

First Serological and Molecular Detection of *Leptospira interrogans* serovar *canicola* Bacteria in Dogs in Some Iraqi Governorates

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Abstract

The aim of present study was to serodetect of canine leptospirosis by using of indirect ELISA, and identification of *Leptospira interrogans* serovar *canicola* in seropositive dogs by application of PCR technique. For this purpose, 218 dogs from urban and rural regions related to three governorates were submitted for blood samples collection. The total results were revealed on 37/218 (16.97%) and 5/37 (13.51%) infected positive dogs by an indirect ELISA and PCR techniques, respectively. According to subjected study's governorates, the positive results of indirect ELISA in Baghdad, Al-Qadisiyah and Dhi-Qar were 23/108 (21.3%), 10/79 (12.66%), and 4/31 (12.9%), respectively; while by PCR assay, the positive results {5/23 (21.74%)} had been detected in Baghdad only. Also, the relationship of positive dogs with some epidemiological risk factors has been discussed in this study. In association to inhabitant type, the rural and urban regions, respectively, were having 16/82 (19.51%) and 21/136 (15.44%) positive dogs by indirect ELISA; whereas, they have 4/16 (25%) and 1/21 (4.76%) positive dogs by PCR, respectively. In regarding to sex factor, the positive infected males and females, respectively, were amounted 13/67 (19.4%) and 24/151 (15.89%) by indirect ELISA; and 2/13 (15.38%) and 3/24 (12.5%) by PCR. In relation to age factor, >2years and ≤2 years groups have taken 36/149 (24.16%) and 1/69 (1.45%) positive dogs by indirect ELISA and 5/36 (13.89%) positive dogs for >2years group, only, by PCR assay.

Statistically, the positive results were reported significant differences at level of $P \leq 0.05$ between the study's regions and between the groups related to each epidemiological risk factor.

Keywords: *Leptospira interrogans*, *Canicola*, dogs, Serological, Molecular, Iraq

الكشف المصلي والجزيئي الاول لبكتيريا *Leptospira interrogans serovar canicola* في الكلاب في بعض محافظات العراق

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الخلاصة

هدفت الدراسة الحالية الى التحري المصلي لداء الليبتوسبايرا الكلابية باستعمال indirect-ELISA، وتأكيـد الاصابة بالنوع المصلي *Leptospira interrogans serovar canicola* في الكلاب الموجبة مصليا باستعمال تقنية PCR. لهذا الغرض، خضع ٢١٨ كلبا تعود الى مناطق ريفية ومدنية تابعة لثلاث محافظات الى جمع عينات الدم . كشفت النتائج الكلية عن ٢١٨/٣٧ (١٦,٩٧%) و ٣٧/٥ (١٣,٥١%) كلبا موجبا للاصابة باستعمال تقنيتي indirect-ELISA و PCR ، على التوالي. اعتمادا على المحافظات الخاضعة للدراسة ، كانت النتائج الموجبة بواسطة indirect-ELISA في بغداد والقادسية وذي قار هي ١٠٨/٢٣ (٢١,٣%) ، و ٧٩/١٠ (١٢,٦٦%) ، و ٣١/٤ (١٢,٩%) ، على التوالي ؛ بينما بواسطة PCR، فقد حددت النتائج الموجبة { ٢٣/٥ (٢١,٧٤%) } في بغداد فقط. كذلك، تمت في هذه الدراسة مناقشة علاقة الكلاب الموجبة مع بعض عوامل الخطر البيئية . فيما يخص نوع الموطن، فقد امتلكت المناطق الريفية والمدنية ، على التوالي ، ٨٢/١٦ (١٩,٥١%) و ١٣٦/٢١ (١٥,٤٤%) كلاب موجبة بواسطة indirect ELISA ، في حين انها بلغت ١٦/٤ (٢٥%) و ٢١/١ (٤,٧٦%) كلاب موجبة بواسطة PCR، على التوالي . فيما يخص عامل الجنس ، بلغت الذكور والاناث الموجبة للاصابة ، على التوالي ، هي ٦٧/١٣ (١٩,٤%) و ١٥١/٢٤ (١٥,٨٩%) بواسطة indirect-ELISA ، و ١٣/٢ (١٥,٣٨%) و ٢٤/٣ (١٢,٥%) بواسطة PCR . فيما يخص عامل العمر، امتلكت مجموعتي < ٢ سنة و ≥ ٢ سنة ١٤٩/٣٦ (٢٤,١٦%) و ٦٩/١ (١,٤٥%) كلاب موجبة بواسطة indirect-ELISA و ٣٦/٥ (١٣,٨٩%) كلاب موجبة لمجموعة < ٢ سنة ، فقط ، بواسطة اختبار PCR .

احصائيا ، سجلت النتائج الموجبة اختلافات معنوية عند مستوى $P \leq 0.05$ بين مناطق الدراسة وبين المجاميع التي ترتبط بكل عامل خطر وبائي .

الكلمات المفتاحية : *Leptospira interrogans* ، *Canicola* ، كلاب ، مصلي ، جزيئي ، العراق

Introduction

Leptospira is a gram negative, aerobic spirochaetal bacterium that infecting many mammals to cause an acute infectious illness for humans and leading to a potential economic losses and public health issues in domestic and wild animals (1). Although, rats consider the main reservoir and source of human infections, dogs may play a role as pathogen reservoir in the leptospirosis cycle (2). There is general consensus that dogs more frequent exposure to diseases risk and they can act sentinels for environmental contamination and as an indicators for human exposure risk (3). All cases of canine leptospirosis are caused by infection with *Leptospira interrogans* that comprised on 23 serogroups and more than 250 pathogenic serovars distributed worldwide (4). The specific serovars are maintained, naturally, by several sub-clinical infected domestic and wild reservoirs that act as sources of exposure and illness for other incidental hosts (5). The most commonly incriminated serovars in canine leptospirosis are including *Canicola*, *Icterohaemorrhagiae*, *Pomona*, *Bratislava*, and *Grippityphosa* (6).

Although, the diagnosis of leptospirosis is based, mainly, on culturing of blood, urine, and cerebrospinal fluid on specific media, most laboratories don't attempt to isolate of *Leptospira* because of their fragile nature, the cost and complexity of the isolation media, and prolonging of incubation period (7). Hence, the serological tests play an important role in the recognition of leptospiral infection with varying degrees in serogroup and serovar specificity (8). However, two serologically diagnostic tests demonstrated a high capability in detection of infections in veterinary field including microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA) (9). ELISA is intended to use in identification of specific IgM or IgG antibodies against sugar *Leptospira* antigens with high specificity, sensitivity, and objectivity by using serum samples. The only disadvantage of ELISA is that a single genus-specific antigen and it does not give an indication for infecting serovar (10, 11). Nonetheless, amplification of leptospiral DNA by PCR technique is required to demonstrate an infective serovar with a high sensitivity and specificity, and it can detect the pathogen from blood or urine of infected animal (12).

To our knowledge, this is the first study carried out in Iraq for serological detection of canine leptospirosis among stray dogs in some Iraqi governorates by using an indirect-ELISA and confirmation of *Leptospira interrogans* serovar *canicola* in seropositive dogs by using of PCR technique. Also, some epidemiological risk factors and their associations with positive dogs were discussed in this study.

Materials and Methods

1. Study's areas and samples collection

From several rural and urban regions within three Iraqi governorates (Baghdad, Al-Qadisiyah, and Dhi-Qar) and during the period from August 2015 to July 2016, a totally of 218 stray dogs from both sexes, selected randomly, were submitted for this study. From each dog, about 6 ml of venous blood samples was drained by using a disposable syringe. Four ml of each blood sample was inserted into plain tube, allowed to clot, and then centrifuged for 10 minutes at 3000 rpm for serum collection. The serum samples were pipetted into 1 ml appendorff - microtubes and stored at -20°C until tested by ELISA (13). While, the rest two ml of blood sample was saved into an EDTA tube for DNA extraction.

2. Indirect ELISA

The serum samples of all study's dogs were tested by using a monoclonal-mediated ELISA to detect the specific IgG-antibodies against canine leptospirosis (Eurovet Veterinaria-Spain). According to manufacturer's instructions of ELISA kit (Catalog No: D1013-AB01), the test protocol was applied, and the results were read at a wave length of 450nm by using a microplate photometer ELISA-reader (BioTek-USA). Also, the validation and interpretation of test results have been discussed in depending on optical densities of these results as detailed in (Table 1).

Table (1): Validation and interpretation of test results

Validation	
Negative Control	OD < 0.350
Positive Control	OD > 0.700
Interpretation	
Negative Samples	OD _{Sample} < mean OD _{Negative Control} + 0.150
Positive Samples	OD _{Sample} > mean OD _{Negative Control} + 0.250

3. PCR

DNA Extraction: According to manufacturer's instructions, (200µL) of EDTA blood samples were employed for *Leptospira* DNA extraction through using of QIAamp DNA blood mini kit (Qiagen-Germany). The eluted DNA was quantified, and the purity was checked by using of spectrophotometer (BioTek-USA).

DNA Amplification: The amplification was carried out by using two pairs of primers targeting to confirm of *Leptospira interrogans* species and *Canicola* serovar, respectively. These primers were amplifying the DNA products of 547 bp and 793 bp and corresponded to hypothetical open reading frame upstream of conserved ribosomal proteins (GenBank accession number AY622662), and included: rr-outer-F (5'-CTCAGAACTAACGCTGGCGGCGCG-3') and rrs-outer-R (5'-GG TTCGTTACTGAGGGTTAAAACCCCC-3') rrs - inner-F (5'-CTTGATAGAAC CACTGGTGGTGCC-3') and rrs-inner-R (5'-CTGGATCGGTTCCATCGCTCAG-3') (14, 15). The amplification was processed by using of thermal cycler (PTC-100/MJ-BIO RAD / USA) and initiated with one cycle (94°C/5 minutes), followed by 45 cycles (94°C/1 minute), (56°C/1 minute), (72°C/ 90 seconds), and with final elongation one cycle (72°C/10 minutes). The PCR reaction was carried out by using of a thermal cycler (ThermoFisher-USA). *L. interrogans* serovar *canicola* genomic DNA was used as a positive control and the DNAase-free water as a negative control in all PCR runs. The amplified products were separated on 2% agarose-gel stained (Qiagen-Germany). The amplified DNA products were stained with ethidium bromide (Qiagen-Germany) and visualized under Ultraviolet.

Data Analysis

All data were ranged and tabled by using a computerized Microsoft Office Excel (2010) program, while, the results were analysed by using of Chi-Square (χ^2) test of IBM/SPSS computerized program (v23) at a level of $P \leq 0.05$ (16). The statistical differences were estimated between seroprevalence of canine leptospirosis and the results of PCR. Also, some epidemiological risk factors (inhabitant area, sex, and age) were discussed in this study.

Results

In (Table 2): The total seroprevalence results of 218 dogs, tested by using of an indirect ELISA revealed on 37 (16.97 %) dogs were seropositives.

Table (2): Seroprevalence of specific IgG-antibodies against canine leptospirosis

Total No.	Seropositives	Seronegative
218	37 (16.97 %)	181 (83.03 %)

In (Table 3): The totally 37 seropositive dogs by an indirect ELISA were tested by using of PCR technique, and the results showed that 5 (13.51 %) of these dogs were positive, molecularly, for *Leptospira interrogans* serovar *canicola*.

Table (3): Results of PCR technique on seropositive dogs

Total No.	Positives	Negatives
37	5 (13.51 %) ^B	32 (86.49 %) ^A

Variation in large letters, horizontally, referred to significant differences at level of $P \leq 0.05$

In (Fig.1) that explain the positive samples in agarose-gel electrophoresis of PCR products. Whereas, Lane M referred to DNA marker (100-1500bp), Lane (1-5) was

represented the positive samples at 547 bp and 793bp PCR product size at 2% agarose, 100 Volt and 80 Am for 1 hour.

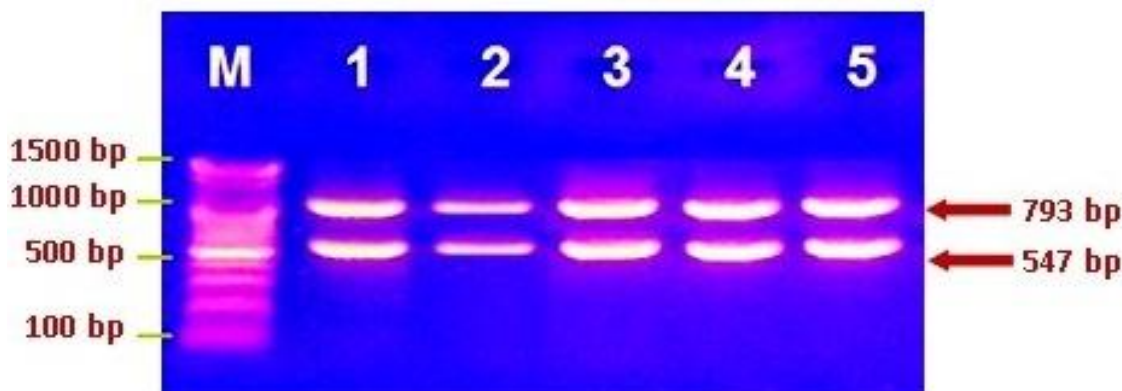


Figure (1): Agarose-gel electrophoresis for PCR products of *L. interrogans* serovar *canicola* positive isolates

In (Table 4): According to governorate, the positive results that detected by indirect ELISA in a totally 108 dogs in Baghdad, 79 dogs in Al-Qadisiyah, and 31 dogs in Dhi-Qar governorates were 23 (21.3 %), 10 (12.66 %), and 4 (12.9 %), while by PCR technique, the positive results were 5 (21.74 %) that showed in Baghdad governorate, only. By both assays, Baghdad governorate were reported a significant increasing in their positive results more than in Al-Qadisiyah and Dhi-Qar governorates ($P \leq 0.05$).

Table (4): Positive results of ELISA and PCR assay, according to study's governorate

Governorate	No.	Seropositives	PCR positives
1 Baghdad	108	23 (21.3 %) ^a	5 (21.74 %) ^a
2 Al-Qadisiyah	79	10 (12.66 %) ^b	0 (0 %) ^b
3 Dhi-Qar	31	4 (12.9 %) ^b	0 (0 %) ^b
Total	218	37 (6.82 %)	5 (13.51 %)

Variation in small letters, vertically, referred to significant differences at level of $P \leq 0.05$

In (Table 5): The associations of positive samples by indirect ELISA and PCR technique with some epidemiological risk factors {inhabitant type (Fig. 1), sex (Fig. 2), and age (Fig. 3)} were detailed as follow:

Table (5): Association of positive dogs by ELISA and PCR to epidemiological risk factors

	Risk factors	No.	Seropositives	PCR positives
1	Inhabitant Type			
	Rural regions	82	16 (19.51%) ^a	4 (25%) ^a
	Urban regions	136	21 (15.44%) ^b	1 (4.76%) ^b
2	Sex			
	Male	67	13 (19.4%) ^a	2 (15.38%) ^b
	Female	151	24 (15.89%) ^b	3 (12.5%) ^a
3	Age			
	> 2 Years	149	36 (24.16%) ^a	5 (13.89%) ^a
	≤ 2 Years	69	1 (1.45%) ^b	0 (0%) ^b

Variation in small letters, vertically, within each factor referred to significant differences at level of $P \leq 0.05$

In (Fig.2) dealt with an inhabitant type risk factor, the animals of study were involved 82 dogs from rural regions and 136 ones from urban regions. The seropositive results by indirect ELISA were 16/82 (19.51%) and 21/136 (15.44%), respectively; while by PCR test, the positive results were 4/16 (25%) and 1/21 (4.76%), respectively. By both tests, the significant differences were reported between rural and urban regions, males and females, and > 2 years and ≤ 2 years at level of ($P \leq 0.05$).

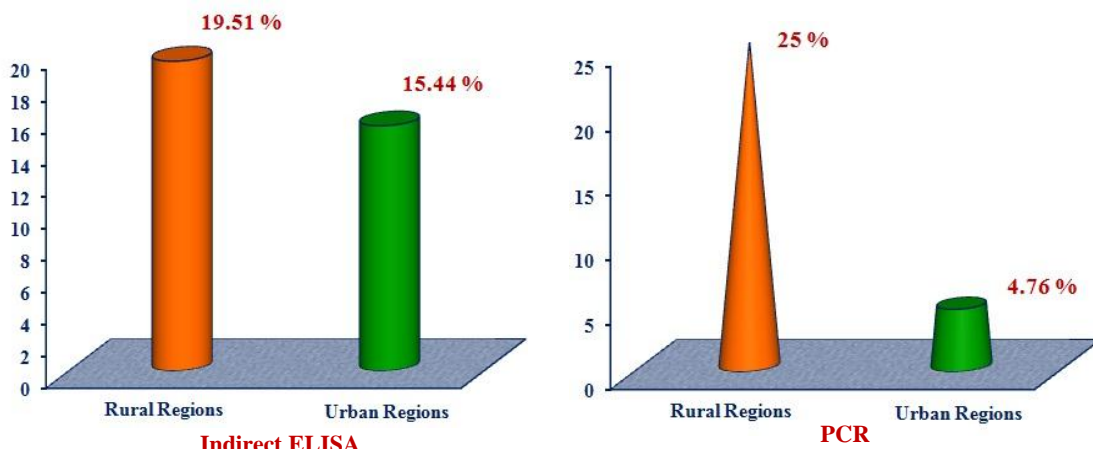


Figure (2): An association of positive dogs by indirect ELISA and PCR to inhabitant type risk factor

In (Fig.2) dealt with the sex risk factor, the study comprised from 67 males and 151 females, and the seropositive dogs by indirect ELISA were 13/67 (19.4%) and 24/151 (15.89%), respectively; whereas by PCR technique, 2/13 (15.38%) and 3/24 (12.5%) positive dogs were detected in males and females, respectively.

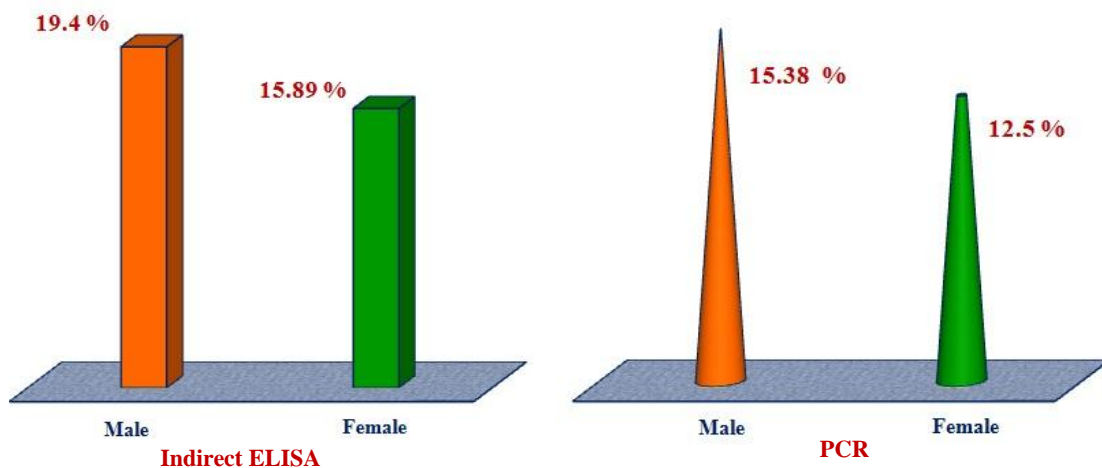


Figure (2): An association of positive dogs by indirect ELISA and PCR to sex risk factor

In (Fig.3) dealt with the age factor, the study's dogs were divided into two age groups, > 2years (149 dogs) and \leq (69 dogs), and the positive results by indirect ELISA were 36/149 (24.16%) and 1/69 (1.45%), respectively; while by PCR technique, 5/36 (13.89%) was the positive result that reported in > 2 years age group, only.

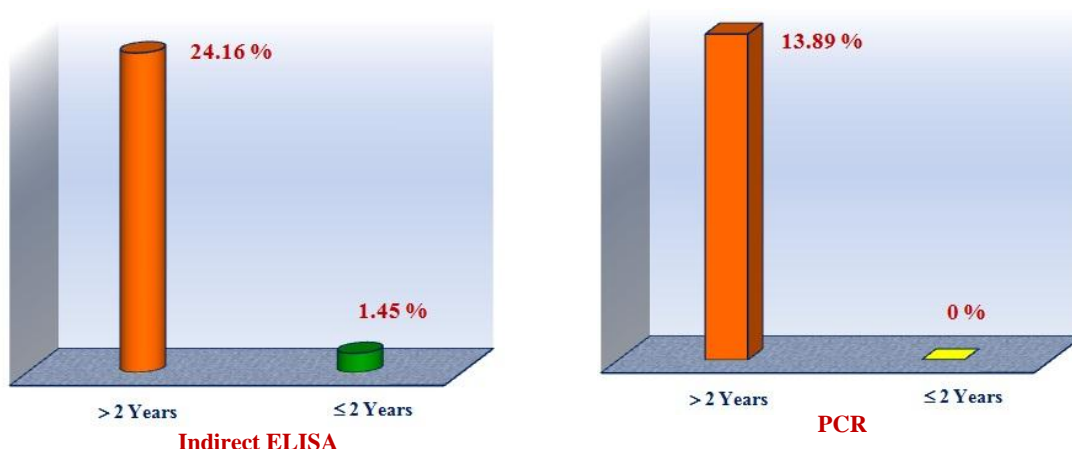


Figure (3): An association of positive dogs by indirect ELISA and PCR to age risk factor

Discussion

In Iraq, this study was performed, firstly, for detection of canine leptospirosis that caused by *Leptospira interrogans* serovar *canicola*. The indirect ELISA and PCR technique have been established that 37/218 (16.97%) and 5/37 (13.51%) of examined dogs were positives, respectively. Nonetheless, this study showed that the positive results of PCR test were less than those reported by indirect-ELISA, and this could be because of low sensitivity of PCR in compared to ELISA test (17), or might be attributable to substances persisted in clotted whole blood samples such as the hemoglobin derivatives, creatinine and urea that could have inhibited for DNA amplification with the leptospiral primers (18). However, several studies demonstrated the importance of PCR test as a complementary test in confirmation of leptospirosis due to the high specificity of it if compared with serological tests and blood culture due to an absence of specific antibodies in early stage of

infection and the fastidious nature of leptospires during cultivation on specific agars (17, 19, 20). In according to regions of samples collection, the positive results that reported in Baghdad governorate {23/108 (21.3%) and 5/23 (21.74%)} were higher than those reported in Al-Qadisiyah {10/79 (12.66%) and 0/10 (0%)}, and Dhi-Qar {4/31 (12.9%) and 0/4 (0%)} by both indirect ELISA and PCR, respectively. Worldwide, the seroprevalence of canine *Leptospira* spp. was varied widely between countries, in Iran (31%) (21), Turkey (43.96%) (22), Egypt (11.3%) (23), Japan (3.9-27%) (5), USA (24.9%) (24), Brazil (7.1-32.2%) (25). As reported by (26), about 10 different canine serovars have been associated with clinical disease, and the most frequently described serovars included *Canicola*, *Icterohaemorrhagiae*, *Pomona*, *Bratislava*, and *Grippotyphosa*. Recently, the serological evidence demonstrated an occurrence of unexpected changes in predominant serovars implicated in canine leptospirosis, and this change has been attributed to widespread use of bivalent *Leptospira* vaccines as well as increased contact between dogs and other animals, especially wild life reservoirs in expanding suburban environments (27, 28). In developing countries, it's thought to that the frequent variation in climatic conditions, especially; high temperature and rainfall during specific periods of the year could be play an effective role in the elevated rates of canine leptospirosis cases (29).

In regarding to the inhabitant type of risk factor, the rural regions reported by indirect ELISA, a significant seropositive prevalence 16/82 (19.51%) higher than urban regions 21/36 (15.44%); and by PCR, 4/16 (25%) positive dogs in rurals and 1/21 (4.76%) positive dogs in urbans. However, the significant increasing of positives in rurals other than urbans could be attributed to many factors as lack of basic sanitation, poor housing conditions, and limited health education that could be represented great risks for human infections, particularly, in rural areas (30). Many studies reported that herding, hounds, stray, and mixed-breed dogs were at highly risk if compared to companion dogs, which presumably, because of increasing the outdoor exposure for contaminated environments (31). Stray dogs that roaming, freely, in cities could be represented an important source in the transmission of infection because of their potential contacts with the infected canines or rodents (32). Also, the widespread using of bivalent vaccines specific, serovarly, for only *Canicola* and *Icterohaemorrhagiae* has been resulted in decreasing prevalence of these serovars, and increasing awareness for infection

with Pomona, Bratislava, and Grippotyphosa serovars particularly in last past 20 years (33). The predominance of canine leptospirosis that associated with these latter serovars was, likely, concerned with an increasing exposure of dogs to wild reservoir hosts in rural or suburban regions (34). In addition, several epidemiological studies demonstrated that the rural areas prone to have a higher risk for infections because of these environments are tend to have larger rates of reservoirs that in contact with the dogs (35, 36).

In related to sex risk factor, the current study showed that in 13/ 67 (19.4%) and 2/13 (15.38%) of males were positives by indirect ELISA and PCR, while, the positive infections rate that reported in females by both assays were 24/151 (15.89%) and 3/24 (12.5%), respectively. Also, the results of age factor were reported that the dogs with > 2 years of age have an elevated positive infection rates by indirect ELISA 36/149 (24.16%) and PCR 5/36 (13.89%); whereas in \leq 2 years group, only 1/69 (1.45%) positive dogs have been detected by indirect ELISA. Worldwide, the associations of sex and age factors with positive canine leptospirosis were controversial (25). Nonetheless, (37) showed the possibility of proportions infected dogs, at any age or sex categories, to be changed over time, and the data that used to identify the potential changes of risk factors could be at different points in time. However, (38) showed that male dogs were at significantly higher risk of leptospirosis than females dogs. In addition, dogs in age groups of 4-10 years of age at a significant greater risk than younger dogs. As reported by (21, 39, 40), Male dogs were more likely to develop leptospirosis than females; probably, due to their natural straying behavior, hormonal influences, increasing their overexposure to environment during socialization process or to temporal gap of immunity. The hypothesis of increasing infection with age might because of reduction the risk that caused by the less outdoor activity, or due to the better immunologic protection as a result of maternally acquired immunity (37, 41).

In conclusion, the results of this study were elicited the high seroprevalence of canine leptospiral infections among the stray dogs of rural and urban tested governorates, with efficacy of indirect-ELISA and PCR techniques in detection of specific antibodies and genes of *L. interrogans* serovar *canicola*. Nonetheless, the further investigations should be continued on canine leptospirosis in Iraq to detect the endemic serotypes of *Leptospira* organisms.

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