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Serodetection and Molecular Confirmation of Mycobacterium tuberculosis Infections in Dogs

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Abstract

The aim of the present study was to detect the prevalence rate of IgG-antibodies specific for M. tuberculosis in dogs by application of indirect ELISA and confirming the infection in seropositive samples by using a molecular test (PCR). For this purpose, 178 stray dogs were selected randomly from some regions of Al-Qadisiyyah governorate / Iraq, for blood samples collection. The total prevalence rate of seropositive was 11 (6.18 %) that subjected for culture, and the suspected samples were tested by PCR technique that revealed on 6 (3.37 %) positive samples. Also, the relationships, between the positive infections with sex and age factors, had been studied. The study revealed that the infection ratio in males and females was 5.41 % and 6.38 % serologically, and 2.7 % and 3.55 % molecularly, respectively. In concerning to age factor, the morbidity rate of tuberculosis in ≤ 1 year and > 1 year by ELISA was 1.09 % and 11.63 %, whilst it was 0 % and 6.98 % with PCR, respectively.

Statistically, at P≤ 0.05 level, the significant differences were reported between positive samples by both (indirect ELISA and PCR) tests, and between age groups, whist, it was not showed between sex groups.

Keywords: Mycobacterium tuberculosis, Serodetection, Molecular confirmation, Dogs

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التحري المصلي والتوكيد الجزيئي لأصابات عصيات السل في الكلاب

احلام علي صخي الغالبي ، مثال كريم عباس الحسني ، ابتسام ثامر جعاز

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الخلاصة

هدف الدراسة الحالية كان لتحديد مدى انتشار الاجسام المناعية - IgG الخاصة ببكتريا عصيات السل في الكلاب باستعمال الاليزا غير المباشر وتوكيد الاصابة في العينات المصلية الموجبة باستخدام الاختبار الجزيئي (تفاعل البلمرة المتسلسل) . لهذا الغرض ، تم اختيار 178 كلب سائب عشوائيا من بعض المناطق في محافظة القادسية / العراق ، لجمع عينات الدم . المعدل الكلي للانتشار المصلي الموجب كان 11 (6.18 %) التي خضعت للزرع ، والعينات المشكوكه اختبرت بواسطة تقنية تفاعل البلمرة المتسلسل الذي كشف عن 6 (3.37 %) عينة موجبة . ايضا ، تمت دراسة العلاقات بين الاصابات الموجبة مع عاملي الجنس والعمر . كشفت الدراسة ان نسبة اصابة الذكور والاناث كانت 5.41 % و 6.38 % مصليا ، و 2.7 % و 3.55 % جزيئيا ، على التوالي . فيما يتعلق بعامل العمر ، كانت نسبة الاصابة بالسل في الكلاب 1 ≤ سنة و 1 < سنة بواسطة اختبار الاليزا كالتالي 1.09 % و 11.63 % في حين انها كانت 0 % و 6.98 % في اختبار تفاعل البلمرة المتسلسل ، على التوالي .

احصائيا ، عند مستوى $P \leq 0.05$ ، سجلت الاختلافات المعنوية بين العينات الموجبة بكلا الاختبارين (الاليزا غير المباشر وتفاعل البلمرة المتسلسل) ، كذلك بين كلا من مجموعتي العمر ، في حين انها لم تلاحظ بين مجموعتي الجنس .

الكلمات المفتاحية : عصيات السل ، الكشف المصلي ، التوكيد الجزيئي ، كلاب

1- Introduction

Mycobacterium tuberculosis, first described by Robert Koch in 1882, is an obligate, opportunistic pathogenic bacterium of Actinobacteria phylum relating to Mycobacteriaceae family (1). Although, the organism is considered as the primary human pathogen, natural infection by it can occur in a wide variety of animal hosts such as dogs, cats, cattle, monkeys due to close and prolonged contact with infected humans as a result of repeated aerosol exposure or cosuming of contaminated sputa, milk or tissues (reverse zoonoses) (2, 3). Whilst dogs have a natural resistance to infection, several studies reported that M. tuberculosis was responsible on approximately 75% of reported canine mycobacterium infections (4). However, most canine tuberculosis infections are prevalent in resource-poor settings in which the sophisticated veterinary services are gradually unavailable and where cases of canine tuberculosis remained largely undetectable (5). As well as, the susceptibility of dogs to infection was studied, naturally and experimentally, and showed that in almost cases the disease runs subclinically with pathological changes or lesions localized, mainly, in the lungs, small intestine, liver, heart, spleen, lymph nodes and skin (6). In fact, the diagnosis of M. tuberculosis in dogs is very difficult due to lack of clinical symptoms in early and middle stages, absence of practical effective assays in detection of clinical (active) and subclinical (latent) infections, and the false-negative or false-positive reactions that can be resulted after repeated using of tuberculin skin test or other classical methods as culture and microscopy. This fact is increased an importance of application the advanced laboratory diagnostics (7, 8). Currently, many commercial serological tests are available, mainly enzyme-linked immunosorbent assays (ELISA), in depending on detection of IgG antibodies to M. tuberculosis antigens (9). In comparison to other methods, ELISA can potentially provide a rapid diagnosis within hours, technological simplicity, and modest training requirement. In addition, this test can be adapted to point-of-care formats and performed at peripheral health facilities without onsite microscopy services (10). Several studies viewed that, though ELISA is accounted as one of the best diagnostic methods, but it has a greatly variable sensitivity and specificity results, very expensive and unreliable to be applied, alone, as a test of tuberculosis gold standard (10, 11, 12). Recently, PCR-based species identification has become useful in taxonomic classification and molecular epidemiology of Mycobacterium infections in both humans and animals. Also, this test is known by its sensitivity, specificity, simplicity of performance and rapidity, reducing the time of research and increasing the sureness of the obtained results by other diagnostic methods (13).

The aim of present study was to detect the prevalence of specific IgG antibodies for M. tuberculosis in dogs by using an indirect ELISA test and confirmation the infection in, only, seropositive samples by application of a molecular PCR technique.

2- Material and Methods

Region, Animal and Sample: From different regions of Al-Qadisiyyah governorate / Iraq, a totally of 178 stray dogs were selected, randomly, for this study during the period (March - November / 2015). In accordance to their sex and age, the study's dogs divided into two sex groups (37 males and 141 females) and two age groups (less than and more than 1 year). By using a disposable syringe, about 6 ml of blood sample was drained from each dog, and packaged into two tubes; 2 ml in a tube with anticoagulant (sodium heparin) for culture and PCR amplification with 4 ml in free-anticoagulant tube that centrifuged in laboratory at 3000 rpm for 15 minutes and, then, each pipetted serum sample was kept in numbered microtube at -20°C until serological ELISA testing (14, 15).

Serology by ELISA: Canine serum samples were analyzed for detection of M. tuberculosis IgG antibodies by using a commercially ELISA kit (MyBioSource, Canada) with Catalogue No. MBS9381233). All serum samples were tested according to manufacturer's instructions (MyBioSource, Inc.), measured at 450 nm, and the interpretation of results had done as follow:

Table (1): Results interpretation as mentioned by manufacturer

	Results	Final Estimation
1	< 8	Negative (Non-Infected)
2	> 12	Positive (Infected)

Molecular confirmation by PCR: All seropositive samples were subjected for culture and the suspected colonies were examined for DNA extraction. M. tuberculosis DNAs was extracted as described by (16) by using of a DNeasy PCR kit (Qiagen, Germany), under the catalogue No. 4555265. Two sets of primers were used in this study, the first set was IS43 (5' TCAGCCGCGTCCCCGCCA 3') and IS41 (5' CCTGCGAGCGTAGGAGTCGG 3') for IS6110 gene, while the second set of primers was TB-1F (5' GAACAATCCGGAGTTGACAA 3') and TB-1R (5' AGCACGCTGTCAATCATGTA 3') for MPB70 gene. The expected size for amplification of DNA fragments were 317 bp and 372 bp for first and second set of primers, respectively. The M. tuberculosis double stranded probes for both sets were prepared and labeled with PCR amplification kit (Sigma-Aldrich, USA) (17, 18). The reaction condition were one cycle of 96°C for 3 minutes, 30 cycles for 96°C for 30 seconds for denaturation, 65°C for 30 seconds for annealing, 72°C for 30

seconds for extension and, finally, a cycle of 7 minutes at 72°C using a thermocycler (Qiagen, Germany), and the PCR products was visualized by on 2.5 % agarose gel under ultraviolet light.

Statistical analysis: All received data was tabled and analysed by application of computerized Microsoft Excel 2010 and IBM-SPSS (v.23) programms to evaluate the prevalence of M. tuberculosis infections in dogs. The significant differences between and within serological and molecular results by Chi-Square (X^2) test at $P \leq 0.05$ level (19).

3- Results

In Table (2): the serum samples of all study's dogs were submitted for serological indirect ELISA that revealed on 11 (6.18 %) seropositive samples which cultured and examined by a molecular PCR technique and showed that 6 (3.37 %) of these samples were positives.

Table (2): Application of serological and molecular tests on (178) dogs

	Diagnostic Test	Positives	Negatives
1	Serological ELISA	11 (6.18 %) [a]	167 (93.82 %)
2	Molecular PCR	6 (3.37 %) [b]	172 (96.63 %)

Difference in small letters, vertically, referred to a statistical significant difference at $P \leq 0.05$

Figure (1): An identification of mycobacterial DNAs in blood samples of all tested dogs (178) by PCR method. The amplification of primers that directed to A (MPB70 / 372 bp) and B (IS6110 / 317) demonstrate a presence of tuberculosis infection in, only, 6 dogs as numbered (1-6). Lane (S) was used as a standard size for mycobacterial DNA.

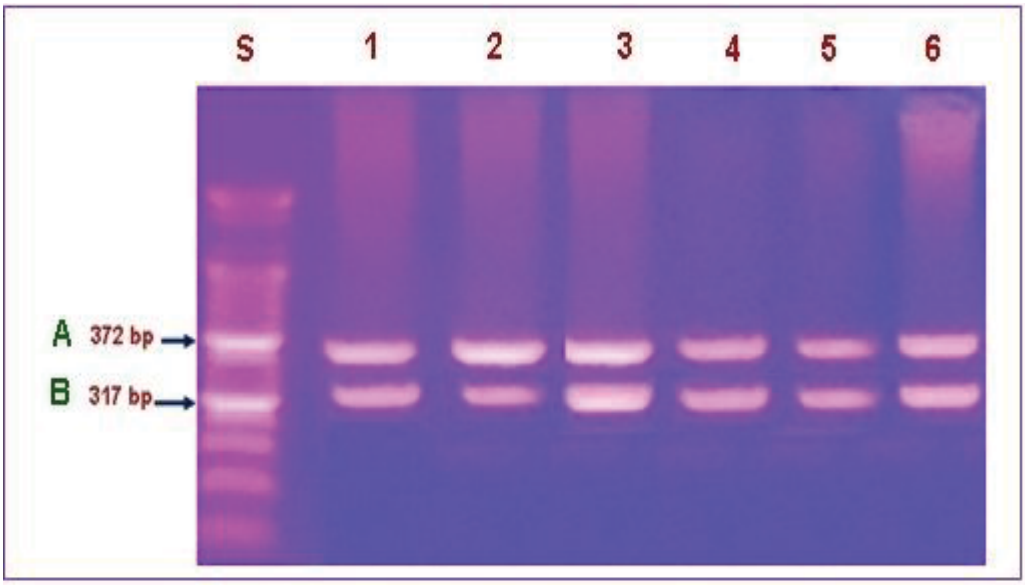

Figure (1): Positive M. tuberculosis dogs by molecular PCR test

The tested dogs were divided, in this study, according to their sex, into two groups that involved 37 males and 141 females. By ELISA, the seropositive results were 2 (5.41%) in males and 9 (6.38%) in females, whereas by PCR, the positive results were 1 (2.7%) and 5 (3.55%) in males and females, respectively, Table (3).

Table (3): Association of sex factor with positive results by both diagnostic tests

	Sex	No.	Serological ELISA	Molecular PCR
1	Males	37	2 (5.41 %) [b]	1 (2.7 %) [b]
2	Females	141	9 (6.38 %) [b]	5 (3.55 %) [b]

Difference in small letters, vertically, referred to a statistical significant difference at P≤ 0.05

In association to age factor, the study's dogs were divided into two age groups that included less than or equal to 1 year (92 dogs) and more than 1 year (86 dogs), Table (4). In less and more than 1 year age groups, the seropositive results were 1 (1.09 %) and 10 (11.63%), respectively; whilst they were 0 (0%) and 6 (6.98%) by PCR test, respectively.

Table (4): Association of age factor with positive results by both diagnostic tests

	Age	No.	Serological ELISA	Molecular PCR
1	≤ 1	92	1 (1.09 %) [b]	0 (0 %) [b]
2	> 1	86	10 (11.63 %) [a]	6 (6.98 %) [a]

Difference in small letters, vertically, referred to a statistical significant difference at P≤ 0.05

4- Discussion

In recent decades, despite the considerable progress towards control and/or eradication strategies, M. tuberculosis remained with highly prevalence in humans as well as domesticated and wild animals in several countries of the world (20). Approximately, one-third of world human population is infected or exposed largely for tuberculosis pathogens; and about 1.5 to 2 million of peoples die every year as tuberculosis victims (21). While in animals, the global prevalence of canine tuberculosis was varied between 0.1 to 6.7% and the potential of diseased dogs to act as reservoirs for human infections has been highlighted by several studies (22). As well as, many European surveys had been done in last century, detected that about 2-10% of dogs found to be infected with tuberculosis (4).

In various countries, involving Iraq, there is little information regarding to animal tuberculosis with full absence for epidemiological studies whether clinically or by bacteriology, serology and molecular techniques related to canine tuberculosis. Thus, the present study was the first one that detected the IgG antibodies against canine tuberculosis, serologically, and confirmed the infection by molecular PCR technique. The total seropositivity and molecular results as showed by this study were 6.18% and 3.37%, respectively. Worldwide, the epidemiological investigations of M. tuberculosis infections have evidenced high levels of tuberculosis transmission between people and it could be expected that dogs living in such environments would be at a particular risk for infection by this bacilli (23). Parsons, (2010) indicated that high levels of M. tuberculosis transmission to dogs; were occurred in those living in contact with smear-positive tuberculosis human patients, and the risk of progression to clinically active tuberculosis is lower than 10%v (24). In general, the persistence of active clinical signs is depending mainly on rout of exposure and degree of localization or dissemination (25). M. tuberculosis is not an easy disease to diagnosed in dogs, especially in early and middle stages of disease which could developed without any clinical signs, and even animals with active and open lesions could remained without signs for long periods, and even the clinical signs are present, they are usually non-specific (17). Thus, different alternative approached have been used in detection of canine mycobacterial infections involving pathological, microbiological, histological, cytological, serological and molecular methods (18). Although, serological tests (include ELISA) have a long successful history in diagnosis of many diseases, several precautions had be taken to restraint the utilizing of these techniques in routine practice (8). Attractively, ELISA test was utilized broadly during the recent years because of it is safe to used, fast to applied, simple to performed, computerized in read, economic in cost and high in accuracy (10). Also, the test has high negative predictive value (high specificity) that making it potentially useful as a screening test but low in sensitivity whereas the prevalence of latent tuberculosis infection is high (14). The results of ELISA test didn't have the dependable practical reliability and required to a confirmatory rapid test that has an enhanced diagnostic predictability with high sensitivity and specificity. Although, microbiological diagnosis (culture) was considered as definitive gold standard to confirm of tuberculosis, it might be required a few weeks for isolation and specific biochemical identification. In comparison to molecular methods, PCR technique had been used to detect both experimental and natural M. tuberculosis infection, and can be applied in several body fluids or tissue specimens to detect the Mycobacterium strains that grow, slowly, in conventional culture media (18, 26, 27). In this study, ELISA reported a significant increasing in its results where compared to PCR and this could be attributed to high prevalence of tuberculosis infection or due to a recurrent previous

exposure to low level of M. tuberculosis. The increasing or decreasing of antibodies levels doesn't necessarily support the actual rate of infection due to frequent reactivation of the causative pathogen (28). In relating to sex factor discussed in the present study, the positive results, reported by both ELISA and PCR tests, showed an absence of significant differences between males and females groups, which might be referred to that both sexes were exposure to M. tuberculosis infections at the same level. While, in concerning to age factor, the study detected a clearly rising in an infected dogs after 1 year of age. Worldwide, no epidemiological data available that could be clarified the actual reasons for heightening canine tuberculosis with age increasing. However, there are a variety of factors that can increase a dog's susceptibility to be diseased with age increasing, mainly the chronic nature of infection particularly in dogs that have a natural resistance to infect with tuberculosis, immune suppression as a result of age's advancing, concurrent exposure to causative agent especially in private dogs lived in contaminated environment with tuberculosis, or due to other factors such as stress and starvation (29, 30, 31).

In conclusion, the present study was the first Iraqi study documented the seroprevalence of M. tuberculosis IgG antibodies in dogs by using a commercially indirect ELISA test, and confirmed the infection by amplification of specific DNAs in blood samples with PCR technique. The study suggested that the extent of undiagnosed, subclinical tuberculosis infection remains unknown and further investigations in other regions is required

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