Seroprevalence and bacteriological study of canine leptospirosis in Tehran and its suburban areas

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Summary
To determine the seroprevalence of canine leptospirosis in Tehran and its suburban areas, 300 blood samples were collected from dogs referred to the Small Animal Teaching Hospital of Faculty of Veterinary Medicine at University of Tehran, Iran, between October 1998 and October 2000. Following separation of sera, they were kept at -30 °C until the time of laboratory examinations at Razi Institute. All referral cases were selected from the non-vaccinated dogs against leptospirosis. They were 3 months to 11 years old. All of the sera were examined by microscopic agglutination test (MAT) and indirect fluorescent antibody (IFA) techniques in serial two-fold serum dilutions from 1:50 to 1:1600. A titer of 1:100 or more was considered positive. All sera were examined against 16 serogroups of live leptospiral antigens as recommended by world health organization (WHO). Seroprevalence analysis in this study indicated that 93 out of 300 (31.0%) serum samples were positive against one, two or three leptospiral serogroups of canicola (9.0%), icterohaemorrhagiae (5.7%), grippotyphosa (3.7%), canicola+grippotyphosa (3.3%), canicola+icterohaemorrhagiae (3.0%), grippotyphosa+icterohaemorrhagiae (0.7%) and canicola+icterohaemorrhagiae+grippotyphosa (5.7%) using MAT at a titer of 1:100 or more. No leptospiral organism was isolated from 93 urine samples of reacted dogs in this study. Seventeen out of 93 (18.3%) reacted dogs showed clinical symptoms of leptospirosis.

The rate of positive reaction was 36.9% (31 out of 84) in farmer dogs and 31.0% (67 out of 216) in urban dogs. The rate of positive reaction was 42.3% (60 out of 142) in male and 34.4% (54 out of 158) in female dogs. The statistical analysis using McNemar test, however showed no significant difference between urban and farmer as well as between male and female dogs in this study (p = 0.076). The prevalence rate of reacted dogs examined by IFA technique at 1:100 dilution of serum samples was 34.3% (103 out of 300). The same rate using MAT was 31.0% (93 out of 300). The proportion of reacted dogs examined by IFA technique was the same as that examined by MAT at a titer of 1:400 or more. If MAT is considered as the gold standard test for diagnosis of leptospirosis, then the IFA at a titer of 1:100 or more would have a sensitivity of 95%, a specificity of 92%, a false positive rate of 8% and a false negative rate of 5%. In conclusion, with the given sensitivity and specificity of IFA technique, we suggest it as a supportive diagnostic procedure to be used for detection of canine leptospirosis in the field. To prevent and control of canine leptospirosis as a potential zoonotic disease in Iran, use of potent vaccines against the three major serogroups of canicola, icterohaemorrhagiae and grippotyphosa in all urban (pet) and farmer (shepherd) dogs older than 3 months is highly recommended.

Key words: Canine leptospirosis, Seroprevalence, IFA, MAT, Tehran, Iran

Introduction
Leptospirosis is a complicated infectious disease that affects most of warm-blooded animals and human beings (Rad, 1979; Rad, 1985; Rad, 1990; Rad et al., 1999). The animals affected include rats, mice, squirrels, domestic animals such as cows, sheep, goats, camels, pigs, dogs, cats and some wild animals such as raccoons, coyotes, monkeys, fox, wolves and skunk (Weaver, 1962; Sebek, 1986; Woolf and Gremillion, 1986; Zarnke and Ballard, 1987; Smith, 1990; Morgan, 1992; Rim et al., 1996). Leptospirosis is one of the most important zoonotic diseases. It has a wide range of hosts in different geographical regions all over the world. Dogs are very
important hosts, because they can transmit the disease to human beings and animals by means of urination in waters of the recreation areas and/or through direct contact of hosts with the infected urine shedding of dogs.

The infected dogs may be carriers of leptospiral organisms for 1–2 years in a subclinical form or with presentations of renal failure. Within this period, the dogs are very important hazardous carriers for susceptible human and animal hosts. Canine leptospirosis has been reported from many countries round the world. There are reports from Brazil (Rubel et al., 1997), Philippines (Weekes et al., 1997), USA (Gese et al., 1997), Chile (Pineda et al., 1996), Argentina (Brihuega and Huter, 1994), India (Venkataraman and Neduncheelliyan, 1993), Italy (Valpreda et al., 1982), Proturia (Myburg et al., 1993), Germany (Timoney et al., 1974), New Zealand (Hilbink, 1989), Australia (Sullivan, 1974), Finland (Jarvinen et al., 1986), Netherlands (Hartman et al., 1986), France (Barrat et al., 1985), Iran (Rad, 1979; Abdullahpour, 1987), Tanzania (Mugarula, 1984), Portrico (Farrington and Sulzer, 1982), England (Pritchard et al., 1986), Taiwan (Liu and Wang, 1977), China (Zeng and Lei, 1987), Canada (Prescott et al., 1991), USSR (Sebek and Treml, 1978), Swiss (Rey, 1987), Greece (Dabalis, 1986), Turkey (Ulgen et al., 1987), Belgium (Desmecht et al., 1986), Japan (Ryu et al., 1974) and Guatemala (Green, 1996).

Leptospirosis is called Weil’s disease in human medicine. Leptospira serotypes canicola, icterohaemorrhagiae, and grippotyphosa are the most common prevalent serotypes in dogs of most of the countries in the world (Rad et al., 1999). Canine leptospirosis has been reported from different geographical areas of the world (Sebek and Treml, 1978; Faine, 1982) as well as Iran (Rad, 1979; Abdullahpour, 1987; Rad, 1990). Considering the importance of canine leptospirosis in epidemiology of human leptospirosis as a zoonotic disease in Iran, the study of serologic and bacteriologic evaluation in dogs seems crucial. This study was conducted on referred dogs to the Small Animal Teaching Hospital of Faculty of Veterinary Medicine at University of Tehran, which is the most famous and largest small animal clinic in Iran with 15,000 referral cases annually.

The statistical analysis of this study is comparable with the previous study that was conducted about 14 years before using a macroscopic agglutination test (slide test) for screening purposes in Iran (Abdullahpour, 1987). The results indicated that the prevalence rate of canine leptospirosis among the referral cases to the aforementioned center at University of Tehran had a 12-fold increase within 14 years (1987 to 2000). This significant difference might be due to applying the most sensitive and accurate procedures such as IFA and MAT techniques for diagnosis of canine leptospirosis in the field as well as the possibility of increased naturally infected canine leptospirosis prevalence rate among the studied population of dogs in Tehran and its suburban areas.

Considering the importance of the rate of infected dogs in the epidemiology of leptospirosis in both human and animal populations, this project was designed to determine the frequency rates of leptospirosis in dogs that have close contact with their owners.

Materials and Methods

Serum samples
To determine the prevalence of canine leptospirosis in Tehran and its suburban area, 300 blood samples were collected from dogs referred to the Small Animal Teaching Hospital of the Faculty of Veterinary Medicine at University of Tehran, from October 1998 to October 2000. All cases were selected from the dogs which have not been vaccinated against canine leptospirosis.

All clinical observations were recorded in a data sheet for each case before blood collection. The dogs were 3 to 132 months (11 years) old. Out of 300 dogs, 142 were male and 158 were female. Out of 300 dogs, 216 were urban (pets) and 84 were non-urban (farmer) dogs. Among the 93 dogs that were positive serologically, 17 cases (10 males and 7 females) showed clinical symptoms of mild fever, anorexia, epistaxis, vomiting, transient hematuria, hematochezia, renal failure and
scleral congestion. Two-hundred and eighty three dogs (132 males and 151 females) out of 300 did not show any clinical symptoms of canine leptospirosis. Five ml of blood was collected from radial or saphenous vein of each dog in a venoject tube with no anticoagulants. After one hour at room temperature, to separate the sera, the blood samples were centrifuged at 2500 rpm for 10 min. Appropriate numbers were assigned to each microfuge tube. The serum samples were kept at -30°C until the time of examination at Razi Institute.

Serologic methods

All the serum samples were examined by standard methods of microscopic agglutination test (MAT) and indirect fluorescent antibody (IFA) techniques for serogrouping. The sera were titrated against 16 serogroups of live leptospiral antigens according to World Health Organization (WHO) guidelines (Faine, 1982; Zeinali, 2000) in the Research and Diagnostic Lab of Leptospirosis in Razi Institute of Iran. The following standard live leptospiral serogroups, provided by Pasteur Institute of France, were used in this study:

1- Australis 9- Icterohaemorrhagiae
2- Autumnalis 10- Javanica
3- Ballum 11- Panama
4- Bataviae 12- Pomona
5- Canicola 13- Pyrogens
6- Cynopteri 14- Semaragna
7- Grippotyphosa 15- Tarassovi
8- Hebdomadis 16- Sejroe

In the IFA technique, we used five leptospiral serogroups of canicola, icterohaemorrhagiae, grippotyphosa, pomona, and sejroe-harjo, that were prevalent in outbreaks of Iran. They were used as polyvalent as well as monovalent live antigens in IFA techniques. These leptospiral antigens were prepared according to the recommendations of WHO (Faine, 1982). Conjugated antibody was prepared and titrated in the section of Biotechnology of Razi Institute of Iran in the form of rabbit anti-dog IgG. It was used as conjugated antiserum in IFA technique. This antiserum was titrated with former positive control serogroups of leptospiral organisms that were kept live in the stocks of Research and Diagnostic Laboratory of Leptospirosis in Razi Institute of Iran. The results were observed under immuno-fluorescent microscope. Fluorescent isothiocyanate was used for conjugation of antibodies in these processes. For titration of monovalent antigens, five two-fold serial dilutions of serum samples were accomplished from 1:50 to 1:1600 dilutions for each of the canine serum samples that were examined by IFA technique. The original serovars of antigens were mostly prepared from L. biflexa (serovar patoc) and L. interrogans (serovar copenhageni, strain winjberg) (Faine, 1982).

Urines samples

One to two ml of urine samples were collected from each dog that showed positive reactions against either one, two or three leptospiral serogroups of canicola, icterohaemorrhagiae, grippotyphosa in MAT at a titer of 1:100 or more by either cystocentosis procedure or using sterile Nelaaton catheter in aseptic conditions. After collecting the urine samples, they were neutralized by 1:10 normal NaOH (Green, 1996). Then, 0.25 ml of urine samples were added to a special tube contained 5 ml specific sterile preserving tampon for transporting leptospira, prepared by Leptospirosis Laboratory of Razi Institute (Green, 1996). The urine samples were transported within 2–3 days from Small Animal Clinic, located in Tehran city to the research laboratory of leptospirosis in Razi Institute, located about 40 km West of Tehran in Hesarak, Karadaj.

The urine samples were cultured in the specific broth media such as Ellinghausen, Gardner, Fletcher and Gardner-Razi culture mediums prepared according to Jokit et al., (1992) and Vand-E-Yousofi et al., (1996).

Results

Seroepidemiological analysis in this study indicated that 93 out of 300 serum samples (31.0%) were positive for leptospiral serogroup of canicola; 27 out of 300 (9.0%) for icterohaemorrhagiae; 17 of 300 (5.7%)
for *grippotyphosa*; 11 of 300 (3.7%) had mixed positive reactions against leptospira serotypes of *caneloca* and *grippotyphosa*; 10 of 300 (3.3%) against *caneloca* and *icterohaemorrhagiae*; 9 of 300 (3.0%) against *grippotyphosa* and *icterohaemorrhagiae*; 2 of 300 (0.7%) against *caneloca* and *icterohaemorrhagiae* and *grippotyphosa*; and 17 of 300 (5.7%) using MAT at a titer of 1:100 or more. Although the rate of leptospiral infection as shown by IFA technique (34.3%) was slightly more than that of MAT (31.0%) at a titer of 1:100 or more, the statistical analysis using Chi square test showed no significant difference between the results of MAT and IFA techniques (P=0.05).

Based on a MAT at a titer of 1:100 or more, the seroepidemiological analyses revealed three distinct serogroups of leptospiral infections in dogs of Tehran and its suburban areas. These serogroups were *caneloca* (9.0%), *icterohaemorrhagiae* (5.7%) and *grippotyphosa* (3.7%). The *L. interrogans* serotype of *caneloca* was the dominant serovar of Leptospira genus in Tehran and its suburban areas (Fig. 1).

The frequency of positive reactions in farmer dogs (31 out of 84 [36.9%]) was more than that of urban dogs (67 out of 216 [31.0%]). The frequency of positive reactions was 43.2% (60 out of 142) in male and 34.4% (54 out of 158) in female dogs. The frequency of leptospiral infection in the reacted dogs under one year old (45 out of 93 positive cases [49.0%] using MAT at a titer of 1:100 or more) was slightly less than the reacted dogs aged more than one year (48 out of 93 positive cases [51.0%]). However, statistical analysis using McNemar test showed no significant differences between urban and farmer, male and female, age less than and more than one year old dogs in this study (P>0.076).

The prevalence rate of reacted dogs examined by IFA technique at a titer of 1:100 or more was totally 103 out of 300 cases (34.3%), while it was 93 out of 300 cases (31.0 %) using MAT at a titer of 1:100 or more. Nonetheless, statistical analysis using McNemar test showed no significant difference between the results of these two serological techniques.

Interestingly, the proportion of reacted dogs examined by IFA technique was exactly the same as examined by MAT at a titer of 1:400 (23 out of 300 [7.7%]). Statistical analysis using McNemar test indicated that if MAT at a titer of 1:100 or more is considered as the gold standard test for the diagnosis of leptospirosis, then IFA technique would have a sensitivity of 95% and a specificity of 92%. The false positive and false negative rates then will be 8% and 5%, respectively.

Seventeen out of 93 dogs (18.3%) that were positive in MAT at a titer of 1:100 or more presented with clinical signs of leptospirosis. The clinical symptoms that were observed during the clinical examinations included fever, anorexia, vomiting, polyuria, polydipsia, icterus and scleral congestion.

None of the 93 urine samples that were collected from the positive cases (MAT at a titer of 1:100 or more) showed leptospiral organisms following culture in specific broth media such as Elinghausen, Fletcher, Gardner and Gardener-Razi. In spite of searching leptospiral organisms in all of the urine culture media by dark field microscope, none of them was positive. The culture media were examined once a week for at least two months following the day of the urine sample was cultured.

**Discussion**

Leptospirosis is one of the complicated zoonotic diseases that infects most of the warm-blooded animals and human beings (Green, 1996). Canine leptospirosis has been reported from different geographical areas of the world (Faine, 1982; Hartman, 1984; Green, 1996; Zeinali, 2000) as well as Iran (Abdullahpour, 1987; Rad et al., 1999).

Comparison of the results of two studies performed on seroprevalence rates of canine leptospirosis in Tehran area and its surroundings revealed an increasing rate of positive reaction in serological findings from 2.5% in 1987 (Abdullahpour, 1987) to 31.0% in 2000 (Zeinali, 2000). This finding may imply the possibility of increasing risk of distribution of leptospiral infection in the dogs of Tehran and its suburban areas (Fig. 2).
Fig. 1: Comparative seroprevalence rates of leptospiral serogroups in the studied population of dogs in Tehran and its suburban areas (year 2000) by MAT at a titer of 1:100 or more

Fig. 2: Comparative seroprevalence rates of canine leptospirosis in Tehran and its suburban areas within 14 years by MAT at a titer of 1:100 or more
The most prevalent leptospiral serotypes isolated from the dogs of Tehran area were *grippotyphosa*, *icterohaemorrhagiae* and *ballum* as reported earlier (Abdullahpour, 1987). The present investigation indicated that the prominent leptospiral serotypes are *canicola*, *icterohaemorrhagiae* and *grippotyphosa*, respectively (Zeinali, 2000). Ulgen et al., (1987) have reported five serotypes of *icterohaemorrhagiae*, *grippotyphosa*, *ballum*, *pomona*, and *canicola* in their study on seroprevalence of canine leptospirosis in Turkey.

The external envelope of leptospiral organisms contains most of its active and protective antigens. They are mucopolypeptides. The relative serotypes of leptospiral organisms, which are common in antigenicity characteristics, are classified as one serogroup (Green, 1996). Based on this serogrouping identification, we could identify three major serogroups of *canicola*, *icterohaemorrhagiae* and *grippotyphosa* by MAT. *Canicola* serogroup was the dominant leptospiral serogroup in this study and it was more prevalent in male dogs. According to our results, the frequency of leptospiral infection with *canicola* serotype in male and female dogs was 21.8% and 19.7%, respectively (Zeinali, 2000). This finding was correlated with the results of other investigators in Iran (Abdullahpour, 1987).

In most of cases infected with leptospirosis, the organisms remain in the mucosal tubular cells and in spite of the high level of blood serum titers in patients, the immune complex of leptospiral organisms persist for months (Rad, 1985). Some investigators have called this period as the “secondary period” of infection in leptospirosis and that is the time that one can isolate leptospiral organisms from the urine samples of infected dogs (Faine, 1982; Hartman, 1984; Morgan, 1992). In spite of collection of 93 urine samples of serologically-reactive dogs (MAT at a titer of 1:100 or more), we could not isolate leptospiral organisms from any of these cases even from those of them which presented with clinical signs of leptospirosis (n=17). This might be occurred due to some technical problems and/or delay in culture of urine samples. Therefore, research should be continued to evaluate the bacteriologic and epidemiologic status of canine leptospirosis in different provinces of Iran to isolate the endemic serotypes of leptospiral organisms for preparing the original and polyvalent vaccine against local leptospiral serotypes in the dogs.

**Conclusions**

According to this study, the prominent incidence of canine leptospiral serotypes in Tehran and its suburban areas are *canicola*, *icterohaemorrhagiae* and *grippotyphosa*.

Research should be continued on canine leptospirosis in Iran to isolate the endemic serotypes of leptospiral organisms for preparing a polyvalent effective vaccine. Due to some technical difficulties in isolation of leptospires, the authors suggest that the urine and blood samples collected for bacterial culture should be cultured immediately soon after sampling. The culture should be even done at the clinic, if possible.

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