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Congo Red Staining : An Effective Affordable Tool for Diagnosis of Bovine Leptospirosis

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Abstract

The present study involves evaluation of dark field microscopy and Congo red staining of urine sediments for diagnosis of leptospirosis in clinically suspected cases. A total of 24 urine samples were collected from cattle with clinical signs *viz;* hematuria, jaundice, hemagalctia and abortion. Mid stream urine samples were collected in sterile containers and transported immediately to laboratory. Smears were prepared from urine sediments and air dried. Methanol fixed smears were stained with 1% Congo red solution and observed under 100X magnification. Negatively stained leptospires could be observed in two cases. These samples were later confirmed with polymerase chain reaction. Hence congo red staining can be used as a low cost diagnostic tool for leptospirosis.

Key words : Leptospirosis, diagnosis, Congo red stain

Leptospirosis is a major zoonotic disease particularly in tropics and subtropics. It is a disease with considerable economic importance in livestock due to manifestations like infertility and abortion. Clinical presentation of leptospirosis in livestock includes fever, jaundice, hematuria, hemagalactia and reproductive failure. Diagnosis of leptospirosis in livestock is difficult because of the low specificity, sensitivity and vague interpretation of various diagnostic tests due to the absence of specific clinical signs (Radostits *et al.,* 2017).

India is endemic for leptospirosis, Balakrishnan *et al*. (2016) reported 75.66% seroprevalence for leptospirosis in buffaloes of Tamilnadu. The appraised productivity cost of leptospirosis in 2019 was 4.6 billion $ in India (Agampodi *et al.,* 2023). From 2005 to 2021, there were 21215 leptospirosis cases in Kerala as retrieved by Antima and Banerjee (2023). Seroprevalence in animals was; cattle-20.68% (Divya *et al*., 2021), goats- 25.81% (Divahar *et al*., 2019) and pigs -35.92% (Reshma, 2018) from different parts of Kerala .

Microscopic agglutination test is considered as the gold standard test for diagnosis of leptospirosis and it provides a useful epidemiological data. However it is not suitable for routine diagnosis as it demands maintenance of live pathogenic stock cultures and paired serum sample is required to verify the diagnosis. Immunological assay like Enzyme linked immunosorbent assay (ELISA) and molecular diagnostic tests like Polymerase chain reaction (PCR) targeting various genes are widely used nowadays for the confirmatory diagnosis of leptospirosis. However, these assays required sophisticated laboratory equipments and skilled personal to perform the test and it is difficult to perform these test inresource limited settings. In this context, this study aims at evaluation of a conventional staining technique for demonstration of leptospires from clinically ill animals. This study aims at evaluation of a conventional

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staining technique for demonstration of leptospires from clinically ill animals.

Materials and Methods

The study was conducted at the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Pookode during the period from January 2022 to November 2023. As per CPCSEA guidelines, study involving clinical samples does not require the approval of Institutional Animal Ethics Committee and authors were permitted by animal owners for sampling.

Collection of samples

A total of 24 urine samples were collected from cattle with clinical signs *viz;* hematuria, jaundice, hemagalctia and abortion from four northern and two central districts of Kerala. Mid stream urine samples were collected in sterile containers and transported immediately to laboratory. In case of samples with transportation time of more than two hours samples were collected with equal quantity of 1X sterile PBS (pH 7.2). Two ml of urine sample was transferred to a microcentrifuge tube and centrifuged at 12000 rpm for 3 minutes. Smears were prepared from urine sediments on clean grease free slides and air dried. Air dried smears were fixed using methanol. DNA was extracted from the urine samples using Genomic DNA kit (Origin™), Kollam, Kerala.

Staining procedure

One percent aqueous solution of Congo red (0.5g Congo red powder in 50 ml distilled water and add 50 ml 100% ethanol) was poured on the fixed smear and allowed it to get dried. Dried smear was washed in a decolouriser (95% ethanol containing two parts of concentrated hydrochloric acid) for 45 seconds. Smear was washed with distilled water and air dried. Dried smear was observed under oil immersion microscope (100X) (Collee *et.al*, 1996). Leptospira emerged unstained against the dark back ground.

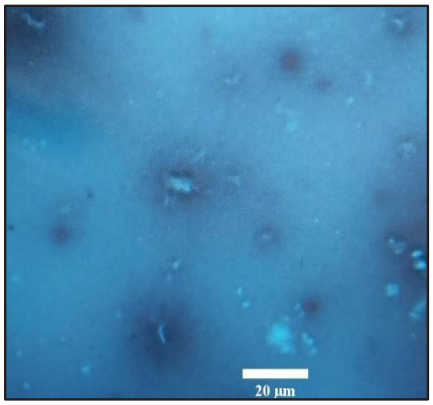
Polymerase chain reaction

Extracted DNA was subjected to PCR targeting *lipl*32 gene to confirm the diagnosis (Krishna *et.al,* 2013). Electrophoresis of PCR products was carried out to visualize the amplicons at 790bp.

Results and Discussion

Out of the 24 urine samples examined two samples showed presence of negatively stained leptospires as colourless spirochetes against dark blue back ground. These organisms were measured using micrometry and found to be 6-8 µm in size. The DNA was extracted from these two urine samples and subjected to polymerase chain reaction and yielded positive amplicons at 790bp.

Leptospires are corkscrew-shaped bacteria, which differ from other spirochaetes by the presence of end hooks. They belong to the order of Spirochaetales, family Leptospiraceae, genus Leptospira, about 0.1 µm in diameter by 6–20 µm in length (Faine *et.al,* 1999). Leptospires are mobile, their bodies are small diameter requiring the use of dark field microscopy (DFM) or phase contrast for observation of organisms from infected urine or serum. Even though it



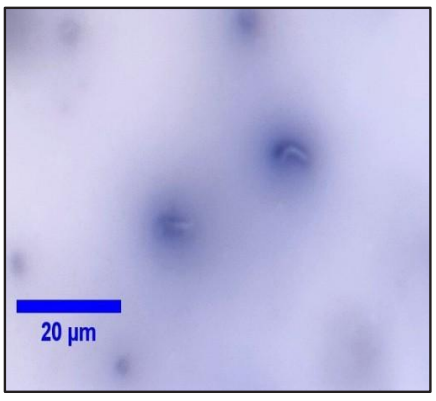


Fig 1 & 2 : Negatively stained leptospires of 6-8 µm at 100 X magnification

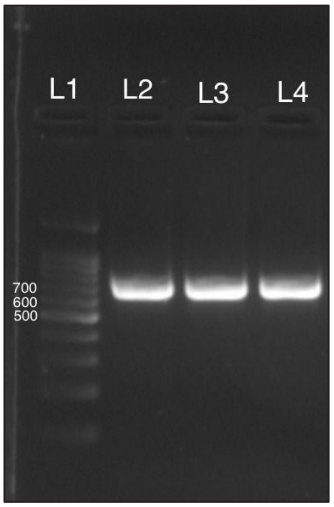


Fig 3 : L1- Ladder, L2, L3- urine samples, L4- positive control (790 bp)

is a simple low cost technique its sensitivity and specificity are questionable because of the chance for non specific observations. Leptospires are poorly visible by conventional gram staining techniques. Leptospirae are not visible through the optical microscope on a light field, but on a dark field they show up quite brilliantly. They can be observed, as negative images, on a glass slide. For this purpose the most suitable procedure is the Congo red method proposed by Hoyer (1956). Ajaj and Al-Farwachi (2013) reported 33 out of 35 samples were positive in Congo red staining from suspected bovine urine samples. Ryu (1963) demonstrated a modified Gram staining for identification of leptospires. But Babudeiri (1961) used Congo red staining for demonstration of leptospires, considering the invisibility of thin spirochetes in Gram staining. Compared to other staining techniques in congo red staining the back ground get stained to dark and so thin leptospira can emerge as bright against it (Benians, 1961). Chance of taking colour by nonspecific objectives and giving false positive result is less in Congo red staining.

In the present study negatively stained organisms with hooked ends of 6-8 micrometre could be observed in two urine samples (8.3 per cent) and later confirmed as leptospira through PCR. Motility suggestive for spirochetes could not be observed on DFM in these samples. Hence Congo red staining is a better, simple, rapid and cost effective direct microscopic identification method for diagnosis of leptospirosis.

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