*Original Research*

# Diagnosis of Leptospirosis in Animals and Human by Dark Field Microscopy and Polymerase Chain Reaction

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| **Rec. Date:** | Nov 08, 2017 10:32 |
| **Accept Date:** | May 02, 2018 16:17 |
| **DOI** | 10.5455/ijlr.20171108103203 |

**Abstract**

*Leptospirosis is an economically important disease affecting the most of domestic animals. A study was conducted to investigate the prevalence of leptospirosis in animals and human by Dark field microscopy and Polymerase chain reaction targeting the Lip L32 gene. A total of 775 samples comprising of 118 canine (108 sera and urine 10), 161 bovine (102 sera, 49 urine, 3 milk and 7aborted contents), 153 rodent (60 sera, 33 urine and 60 kidney tissues), 177 swine sera and 166 human sera were collected with the unknown disease status. DFM revealed that canines (2.7 % sera and 40 % urine), bovines (4 % urine and 0.9 % sera), rodents (5 % sera and 9 % urine) and human (0.6% sera) samples were positive. PCR revealed that in canine (19.4 % sera and 60 % urine), bovine (5.8 % sera, 10.2% urine, 100% milk and 14.2% aborted contents), rodent (8.3% sera, 12.1 % urine and 13.3% kidney tissues), and human (0.6% sera) positivity for leptospirosis. Dark Field Microscopy can be used as a screening but when combine with Polymerase Chain Reaction can be utilized efficiently for detection of pathogenic leptospires.*

**Key words:** Animals, Human, DFM-PCR, Leptospirosis, Samples

**How to cite:** Bojiraj, M., Porteen, K., Gunaseelan, L., & Kannan, S. (2018). Diagnosis of Leptospirosis in Animals and Human by Dark Field Microscopy and Polymerase Chain Reaction. International Journal of Livestock Research, 8(10), 172-183. doi: 10.5455/ijlr.20171108103203

## Introduction

Leptospirosis is an emerging anthropozoonotic bacterial infection which occurs worldwide caused by various serovars of the genus *Leptospira* Faine *et al*. (2000). The disease has gained extreme zoonotic importance, especially in countries like India, where large numbers of livestock, rodents, wild animals, poor sanitary conditions, poor animal management and close association between man and animals have

provided the environment for spread of the disease Venkatesha and Ramadass (2001). Leptospirosis causes life threatening infections in almost all the domestic animals (cattle, sheep, goat, horse, pig, dog and cat), several wild animals and human (Sofia *et al.*, 2009). Successful laboratory confirmation of leptospirosis depends on the samples available and the stage of illness. There are many techniques available for the detection of leptospires which includes conventional, molecular and serology methods. Direct examination of leptospires in urine and blood samples can be performed with dark field microscopy. The technique is considered cheap, economic and rapid however, there is some limitation as the concentration of leptospires presence may be too low and insensitive (Sharma and Kalawat, 2008). Polymerase chain reaction (PCR) for leptospirosis was done in recent years by targeting the various genes like Lip L 21 and Lip L 32 for identification of pathogenic *Leptospira*. PCR is a useful tool for rapid and early diagnosis of leptospirosis from clinical samples (Cheema *et al.*, 2007). Detection of leptospires, from kidney tissues of rodents and livestock by PCR has also proved as a tool for the screening of leptospires in endemic areas (Meenambigai *et al.*, 2007). Therefore, the aim of the study was to identify the prevalence of leptospirosis in animals and human by Dark field microscopy and Polymerase chain reaction based on targeting the Lip L32 gene.

## Materials and Methods

### Sampling Area

Samples for the present study was collected from various urban parts of Tamil Nadu *viz*. Madras Veterinary College Teaching Hospital, Chennai, Postgraduate and Research Institute in Animal Sciences, Kattupakkam, Society for Prevention of Cruelty to Animals at Chennai, District Livestock Farm at Hosur, unorganized small piggery units from Thanjavur, Thiruvarur and Erode districts.

### Sample Collection

A total of 613 blood samples was collected from canine (108), bovine (102), rodent (60), swine (177) and human (166). Aseptically, 3-5 ml of blood was collected by intravenous puncture using disposable hypodermic needle (Dispovan) into 6 ml plain vacutainar tubes. Serum was harvested from clotted blood by centrifugation at 2000 rpm for 2 min and transferred in 2 ml capped plastic vials (Eppendorf), tested immediately under dark field microscope and stored at -20°C until tested by Polymerase Chain Reaction. A total of 92 urine samples were collected from canine (10), bovine (49) and rodent (33). Urine sample were collected aseptically from urinating bovine, using catheter in canine and collecting directly from bladder in rodent. The urine sample was then transferred into 10 ml collection tubes (Tarsons), tested immediately under dark field microscope and stored at -20°C until tested by polymerase chain reaction. A total of 60 kidney tissue samples was harvested aseptically from rats (23 male and 37 female) after euthanizing by carbon dioxide ($CO_2$). The samples were stored in eppendorf tubes and at -20°C until tested





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by polymerase chain reaction. A total of 3 cow milk samples suspected for leptospirosis were collected and tested immediately under dark field microscope and stored at -20°C until tested by polymerase chain reaction. A total of 7 aborted content samples were collected and stored at -20°C until tested by polymerase chain reaction.

## Materials and Methods

### Dark Field Microscopy

Sera, Urine, milk samples tested for leptospirosis as per Faine *et al.* (1999).

### Polymerase Chain Reaction

Polymerase chain reaction was done in the present study targeting Lip L 32 gene which is a conserved outer membrane protein gene that has the ability to distinguish pathogenic and non- pathogenic leptospires based on Aswar (2014). DNA was extracted from all samples by using M/s Bio Basic DNA extraction Kit (Canada) as per the manufacturer instructions.

### Statistical Analysis

Percentage analysis, Chi-squared test, kappa statistics, sensitivity and specificity were calculated as per Thrus field (2005) using MS office 2007 Excel spread sheet, coded and analyzed by SPSS version 17.

## Result and Discussion

### Dark Field Microscopy

In the present study, 2.77 percent (3 out of 108) of canine sera samples were positive by DFM, however similar studies conducted by Pawar (2013) and Aswar (2014) revealed a higher percentage positivity of 5.52 per cent and 29.3 per cent. The lower positivity in our study might be attributed to the fact that samples of the present study were collected from the general population irrespective of disease status and also may be due to presence of lower number of organism in the samples screened (Turner, 1970). Our study showed 40 percent (4 out of 10) of urine samples were positive from canines. The results are in concordance with the Krishna *et al.* (2012) who also reported 50 percent positivity in urine samples. The higher level of positivity in urine sample compared to serum samples might be due to shedding of the organism in urine samples (Feigin *et al.*, 1973).

Screening of bovine samples revealed that only 0.9 per cent (1 out of 102) of sera and 4 per cent (2 out of 49) of urine sample were found positive by DFM**.** Similarly, Magajevski *et al.* (2005) observed that of the 18 bovine urine samples screened, none of the samples were positive by DFM. The lower percentage positivity could be due lower sensitivity of DFM and low incidence of disease in bovine population Sharma and Kalawat (2008). In rodents 5 per cent (3 out of 60) of sera samples were found positive by DFM,





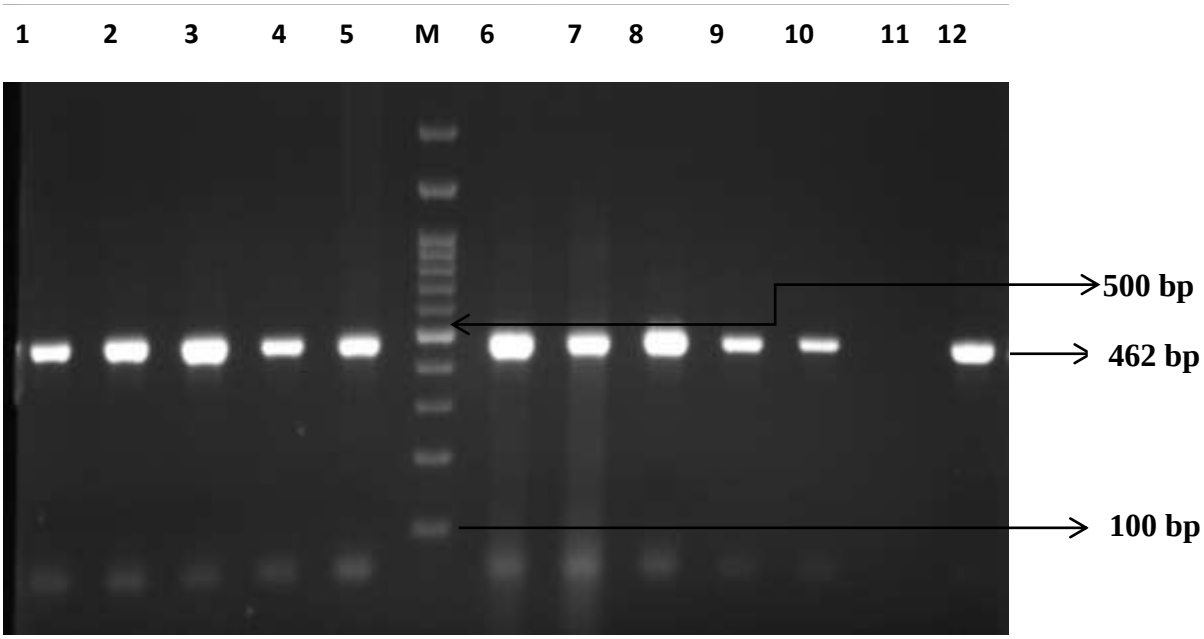
 DOI 10.5455/ijlr.20171108103203

however Dhannia *et al.* (2011) reported 2.9 percent positivity and Aswar (2014) reported 33.3 percent positivity by DFM in rodent sera samples. The variation in results may be due low sensitivity of DFM and low number of organisms in serum sample. Of the 33 urine samples, 3 (9%) samples were positive. The results were in accordance with Vinodkumar *et al.* (2011) who reported 8.8 percent positivity in urine samples from field rats. Out of 177 swine sera samples screened none of the samples was found positive. Poor sensitivity and delay in the processing of the samples, since samples were collected from different study areas are some contributory factors for absence of leptospira organism by DFM OIE (2008).

Present study 0.6 per cent (1 out of 166) of human sera sample was found positive. The lower percentage of positivity in the present study might be due to collection of samples from patients with unknown disease status. However, Arumugam (2011) evaluated the serum samples from patients with known history of leptospirosis and observed 80 percent positive by DFM (598 out of 748 positive cases).

**Polymerase Chain Reaction (Lip L 32 gene)**

Canine 108 sera and 10 urine samples subjected to PCR targeting Lip L 32 gene revealed 19.4 percent (21/108) and 60 per cent positivity (6 out of 10) positivity respectively (Fig. 1).




1, 2, 3, 4, 5- canine urine samples
M- 100 bp Marker
6, 7, 8, 9, 10 - canine serum samples
11- Negative control
12- Positive control (462 bp)

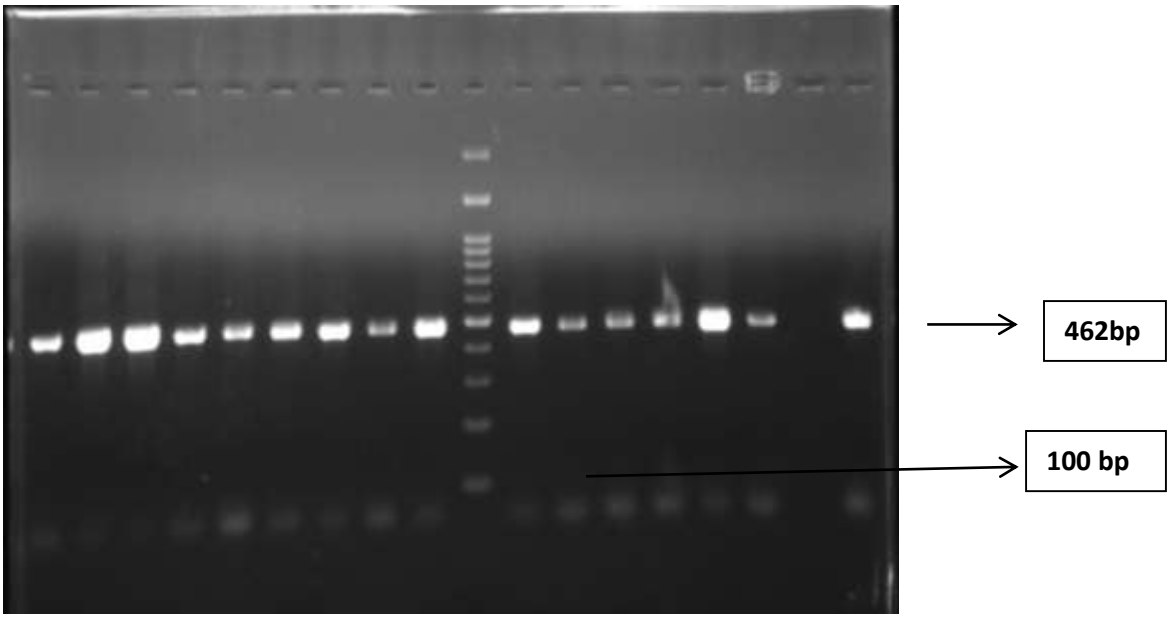
**Fig. 1:** Gel electrophoresis of PCR products with Lip L 32 gene (Canine)





DOI 10.5455/ijlr.20171108103203

The sera results were in concurrence with Pawar (2013) and Oliveira *et al.* (2012) who have reported 12.5 and 14.5 percent positivity in canine sera samples by PCR targeting secY and flab genes. Aswar (2014) reported 26 per cent positivity in canine urine samples targeting Lip L 32, whereas Oliveira *et al.* (2012) observed a positivity of 14.2 per cent in canine urine by PCR targeting secY and flab genes. Results of our study indicate that PCR targeting Lip L 32 gene for screening urine samples has higher sensitivity when compared to other gene targets. Bovine 102 sera samples were screened by PCR targeting Lip L 32 gene and 6 samples were found positive with 5.8 per cent positivity (Fig. 2).




1, 2, 3 -Bovine milk samples
4-Aborted content
5, 6, 7, 8, 9-Bovine urine samples
M-100 bp Marker
10, 11, 12, 13,14, 15-Bovine serum samples
16-Negative control
17-Positive control(462 bp)

**Fig. 2:** Gel electrophoresis of PCR products with Lip L 32 gene (Bovine)

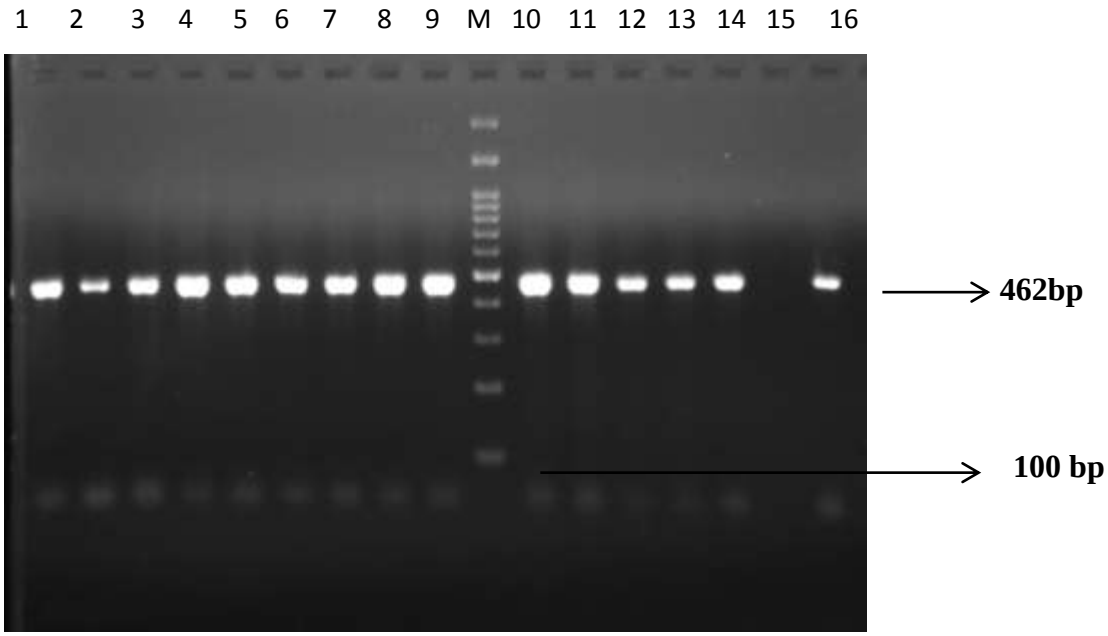
Similar study conducted by Felt *et al.* (2011) revealed that none of the serum samples tested were positive by PCR targeting Lig 1 and Lig 2 genes. The variations in the results could be attributed to high sensitivity of Lip L 32 in comparison to other gene targets. Out of 49 bovine urine samples 5 (10.2 %) were positive. The results are in accordance with reports of Shi *et al.* (1997) and Pawar (2013), who reported 13.2 and 7.14 per cent positivity in bovine urine samples by PCR. Milk samples (3) collected from cows showing signs of hemorrhagic mastitis subjected to PCR and revealed 100 per cent positivity (3 out of 3) indicating high sensitivity and specificity of Lip L 32 gene in diagnosis. Aborted contents (7) collected and subjected





DOI 10.5455/ijlr.20171108103203

to PCR showed (1 out of 7) 14.2 per cent positivity. Similarly Doosti and Tamimian (2011) observed 14.1 percent positivity by PCR targeting 16SrRNA gene to identify Leptospira organisms in aborted contents. A total of 60 rodent sera samples were screened and 5 samples were found positive with 8.3 per cent positivity (Fig. 3) by PCR. Other scientists have also reported similar results of 13 percent (Latifah *et al*., 2012) and 3.33 percent positivity (Chowdhary *et al.*, 2014) in rodent sera samples by PCR. A total of 33 rodent urine samples subjected to PCR revealed that only 4 samples (12.1%) were positive. Vinodkumar *et al.* (2011) also reported 17.65 per cent positivity in rodent urine samples from Bangalore. Out of 60 kidney tissues samples screened 8 were positive with 13.3 per cent positivity. Our results are in agreement with the results of various authors who have also showed 10 percent (Mayer –Scholl *et al.*, 2014) and 11.3 percent positivity (Esfandiari *et al.*, 2015) in rodent kidney tissues samples.



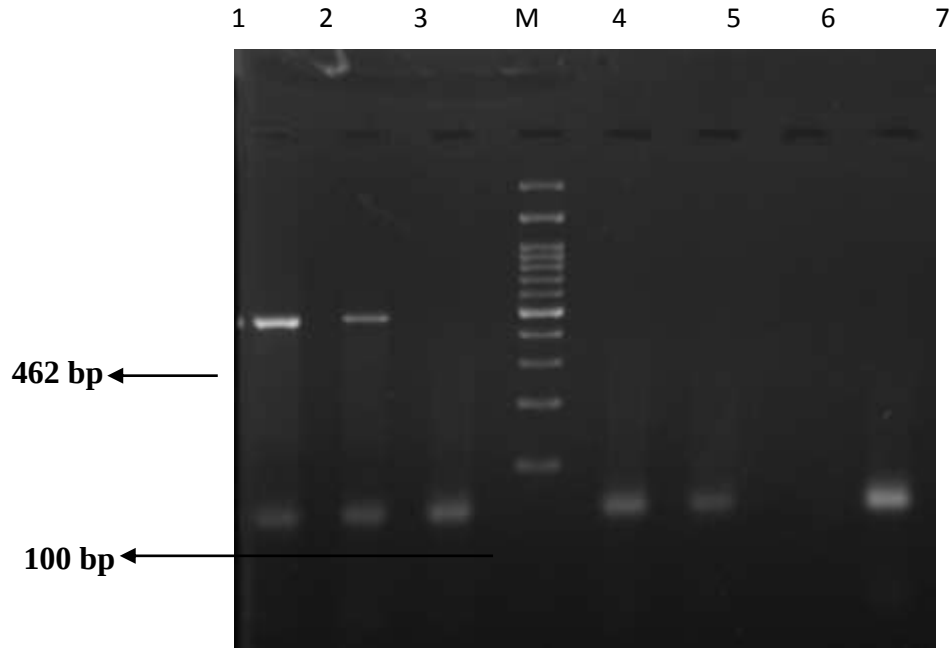

1, 2, 3, 4- Rodent urine samples
5, 6, 7, 8, 9, - Rodent serum samples
M- 100 bp Marker
10, 11, 12, 13, 14- Rodent kidney samples
15- Negative control
16-Positive control (462 bp)

**Fig. 3:** Gel electrophoresis of PCR products with Lip L 32 gene (Rodent)

A total of 177 swine sera samples screened for the presence of Leptospira antigen by PCR revealed that none of the samples were positive. Negative results of our study may be attributed to the unknown disease status of the animals from which the samples were collected and that studies needs to be done to utilize other samples like urine, kidney for the diagnosis of Leptospirosis (Oliveira *et al.*, 2007). Out of 166 human sera samples screened 1 sample was positive with 0.6 per cent positivity (Fig. 4).




DOI 10.5455/ijlr.20171108103203




1- Positive control(462 bp)
2- Positive human serum sample
3, 4, 5, 7 - Negative human serum samples
6- Negative control
M- 100 bp Marker

**Fig. 4:** Gel electrophoresis of PCR products with Lip L 32 gene (Human)

However, other researchers have reported 18 per cent positivity (Shekatkar *et al.*, 2010) and 30 per cent positivity (Ooteman *et al.*, 2006) in human screened by PCR. The higher positivity in their studies may be due to screening of human patients suspected with signs of Leptospirosis (Table 1).

**Table 1:** Diagnosis of leptospires in animals and human by DFM and PCR

| Species | Category of Sample | DFM | | | PCR (Lip L 32) | | |
|---|---|---|---|---|---|---|---|
| | | No. of Samples Tested | Positive | Positive (%) | No. of Samples Tested | Positive | Positive (%) |
| Canine | Serum | 108 | 3 | 2.7 | 108 | 21 | 19.4 |
| | Urine | 10 | 4 | 40 | 10 | 6 | 60 |
| Bovine | Serum | 102 | 1 | 0.9 | 102 | 6 | 5.8 |
| | Urine | 49 | 2 | 4 | 49 | 5 | 10.2 |
| | Milk | 3 | - | - | 3 | 3 | 100 |
| | Aborted contents | - | - | | 7 | 1 | 14.2 |
| Rodent | Serum | 60 | 3 | 5 | 60 | 5 | 8.3 |
| | Urine | 33 | 3 | 9 | 33 | 4 | 12.1 |
| | Kidney | - | - | - | 60 | 8 | 13.3 |
| Swine | Serum | 177 | - | - | 177 | - | - |
| Human | Serum | 166 | 1 | 0.6 | 166 | 1 | 0.6 |






DOI 10.5455/ijlr.20171108103203

**Comparison of DFM with PCR Using Various Samples**

**Canine**

The positivity for leptospirosis in urine of dogs detected by DFM and PCR was 40 per cent and 60 per cent respectively. Senthilkumar *et al.* (2001) reported that the DFM and PCR of urine sample showed 50 per cent and 75 per cent positivity, respectively which indicated that PCR assay was highly sensitive and specific assay for the detection of leptospiral infection compared to DFM. The sensitivity and specificity of DFM on comparison with PCR was 66.67 per cent and 100 per cent respectively. However, Brown *et al.* (1994) reported a sensitivity of 100 per cent and specificity of 88.3 per cent by PCR of urine. These studies suggested that combination of test should be done to confirm leptospirosis in dogs. In sera samples of dogs 2.7 per cent and 19.4 per cent were positive for leptospirosis by DFM and PCR. Senthilkumar *et al.* (2001) reported that 43 and 82 per cent of sera samples of dogs were positive by DFM and PCR which indicates that PCR is a more sensitive method for detection when compared to DFM. The sensitivity and specificity of DFM on comparison with PCR was 14.29 per cent and 100 per cent respectively whereas, Krishnaveni (2010) reported that PCR had a sensitivity of 75 per cent and specificity of 93.75 per cent while testing sera of dogs (Table 2 & 3).

**Table 2:** Comparison of DFM with PCR (Lip L 32) in urine samples of Canine

| DFM | PCR (Lip L 32) | | Total | Chi-square Test | Sensitivity | Specificity | Kappa Value |
|---|---|---|---|---|---|---|---|
| | Positive | Negative | | | | | |
| Positive | 4 | - | 4 | $2.10^{NS}$ | 66.67 | 100 | 0.615 |
| Negative | 2 | 4 | 6 | | | | |
| Total | 6 | 4 | 10 | | | | |

**Table 3:** Comparison of DFM with PCR (Lip L 32) in serum samples of Canine

| DFM | PCR (Lip L 32) | | Total | Chi-square Test | Sensitivity | Specificity | Kappa Value |
|---|---|---|---|---|---|---|---|
| | Positive | Negative | | | | | |
| Positive | 3 | - | 3 | $8.04^{**}$ | 14.29 | 100 | 0.223 |
| Negative | 18 | 87 | 105 | | | | |
| Total | 21 | 87 | 108 | | | | |

**Bovine**

DFM and PCR were able to detect 4 per cent and 10.2 per cent positivity in bovine urine samples respectively. On comparison it was noticed that the sensitivity and specificity of DFM on comparison with PCR was 40 per cent and 100 per cent respectively. The results are in concordance with the results of various authors who have also opined the PCR was a highly sensitive and specific technique Rodriguez *et al.* (2011) and Faber *et al.* (2000). There are no much studies on screening of bovine sera for leptospira detection in the past. We made an attempt to compare DFM and PCR for detection of leptospirosis in sera of bovine. Our study showed that 0.9 per cent and 5.8 per cent sera samples were positive by DFM and





DOI 10.5455/ijlr.20171108103203

PCR and the sensitivity and specificity of DFM on comparison with PCR was 16.67 per cent and 100 per cent respectively. Our results suggest that combination of tests should be performed to identify positive cases of leptospirosis (Table 4 & 5).

**Table 4:** Comparison of DFM with PCR (Lip L 32) in urine samples of Bovine

| DFM | PCR (Lip L 32) | | Total | Chi-square Test | Sensitivity | Specificity | Kappa Value |
|---|---|---|---|---|---|---|---|
| | Positive | Negative | | | | | |
| Positive | 2 | - | 2 | $9.55^{**}$ | 40 | 100 | 0.545 |
| Negative | 3 | 44 | 47 | | | | |
| Total | 5 | 44 | 49 | | | | |

**Table 5:** Comparison of DFM with PCR (Lip L 32) in serum samples of Bovine

| DFM | PCR (Lip L 32) | | Total | Chi-square Test | Sensitivity | Specificity | Kappa Value |
|---|---|---|---|---|---|---|---|
| | Positive | Negative | | | | | |
| Positive | 1 | - | 1 | $3.55^{*}$ | 16.67 | 100 | 0.274 |
| Negative | 5 | 96 | 101 | | | | |
| Total | 6 | 96 | 102 | | | | |

**Rodent**

In rodents showed 9 per cent (3 out of 33) and 12.1 per cent (4 out of 33) urine samples positive by DFM and PCR. Vinodkumar *et al.* (2011) who have also reported 8.82 percent and 17.65 percent positivity by DFM and PCR. A comparison was made between the tests to ascertain the efficiency and the sensitivity and specificity of DFM on comparison with PCR was 75 per cent and 100 per cent respectively. The less positivity by DFM in the present study may be attributed to lower concentration and intermittent shedding of the organisms in urine sample. The positivity for leptospirosis in sera of rodent detected by DFM and PCR was 5 per cent (3 out of 60) and 8.3 per cent (5 out of 60) respectively. The sensitivity and specificity of DFM on comparison with PCR was 37.5 per cent and 100 per cent respectively. Dhannia *et al.* (2011) opined that PCR is a rapid and reliable method for diagnosis of leptospirosis in rodents (Table 6 & 7).

**Table 6:** Comparison of DFM with PCR (Lip L 32) in urine samples of Rodent

| DFM | PCR (Lip L 32) | | Total | Chi-square Test | Sensitivity | Specificity | Kappa Value |
|---|---|---|---|---|---|---|---|
| | Positive | Negative | | | | | |
| Positive | 3 | - | 3 | $15.77^{**}$ | 75 | 100 | 0.841 |
| Negative | 1 | 29 | 30 | | | | |
| Total | 4 | 29 | 33 | | | | |





DOI 10.5455/ijlr.20171108103203

**Table 7:** Comparison of DFM with PCR (Lip L 32) in serum samples of Rodent

| DFM | PCR (Lip L 32) | | Total | Chi-square Test | Sensitivity | Specificity | Kappa Value |
|---|---|---|---|---|---|---|---|
| | Positive | Negative | | | | | |
| Positive | 3 | - | 3 | 13.39** | 37.5 | 100 | 0.51 |
| Negative | 5 | 52 | 57 | | | | |
| Total | 8 | 52 | 60 | | | | |

***highly significant (p<_=0.01), *significant p<_=0.05, NS-Non significant*

## Conclusion

Leptospirosis disease symptoms are nonspecific in animals and human. The early identification of carrier animals and information on the shedding state are crucial to prevent the spread of leptospiral infection to other animals and humans. Based our study results Dark Field Microscopy (DFM) can be used as a screening test for leptospires but when combine with Polymerase Chain Reaction (PCR-Lip L 32) can be utilized efficiently for detection of pathogenic leptospires (antigen).

## Acknowledgements

We wish to thank Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 051, for funding and allowed this work to do as part of M.V.Sc thesis work. Also we thank Zoonosis Research Laboratory, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai-600 051, helped for conducting MAT test in laboratory.

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DOI 10.5455/ijlr.20171108103203