First Serological and Molecular Diagnosis of Canine *Anaplasma phagocytophilum* Bacterium in Iraq

التشخيص المصلي والجزيئي الاول لبكتيريا *Anaplasma phagocytophilum* الكلابية في العراق

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Abstract

This study was aimed to detect an occurrence and demonstrate the persistence of *Anaplasma phagocytophilum* bacterium in dogs in Baghdad province/Iraq, and an investigation of its relationship with the most important risk factors that involved sex, age and inhabitant region.

For this object, a totally of 267 dogs were submitted for blood samples collection, which examined by using of four diagnostic methods included blood smear test, rapid test, indirect ELISA and PCR technique. Although, the blood smear test was failed in detection of inclusion bodies specific for *A. phagocytophilum* in granulocytes, the rapid test and indirect ELISA have been revealed on 9 (3.37%) and 13 (4.87%) seropositive dogs, respectively; while, the PCR had been detected 7 (2.62%) positive dogs.

Statistically, the significant differences were reported between some applied diagnostic assays, as well as between the groups related to age factor at a level of ($P \leq 0.05$), whereas, they did not detected between the groups associated to both sex and inhabitant factors.

Keywords: *Anaplasma phagocytophilum*, Canine, Serological, Molecular, Diagnosis, Iraq

الخلاصة

هدفت الدراسة الحالية الى تحديد حدوث وبرهنة وجود بكتيريا *Anaplasma phagocytophilum* في الكلاب في محافظة بغداد / العراق ، وتقصي علاقتها مع اهم عوامل الخطر التي تضمنت الجنس ، العمر ومنطقة السكن. لهذا الغرض ، بالاجمال 267 كلبا خضعت لجمع عينات الدم ، التي فحصت باستعمال أربع طرق تشخيصية تضمنت اختبار المسحة الدموية ، الاختبار السريع ، اختبار الاليزا الغير مباشر وتقنية تفاعل البلمرة المتسلسل .

بالرغم من فشل اختبار المسحة الدموية في تحديد الاجسام الاشتمالية الخاصة *A. phagocytophilum* في الخلايا الحبيبية للكلاب التي خضعت للاختبار ، كشف الاختبار السريع واختبار الاليزا الغير مباشر عن 9 (3.37 %) و 13 (4.87 %) كلاب موجبة مصليا ، على التوالي ، في حين تمكن اختبار تفاعل البلمرة المتسلسل من تحديد 7 (2.62%) كلاب موجبة .

احصائيا ، سجلت الاختلافات المعنوية بين بعض الاختبارات التشخيصية وكذلك بين المجاميع المتعلقة بعامل العمر عند مستوى ($P \leq 0.05$) ، في حين ، لم تحدد مابين المجاميع المرتبطة بعاملي الجنس و السكن .

الكلمات المفتاحية : *Anaplasma phagocytophilum* ، كلابية ، مصلي ، جزيئي ، تشخيص ، العراق

Introduction

Anaplasma phagocytophilum are gram negative intracytoplasmic bacteria of *Anaplasmataceae* family that described firstly at California / USA in 1982 **[1, 2].** This organism is defined as an arthropod-borne pathogen that transmitted by a tick of *Ixodes*, especially, *I. persulcatus* and *I. ricinus* that considered as the primary vectors in Asia **[3].** Worldwide, large numbers of domesticated and wild animals can be affected with granulocytic anaplasmosis disease and the zoono-potential the bacteria must accounted, especially to humans at dangerous **[4].** Many canine *A. phagocytophilum* infections were subclinical and self-limiting, and the prevalence might be under-

estimated due to the frequent-nonspecific signals that included several typical and atypical signs **[5].** Globally, the organism was geographically distributed in many continents as Americas, Europe, Africa and Asia, with variable incidence rate between and within their countries **[6].** Several techniques were applied to demonstrate *A. phagocytophilum* infections such as the microscopy, culture, indirect immunoassays and molecular assays **[7, 8].** The serologic tests were used the different methodologies and target antigens, which should be taken into account when attempting to achieve the diagnosis of tested cases **(9).** Recently, the epidemiological surveys that carried out for detection an infection were adopted on applying of one or more serological tests to detect the IgG antibodies and demonstration the infection by using molecular technique in dogs **[10].** To date, number of studies has been pointed on evaluation of canine anaplasmosis incidence among animals or humans, was low at most countries **[11, 12].** Over the last 10 years, the assessment seroprevalence of canine *A phagocytophilum* was facilitated by an availability of screening rapid tests based on p44 antibody testing **[13].** The main goal of this study was to detect specific canine IgG antibodies against *A. phagocytophilum* in stray and herder dogs by using of four diagnostic assays involved blood smear test, rapid test and indirect ELISA, and confirmation the infection by using of PCR technique, in some rural areas of Baghdad province/Iraq.

Materials and Methods

Study's areas and samples

A totally of 267 dogs, stray and herder, were submitted for the present study, which selected randomly from some rural areas of Baghdad province / Iraq, during the period of October 2015 to April 2016. These dogs were selected randomly without clinical testing. From each dog, 4 ml of blood samples were collected by using the disposable syringes and packaged in EDTA tubes that used for blood smears preparation, serological and molecular techniques [14].

Immediately after blood collection, 2 slides (thin and thick) of blood smears were prepared from each dog's sample, stained with Giemsa stain, and examined by microscopically at 1000 x, to detect the specific inclusion bodies (morulae) in granulocytes of infected dogs with *A. phagocytophilum* [15].

The rapid SNAP 4Dx Plus Test (IDEXX / USA) was applied to demonstrate of specific antibodies against pathogen in serum dog's samples. The positive results were interpreted for relying on progress specific color in device's spot [12]. In addition, the fast and simple indirect ELISA (GmbH, Germany) was used to detect the specified IgG antibodies in serum dog's samples. The results of optical density for positive control (OD_{PC}), negative control (OD_{NC}) and samples (OD_{Sample}) were interpreted by using special formulas of the test's manual [16, 17]. While, for molecular test (PCR), a 50 µl of buffy coat was obtained from each blood sample, which used as a template for DNA extraction. According to the instructions of manufacturer of the DNeasy PCR kit (Qiagen GmbH, Hilden, Germany), two primers were used in this study included, GE 9f (5' AACGGATTATTCTTTATAGCTTGCT 3') and GE 2 (5' GGCAGTATTAAAAGCAGCTCCA GG 3') to duplicate the fragments of gene as detailed by [2, 18, 19]. The expected size for amplification of DNA fragments was 546bp, and the products were visualized by 1.5% agarose gel.

Statistical evaluation was carried out by the Microsoft Excel Word and IBM SPSS v.23 program to analyse the relationship between the positive values to microscopic, serological and molecular techniques. Also, this document was studied the association of positive values with the received risk factor's data including the age, sex and inhabitant region if stray or herder dogs. Differences accounted significant at the level of $P<0.05$ [20].

Results

- In (Table 1), a totally of 267 dogs were submitted for this study, examined by four diagnostic assays, and the overall positive results were revealed on 0/267 (0 %) dogs with blood smear test, 9/267 (3.37 %) dogs with rapid test, 13/267 (4.87 %) dogs with indirect ELISA and 7 (2.62 %) dogs with PCR technique.

Table (1): Results of testing 267 dogs by four diagnostic tests

	Test	Positive results	Negative results
1	Blood smear test	0 (0 %) [c]	267
2	Rapid test	9 (3.37 %) [a]	258
3	Indirect ELISA	13 (4.87 %) [a]	254
4	PCR	7 (2.62 %) [b]	260

Variation in small letters, vertically, referred to significant differences at level of P≤0.05

- In (Figure 1): PCR product size at 1.5% agarose gel electrophoresis picture that showed the gene of *A. phagocytophilum* bacterium. Lane (M) DNA marker (1500-100bp), and the Lanes (1, 3, 4, 5, 6, 7, and 8) were represented the positive samples at 546 bp.

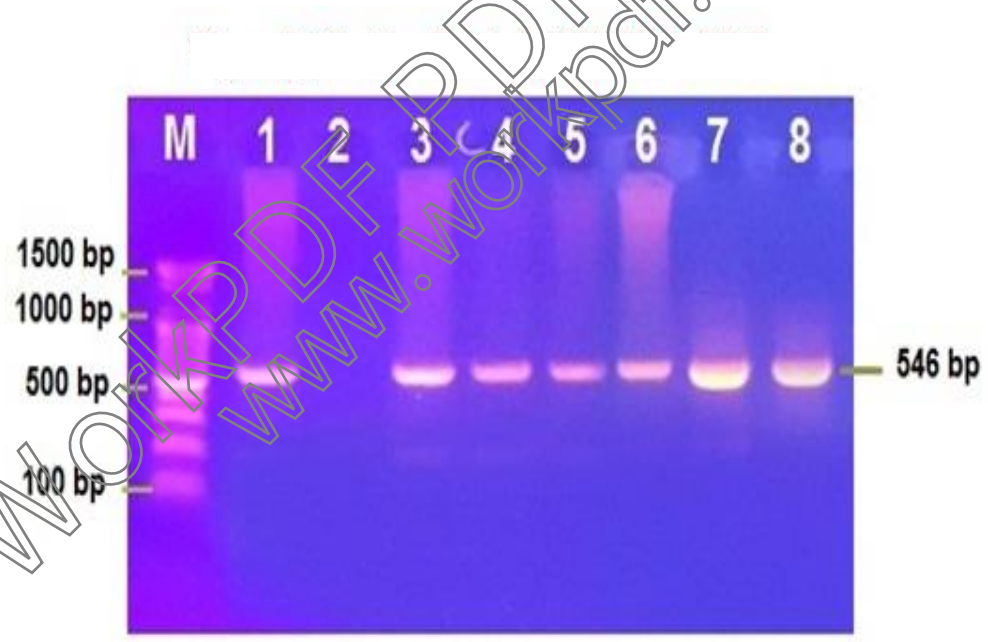

Figure (1): PCR product size at 1.5% agarose gel electrophoresis

- In (Table 2): The relationship of *A. phagocytophilum* positive dogs with most important risk factors that involved sex, age and inhabitant region was discussed in (Figure 2, 3 and 4).

Table (2): Relationship of *A. phagocytophilum* positive dogs with risk factors

Risk factor			No.	Rapid test	Indirect ELISA	PCR
1	Sex	Male	83	2 (2.41%) ^{Bb}	3 (3.61%) ^{Bb}	3 (3.61%) ^{Bb}
		Female	184	7 (3.8%) ^{Bb}	10 (5.43%) ^{Aa}	4 (2.18%) ^{Bb}
2	Age	<2 Years	101	0 (0%) ^{Bb}	1 (0.99%) ^{Bb}	0 (0%) ^{Bb}
		≥2 Years	166	9 (5.42%) ^{Ab}	12 (7.23%) ^{Aa}	7 (4.22%) ^{Ab}
3	Inhabitant region	Strays	94	3 (3.19%) ^{Bb}	5 (5.32%) ^{Ba}	2 (2.13%) ^{Bb}
		Herders	173	6 (3.47%) ^{Bb}	8 (4.62%) ^{Ba}	5 (2.89%) ^{Bc}

Variation in large and small letters, vertically and horizontally, referred to significant differences at level of P≤0.05

* In (Figure 2), according to sex, the study's dogs were classified into 83 males and 184 females, and the results were revealed, respectively, on 2 (2.41 %) and 7 (3.8 %) positive dogs by rapid test, 3 (3.61 %) and 10 (5.43 %) positive dogs by indirect ELISA, and 3 (3.61%) and 5 (2.18 %) positive dogs by PCR.

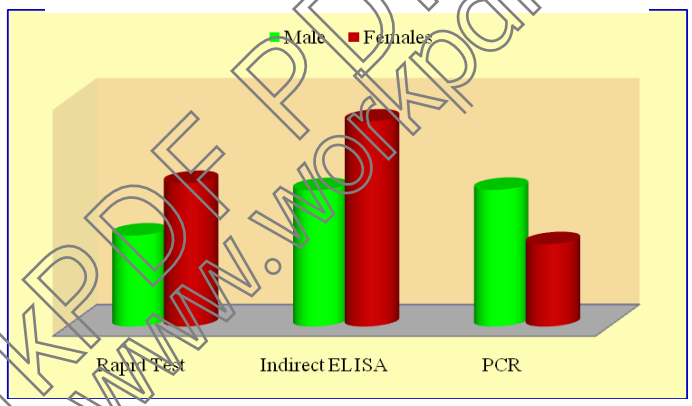

Figure (2): Relationship of positive dogs by diagnostic assays with sex factor

* In (Figure 3), according to their age, the study's dogs were divided into two age groups, < 2 years group involved 101 dogs and ≥ 2 years group involved 166 dogs, and the that reported by rapid test were 0 (0 %) and 9 (5.42 %) positive dogs, by indirect ELISA 1 (0.99 %) and 12 (7.22 %) positive dogs; and by PCR 0 (0 %) and 7 (4.22 %) positive dogs in <2years and ≥ 2 years groups, respectively.

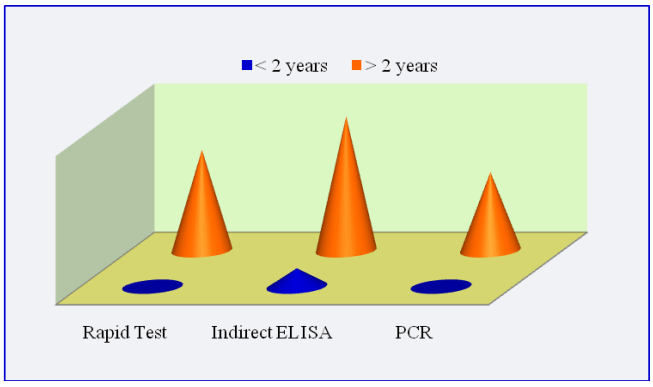

Figure (3): Relationship of positive dogs by diagnostic assays with age factor

* In (Figure 4), according to inhabitant region factor, the study's dogs were involved 94 stray dogs group and 173 herder dogs group, and the positive results that reported, respectively, 3 (3.19%) and 6 (3.46%) by rapid test, 5 (5.32 %) and 8 (4.62 %) positive dogs by indirect ELISA, and 2 (2.13 %) and 5 (2.89 %) positive dogs by PCR.

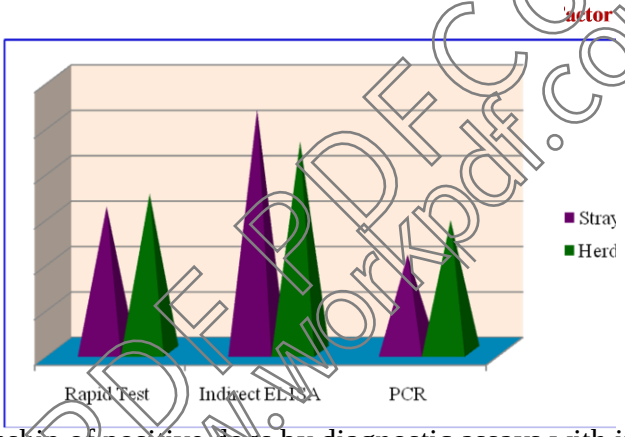

Figure (4): Relationship of positive dogs by diagnostic assays with inhabitant region factor

Discussion

A. Phagocytophilum was known to be animals and human's diseased bacteria among public health, which resulting in sporadic and clustered cases of infections in most countries of United States, Europe and Asia [21]. The presumptive diagnosis of clinical infections is depend mainly on an appearance of the specific signs, blood smear's detection of morulae, geographical situation, exposure's probability to vectors and finally isolation of bacteria by culture [9]. In most natural dog's infection, the incidence of clinical signals was not visible or under-detection due to development of an antibody's titers against *A. phagocytophilum* [22]. Also, because of shared tick-borne as vector with frequent incurrence for numerous vectors, infection's contributing to more than one rickettsial pathogen and other arthropod-borne infections such as *Babesia* spp. and *Bartonella* spp., can occur in dogs commonly to complicate the clinical picture for infection [10]. As well as, the variant signs typified defiance for veterinarians that attempt for diagnosing and determining an effective application of therapies on their diseased animals [23]. Although cell culture isolation is considered as a gold standard for confirming the infections, it's not widely offered or utilized for routine diagnostic purposes because it's required several weeks' incubation, laborious, expensive, and available in specialized research laboratories [24]. The microscopic blood smears examination of present study was significantly failed in detection of inclusions (morulae) within the granulocytes of in all dogs of study (Table 1). As registered by [25], the cytology is low

in sensibility and morulae of *A. phagocytophilum* cannot be distinguished from those of *Ehrlichia ewingii* and it may be confused with cytoplasmic granules or stain precipitate. In addition, the inclusions can't be diagnosed, easily, because it's required high skills to detected, and shortens of the time-appearance [26]. Many veterinary laboratories emerged serological studies for diagnosing of *A. phagocytophilum* in depending on rapid test and ELISA to estimate the IgG antibodies that can be detected, firstly, at 8 days and 2-5days after exposure and appearing of inclusion, respectively [27]. During both acute and convalescent phases of granulocytic anaplasmosis, the serological techniques can be accomplished but not early in the course of illness. The rapid test "IDEXX SNAP 4Dx" that a lateral-flow ELISA device was applied in present study with indirect-ELISA to detect an antibodies versus to *Anaplasma* species in dog serums, and both them reported positive titers with relative priority for ELISA. Generally, those tests are rapid and inexpensive diagnostic method developed and modified in recent years to performed, practically, with high sensitivity and specificity that exceeds on 98% [28, 29]. However, the initial results may be negative in dogs with acute disease and positive results might be reflected to prior exposure instead of effective infection with probability of cross-reactivity occurs between *Anaplasma* spp. [30]. As well as, (31) reported that there was many limitations during serological surveis such as the dogs' history and their import status that could not be recorded, so that, the positive dogs might not be confirmed in specific area that exposed previously to *A. phagocytophilum*. Also, the false-negative results could be occurred due to application of different serological methods or persistence of bacteria in dog or tick that inhabitant at specific geographical region [31]. Generally, a prevalence of positive antibody titers in dogs in some country's regions of Asia, United States, Europe and North America varies from 0.9% to more than 50%, due to geographical location and if the studied dogs are sick or healthy [3, 10]. Because of culture is still not a routine method yet, *A. phagocytophilum* detection is mostly achieved by amplification of DNA specific to 16S rRNA gene [7]. However, PCR assays could be a great important for an application to demonstrate an acute and chronic anaplasmosis in absence of detectable morulae and antibodies [32]. The expansion for where the bacteria could be found in canine tissues and share for chronic infection characteristics had been argumentative, and may be dependent on the infecting strain and host immune response to infection [33]. In regarding to *16S* rRNA gene, [34] reported that the level of bacteraemia could persisted for six months and then, gradually, reduced towards the end of experiment. Also, he was concluded that the cyclic behavior of *A. phagocytophilum* during chronic infection might be influenced by an immunological response, as previously suggested, or by various 16S-rRNA genotypes. The progression in molecular techniques allows for detecting a new "species, strains or genetic variants" and extending the list of microorganisms that able to infect animals or humans [35]. In USA, both canine and human exposure has progressively increased from 2008 to 2010 and the number of reported human cases has increased by 53% during this period [36]. 16S rRNA gene was applied to detect DNA of some bacteria in ticks or reservoirs, in addition to application it in medicine and veterinary as a diagnostic tool; likewise the efficacy of RNA gene fragments varies between reports and depends on the region which is amplified [37]. A molecular 16S rRNA technique considered as extremely sensible, specified and fast test [38]. With microbiology, the test permits to identify the bacteria species that could affect on choosing of drug or its efficacy, whereas in PCR, the positive was used to confirm the infection and the negative response does not always mean that the animal is not infected, because the bacteremia of *A. phagocytophilum* in dogs can be detect during a brief period (<28 days) [39, 40]. for demonstrating of *A. phagocytophilum* persistence in dogs, [2, 41] were reported that it must be accomplished using PCR and serology that allows the detection of IgG class antibodies approximately eight days after the initial exposure. In Asia, North America, Europe and Africa, *A. phagocytophilum* was diagnosed in dogs, cows, human and vectors by using specific primers based on 16S rRNA gene which has tinny hyper-variable regions and nucleotide's sequences that distinguishing between an *Anaplasma* spp. [11, 42]. *A. phagocytophilum* infections were reported, serologically and molecularly in different animal's species as well as humans, and reported variant infection rates, particularly, in neighboring countries as Iran and Turkey [42, 43]. The results of present study associated with the risk factors,

the significant differences were not reported between groups of sex factor (males and females) and between groups of inhabitant regions of dogs (strays and herders) (Table 2, Fig. 2 and 4), while, the increasing in infection rate was showed with advancing of age, and the incidence was, significantly, greater in dogs older than 2 years (Table 2, Fig 3). As referred by [44], the risk of dog's illness was likely to be persists over all life with the possibility of gaining *A. phagocytophilum* infection due to exposing for vectors, and thus, an adult older animals had more times and more chances for becoming diseased. Whereas, [45] reported that the breed, sex and size of dogs wasn't overrepresented, so that, the age of infected dogs ranged from 4 to 13 years, with a mean of 8 years. Whilst, [32, 46, 47] were showed that the bacteria more detected in older dogs and the recurrence of arthropod-borne problems were greater in adult than young, which interpreted by the fact "adult dogs had a higher exposure for vector more than young dogs and the bacteria can found in animals for an addition period post an acute illness". Also, several studies demonstrated the high infection's incidence was not related to the host's age, and the infection required an addition factors for affliction as tick density, geographical prevalence, and effect the spreading of vector-borne diseases [48, 49, 50, 51].

In conclusion, the first serological and molecular incidence of *A. phagocytophilum* infections had been demonstrated by serological and molecular assays in canine population in Iraq. Also, the study reported disability of blood smear test in detection of *A. phagocytophilum* in granulocytes. As well as, the direct relationship between the incidence of canine granulocytic anaplasmosis and age of tested dogs was reported.

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