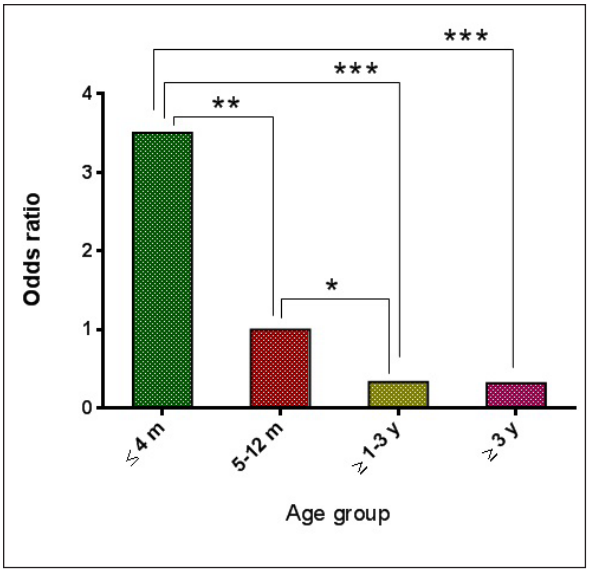

Fig. 3. Results of odds ratio in different age groups.

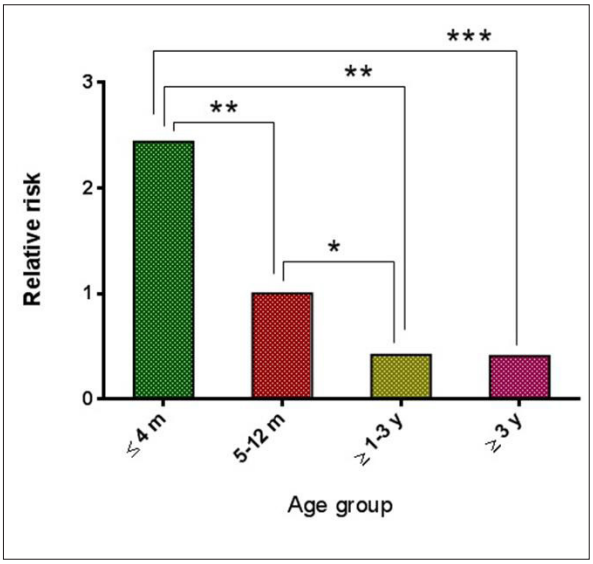

Fig. 4. Results of relative risk in different age groups.

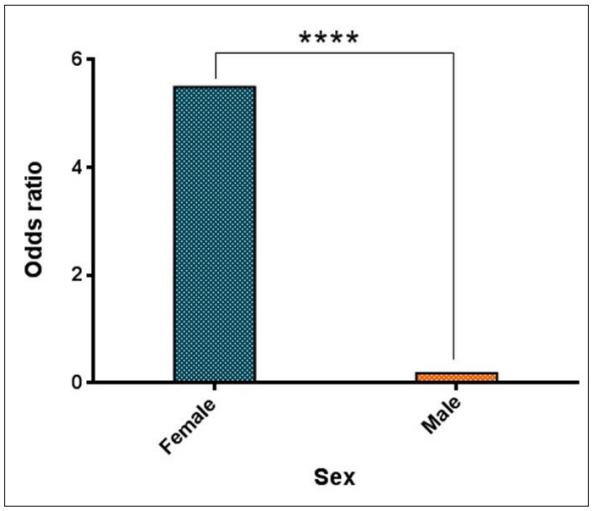

Fig. 5. Results of odds ratio in different sex groups.

40% in Croatia (Poljak *et al.*, 2000; Jurković *et al.*, 2019), 0.6% in Romania (Cazan *et al.*, 2020), 0.9% in China (Wang *et al.*, 2018), 1.09% in France (Pantchev *et al.*, 2009), 1.47% in Italy (Ebani *et al.*, 2014), 2.049% in Latvia (Berzina and Matise, 2013), 2.4%–74.5% in Bulgaria (Angelov *et al.*, 1993; Pantchev *et al.*, 2015), 2.8%–45.3% in Slovakia (Stefancikova *et al.*, 1996; Čabanová *et al.*, 2015), 3.75%–33.7% in Poland (Skotarczak *et al.*, 2005; Cardoso *et al.*, 2012), 6.4% in South Korea (Lee *et al.*, 2020), 6.5%–53.7% in Czech Republic (Sýkora *et al.*, 1990; Pejchalová *et al.*, 2006), 9.52% in Iran (Mosallanejad *et al.*, 2015), 10.2%–27.3% in Japan (Arashima, 1991; Uesaka *et al.*, 2016), 16.8% in Sweden (Egenvall *et al.*, 2001), 17% in Netherlands (Goossens *et al.*, 2001), 21% in Spain (Amusategui *et al.*, 2008), 38.22% in Turkey (Altug *et al.*, 2022), 23–56.9% in USA (Guerra *et al.*, 2000; Wagner and Erb, 2012), 25.81% in Serbia (Savić *et al.* 2010), 31.3% in Brazil (Nascimento *et al.*, 2016), and 35.5% in Germany (Barth *et al.*, 2012). This variation confirmed that the prevalence rate of *B. burgdorferi* in dogs is different between regions, countries, and years, which might be attributed to the diagnostic method used, the presence of vectors as ticks, geographical characteristics, consequence of environmental factors, and diversity in the epidemiology of infection.

Concerning age, we found that there was an increase in the prevalence of *B. burgdorferi* in dogs aged £4 and 5–12 months when compared to older age groups, indicating that younger dogs are more susceptible to *B. burgdorferi* infection than older ones.

Skotarczak *et al.* (2005) reported that the highest percentage of positive dogs was in groups of 2–5 and 5–8 years old, while the lowest was in groups of 0.5–1.5 and >8 years old. Several authors demonstrated that the threshold for dogs developing a stable immune response is their age over 1 year (Stefancikova *et al.*, 1996; Merino *et al.*, 2000). Other researchers indicate that seropositive response in dogs stabilizes itself from their second year of life (Schulze *et al.*, 1987; Lindenmayer *et al.*, 1991; Hovius *et al.*, 1999; Goossens *et al.*, 2001). In contrast, Mosallanejad *et al.* (2015) showed that seropositivity against *B. burgdorferi* was significantly higher in adult dogs above 5 years compared with dogs 1–5 years and <1-year old while, Jung *et al.* (2012) revealed a lack of significant variation between different age groups (<2, 2–5 and >5 years old).

We observed female stray dogs have a higher infection rate and relative risk than males, which might be caused by the greater exposure of females to ticks and

pathogens, physiological burden due to pregnancy and suckling (stress factor), and larger sample size of females than males (Bowman *et al.*, 2009). According to many reports, sex factors do not have an effect on the frequency of disease in dogs infected naturally with *B. burgdorferi* (Stefancikova *et al.*, 1996; Jung *et al.*, 2012; Merino *et al.*, 2000; Skotarczak *et al.*, 2003). In contrast, Mosallanejad *et al.* (2015) showed that seropositivity against *B. burgdorferi* was significantly higher in male dogs than in females, significantly.

Conclusion

To the best of our knowledge, this represents the first Iraqi study on the prevalence of spirochetes, in particular *B. burgdorferi*, in stray dogs in Nineveh province (Iraq). However, additional studies of *B. burgdorferi* infection in other animals and vectors, such as ticks in different geographic areas, appear necessary to detect variation in the distribution patterns of infection. In addition, owners and veterinarians should be aware of zoonotic diseases transmitted from wild and domestic animals, in particular those with tick-bite histories.

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Authors contributions

Both authors contributed to conceptualization. Eva Aisser Ajaj and Zahraa Mustafa Al-Jumaa: Collection of blood samples, molecular testing of blood samples, and statistical analysis of data with approval of the final copy of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Data availability

All data is available in this manuscript.

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