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EVALUATION OF DARK FIELD MICROSCOPY, ISOLATION AND MICROSCOPIC AGGLUTINATION TEST FOR THE DIAGNOSIS OF CANINE LEPTOSPIROSIS

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ABSTRACT

Leptospirosis has emerged as an important zoonotic disease in India and the disease is highly endemic in kerala, affecting wide range of animals and human. The disease is generally underdiagnosed because of wide range of clinical signs and similarity with other diseases. The present study was undertaken to evaluate Dark Field Microscopy (DFM), isolation and Microscopic Agglutination Test (MAT) for diagnosis of canine leptospirosis. A total of 54 samples (48 blood and serum, 6 urine) have been collected from dogs suspected of leptospirosis and presented to the University Veterinary Hospitals, Mannuthy and Kokkalai. Blood and urine samples were examined by DFM. All the samples have been kept for isolation using Ellinghausen-Mc cullough-Johnson-Harris (EMJH) medium with albumin supplement and 5 flourouracil (5- FU) at a concentration of 100 µg /ml of media. Serum samples were screened by the gold standard microscopic agglutination test (MAT). By DFM 31.48 % samples and in isolation 24.07 % samples were found to be positive. Microscopic agglutination test detected a seropositivity of 68.75 %. Although, MAT is laborious and requires handling of live cultures, it could be employed for the serodiagnosis of canine leptospirosis.

KEYWORDS

Leptospirosis, Dark Field Microscopy (DFM), gold standard microscopic agglutination test (MAT)

INTRODUCTION

Leptospirosis is a worldwide underdiagnosed zoonosis caused by spirochaetes belonging to the genus Leptospira. The disease occurs in a wide range of animals and humans that can lead to multiple organ involvement and fatal complications[1]. In dogs, leptospirosis is an important cause of morbidity characterized by fever, vomiting, dehydration, collapse, hepatitis, nephritis and death [2]. Identification of the disease in its initial stage is necessary for initiating proper treatment. Doxycycline therapy adopted soon after the onset of the disease can prevent severe complications and deaths due to leptospirosis [3]. The MAT is considered to be the gold standard test among several serological methods for the diagnosis of leptospirosis [4]. Isolation of the organism gives the definitive diagnosis and is a method of choice to identify circulating serovars in a particular geographical region[5]. Demonstration of organisms by Dark Field Microscopy (DFM) in early stage of the disease is confirmatory but is low sensitive. Therefore an attempt has been made to evaluate DFM, Isolation and MAT for the diagnosis of acute canine leptospirosis.

MATERIALS AND METHODS

A total of 48 blood and serum samples and 6 urine samples have been collected from dogs suspected of leptospirosis and presented to the University

Veterinary Hospitals, Mannuthy and Kokkalai during the period, January 2011 to December 2011. About 3-5 ml of blood has been collected from dogs aseptically in a sterile vial containing 1% sodium oxalate as anticoagulant and urine was collected in sterile phosphate buffered saline of pH 8.0 by cystocentesis and all the samples were screened by dark field microscopy (DFM) as per the method described [6]. Briefly, the blood was centrifuged at 3000 rpm for 5 minutes and the supernatant was collected and examined in DFM. If no organisms were detected the supernatant was subjected to high speed centrifugation at 14,000 rpm for 10 minutes and the pellet was examined by DFM. Subsequently all the samples were inoculated into Ellinghausen-Mc Cullogh-Johnson-Harris medium (EMJH) (DIFCO-USA) with albumin supplement and 5-fluorouracil at a concentration of 100µg/ml of media for isolation. 10 drops of blood and urine was added to 4.5 ml of the culture media by filtering through 0.22µm filter (MILLEX). The culture vials were incubated at 28˚-30˚c for a period of 8-12 weeks and screened every week in DFM before being discarded as negative.

The serum samples were screened by MAT using a battery of 9 serovars of Leptospira interrogans viz., Australis, Autumnalis, Canicola, Gripptotyphosa, Icterohaemorrhegiae, Javanica, Pomona, Patoc and Pyrogenes as per the method described [7]. Briefly, five to seven days old liquid culture of live leptospires with a density of approximately 2 X 10^8 leptospires per millilitre was used as antigen. The test was carried out in a 96 well 'U' bottom microtitre plate. Serum dilutions were made in 96 well microtitre plates for which 196 µl of PBS was mixed with 4 µl (1:50) of serum. From the1:50 diluted serum sample, 30 µl was added to each of the 8 wells in each column of the microtitre plate. In the last column, only 30µl of PBS was added which served as antigen control. Different serovars (30 µl) were added in wells in such a way that each row was charged with only type of antigen including the respective antigen control so that the final serum dilution was 1 in 100. Thus, each row corresponded to each serovar for different serum samples. The plates were closed with lid and incubated at 37 ^{o}C for two to four hours. A drop (10 µl) of the mixture was placed on clean grease-free slide and the wet preparation was examined without coverslip using 20X objective of the dark field microscope (Olympus) for the presence of agglutination or reduction in number of organisms in comparison to the respective antigen control. A 50 per cent reduction in the number of free leptospires in the test sample compared with the respective antigen control was considered as positive with or without agglutination.

Further, quantitative assay was carried out in 96 well microtitre plates against the reacting serovars of leptospires. All the 96 wells were filled with 30 µl PBS. In the first well of each row, 30 µl of 1 in 50 diluted serum samples were added and mixed well. Then, serial double fold dilution was made up to eight wells. From the eighth well, 30 µl was discarded. A constant volume of 30 µl of a particular serovar with a density of 2 X 10^8 per ml was added in each row and incubated at 37 ^{o}C for two to four hours. All the final dilution mixtures (100, 200, 400, 800, 1600, 3200, 6400, 12800) were observed under dark field microscope and the results were recorded. The reciprocal of the highest dilution of the serum which showed 50 per cent agglutination or 50 per cent reduction in the number of free leptospires in comparison to control was considered as the respective titre.

RESULTS

Among 54 samples, 17 (31.48 %) samples (14 blood, 3 urine) were positive by DFM and leptospires could be isolated from 13 (24.07 %) samples (11 blood, 2 urine). The results are presented in **Table 1**. By MAT leptospiral agglutinins could be detected in 33 serum samples against one or more serovars with a titre of 1 in 100 to 1 in 12,800 thus observing a seropositivity of 68.75 % (**Figures 1 & 2**). The entire samples

positive by DFM and in isolation were found positive by MAT.

Table 1. Isolation of leptospires form dogs, from January 2011- December 2011

Specimen	Number of samples	Samples Positive by Dark Field Microscopy	Samples Positive by Isolation
Blood	48	14	11
Urine	6	3	2
Total	54	17	13

Fig.1. Dark field visualization of leptospires (100 X) MAT negative

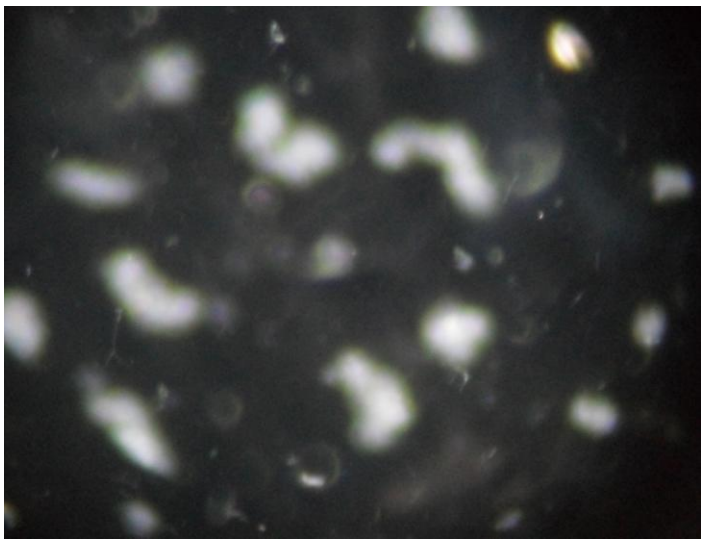

Fig.2. Dark field visualization of leptospires (100 X) MAT positive

DISCUSSION AND CONCLUSION

In the present study, DFM, isolation and gold standard MAT were used for evaluating diagnosis methods for canine leptospirosis. Though isolation of leptospires and DFM gives definitive diagnosis, the per cent positivity was only 24.07 % and 31.48 % respectively as against MAT which detected a seropositivity of 68.25 %. Leptospira has been isolated from urine of 11 of 14 dogs in Brazil employing EMJH medium [8]. The fastidious nature of the organisms and longer generation interval and the possible contamination could be the reason for relatively low number of isolates [4, 9]. Several workers recognized the difficulty in isolating leptospires, despite the presence of leptospires in samples [10, 11]. Approximately 10^4 leptospires/ml of blood are necessary for visualization of one cell per field by DFM [3] and this could be the reason for low sensitivity of DFM compared to isolation and MAT. Serological tests are well documented for the diagnosis of leptospirosis. A total of 68.25 % seropositivity could be detected by MAT. The regular vaccination of dogs in an endemic area like Kerala could be the reason for the high seropositivity. The MAT employing live antigens is the most widely used serological test and it is the reference test against which all other serological tests are evaluated [7]. Though MAT is laborious and time consuming, the relatively high sensitivity of MAT in comparison to DFM and isolation, confirms its diagnostic utility for the detection of canine leptospirosis.

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REFERENCES

1. World Health Organization country office for India. Leptospirosis, Laboratory manual,(2007)
2. Faine, S. Guidelines for the control of leptospirosis. Geneva: World Health Organization. (WHO Offset Publication 67).171p, (1982)
3. Levett, P. N. Leptospirosis. Clin Microbiol Rev, 14: 296-326, (2001)
4. Santa Rosa, C. A. 1970. Diagnostico laboratorial das leptospiroses. Revista de Microbiol, 1: 97-109, (1970)
5. Vijayachari, P., Sugunan, A. P. Umapathi, T. and Sehgal, S. C. 2001. Evaluation of dark ground microscopy as a rapid diagnostic procedure in leptospirosis. Indian J Med Res, 114: 54-58, (2001)
6. Chandrasekaran, S., Pankajalakshmi, VV. Usefulness of dark field microscopy after differential centrifugation in the early diagnosis of leptospirosis in dog and its human contacts. Indian J Med Sc. 51:1-4, (1997)
7. Office International-des-Epizooties. Leptospirosis In: Manual of standards for diagnostic tests and vaccine. 4th Edn. Paris 2008, pp. 251-264
8. Freitas, J.C., Gibson,F., and Claret.R. Isolation of Leptospira spp from dogs, bovine and swine naturally infected. Cienc Rural. 34:853-856, (2004)
9. Thiermann, A. B. Isolation of leptospires in diagnosis of leptospirosis. Modern Veterinary Practice. 5(10):758-759, (1984)
10. Adler,B., S. Faine, W. L. Christopher, and R. J. Chappel. Development of an improved selective medium for isolation of leptospires from clinical material. Vet Microbiol. 12: 377-381, (1986)
11. Bolin, C.A., Zuerner, R. L., Trueba, G. Comparison of three techniques to detect Leptospira interrogans serovar hardjo type hardjo-bovis in bovine urine. Am J Vet Res, 50(7) 1001-1003, (1989)

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