

Research article

Utility of a modified silver staining technique for detection of *Leptospira*

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Abstract

Background: *Leptospira* are spiral thin and highly motile pathogenic bacteria that are best visualized by dark ground microscopy. Although these bacteria are not stained by the Gram's stain, the Fontana stain, which is a silver impregnation staining method, can be used successfully for light microscopy. It is important to investigate the usefulness of Fontana stain method for direct demonstration of *Leptospira* in human body fluids.

Objectives: To determine the usefulness and sensitivity of a modified Fontana silver staining method for microscopic detection of *Leptospira* in clinical specimens.

Methodology: 6×10^8 organisms/ml of *Leptospira interrogans* serovar Icterohaemorrhagiae and Canicola were spiked into PBS (Phosphate Buffered Saline), alkalized urine and serum in triplicate and serial dilutions were made (6×10^6 to 6×10^1 organisms/ml). Smears were prepared using 10 μ l of each dilution. In addition, centrifuged sediment of urine were also used to prepare smears. Slides were stained by modified Fontana method as reported by Gangadhar et al.(1998) and examined. Numbers of leptospire per field ($\times 100$) were recorded.

Results: *Leptospira* spiked in PBS and urine appeared as thin slender bacteria with characteristic hooked ends after Fontana staining under the light microscope. Serum could not be used for the detection of *Leptospira* by this method. Leptospire could be detected by staining the spiked PBS and urine at 6×10^3 – 6×10^6 organisms/ml.


Conclusion: Leptospire could be detected by Fontana staining in spiked PBS, urine (uncentrifuged and sediment). Serum was not suitable for detection of leptospire by Fontana staining. The detection limit of leptospire in PBS and urine by Fontana stain was found to be 6000 organisms/ml.

Keywords: *Leptospira*, Modified Fontana silver stain, Urine

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INTRODUCTION

Leptospirosis is a potentially fatal zoonotic infection with a worldwide distribution, caused by *Leptospira* species.^{1,2} Leptospirosis is endemic in Sri Lanka with outbreaks of disease generally associated with the paddy cultivation seasons and monsoon periods. Many mammalian species can harbor leptospire in their kidneys and act as reservoirs for transmission of infection. Rodents, cattle, buffaloes, horses, sheep, goats, pigs and dogs are the most common reservoirs.³⁻⁵ The disease is transmitted mainly by direct or indirect contact with water sources or soil contaminated with urine of infected animals.⁶ Major life threatening complications of leptospirosis reported in recent outbreaks in Sri Lanka which include renal failure⁷, haemorrhage into most organs and tissues and multi organ failure.⁸

Early diagnosis of leptospirosis is critical as early administration of antimicrobials improve prognosis. Laboratory diagnosis of leptospirosis involves culture, Polymerase Chain Reaction (PCR), Microscopic Agglutination Test (MAT) and detection of leptospire in clinical samples by histological, histochemical or immuno staining techniques.⁹ However, the availability of these diagnostic opportunities are limited to specialized laboratories and are expensive and time consuming. Rapid immunochromatographic assays to detect *Leptospira* specific IgM antibodies are useful in presumptive diagnosis of leptospirosis¹⁰ but are not available in the public sector in Sri Lanka. Due to the unavailability of early diagnostic assays currently in Sri Lanka, the diagnosis of leptospirosis is mainly based on clinical judgment.

Direct demonstration of *Leptospira* in clinical specimens by microscopy can provide an early and rapid diagnostic opportunity. However observation of live motile leptospire require the use of a dark ground microscope and can be unreliable due to presence of leptospira like artifacts in the specimen.¹¹ Leptospire are spiral and thin bacteria which cannot be stained by the Gram stain. However, leptospire in body fluids and tissues can be stained and visualized using silver impregnation methods. The modified Fontana technique can be used for staining of leptospira and is a relatively inexpensive method where stained organisms can be observed using a conventional light microscope which is available in a basic laboratory setting. In this study, we had evaluated the usefulness of a modified Fontana silver staining technique by Gangadhar and Rajsekhar(1998)^{12,13} for detection of leptospire in urine and serum.

Objective

To determine the usefulness of a modified Fontana silver staining method for observation of morphology and enumeration of *Leptospira*.

Materials and Methods:

Bacterial Strains and specimens

Cultures of *Leptospira interrogans* serovar Icterohaemorrhagiae and Canicola were obtained from the National Leptospira Reference Laboratory, Medical Research Institute (MRI), Colombo. Stock cultures were maintained at room temperature in Ellinghausen-McCullough-Johnson-Harris(EMJH) medium.

Preparation of *Leptospira* spiked urine and serum specimens

Confluent bacterial cultures of *Leptospira interrogans* serovar Icterohaemorrhagiae and *Leptospira interrogans* serovar Canicola cultured in EMJH medium were used for the

experiments. Optical Density (OD) of cultures were measured using spectrophotometer at 420 nm and OD was adjusted to McFarland 2 which is equivalent to 6×10^8 organisms/ml. Serial tenfold dilutions of *Leptospira* cultures were prepared in PBS, serum and urine in triplicates.

Fresh urine samples were alkalized using 4 N NaOH until pH was 6-7 and added in triplicate to two sets of tubes for spiking. A serum specimen which tested negative for *Leptospira* IgM and IgG was used for spiking. Tenfold serial dilutions of leptospire were prepared in PBS, urine and serum. Spiked urine specimen was used as follows: one set of spiked urine was used for preparation of smears while the other set was centrifuged at 3000 rpm for 5 minutes. The centrifuged sediments were used for preparation of smears. Smears were prepared using 10 μ l of urine suspension or sediment on pre-labeled clean glass slides and air dried. Experiments were repeated three times to ensure reproducibility.

Modified Fontana silver staining Technique

Smears were stained using modified Fontana silver staining technique as described by Gangadhar and Rajsekhar (1998).^{12,13} Briefly, smears were fixed by dipping in a fixing agent for two minutes containing 1 ml of Glacial acetic acid (Merck) and 2 ml of 40% formaldehyde (Sigma) solution made up to 100 ml in distilled water. The slides were removed using a pair of forceps and blotted onto a tissue paper. Subsequently, the slides were dipped in absolute alcohol for 3 minutes. The under surface of the slides were cleaned using filter paper and the smears air dried. The slides were dipped in the mordant containing 1 g phenol (Sigma) and 5 g tannic acid (BDH) made up to 100ml with distilled water, in a hot water bath pre-heated to 75 °C and kept for 1 minute. The slides were rinsed in distilled water and dipped in ammoniated silver solution, which was pre-heated to 75 °C in a hot water bath and kept for 1 minute. The ammoniated silver solution was prepared by dissolving 0.6g of silver nitrate (Sigma) in 100ml of distilled water. Sixty ml aliquot of this solution was mixed with few drops of 10% ammonia solution and shaken to obtain a brown precipitate. Further ammonia solution was added till the precipitate dissolved. Finally small quantities of silver nitrate solution were added from the 40 ml aliquot to the above ammoniated solution till a stable precipitate reappeared.

After rinsing the slides in distilled water, the slides were air dried and examined under oil immersion objective of a bright field microscope.

Results

Fontana staining method of leptospire resulted in dark brown stained bacteria in a white or pale yellow background. The Fontana stain revealed thin and spiral organisms usually containing one or two hooked ends when observed under 100x. (Figure 1). The morphology of leptospire were clearly visible in smears prepared from spiked PBS and spiked urine specimens. However it was not possible to identify Leptospire spiked to serum using the modified Fontana silver stain method due to the high background staining which was attributed to the high protein content of the serum. Due to the nonspecific staining it was not possible to differentiate the leptospire from the background in serum.

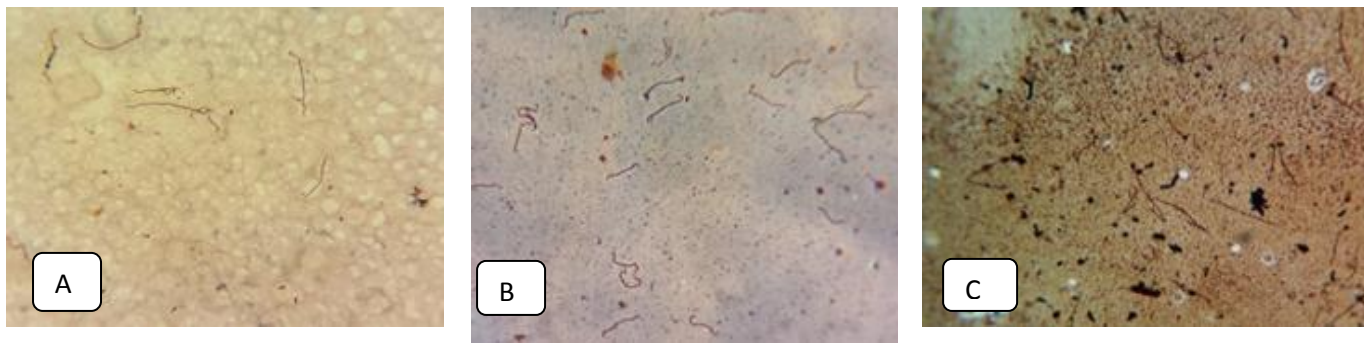


Figure 1: Appearance of leptospire stained with Fontana silver stain (oil immersion); A - in PBS, B - in urine (uncentrifuged) and C - in urine sediment.

Leptospire could be detected in Fontana stained smears made from spiked cultures containing 6×10^3 – 6×10^6 organisms/ml. At a concentration of 6×10^3 organisms/ml, at least 1-3 organisms/smear could be detected in PBS and urine (centrifuged and uncentrifuged), while an average of 5 organisms/field could be detected in a smear made from spiked cultures of 6×10^5 organisms /ml (Table 1). The results indicate that at least 6000 organisms/ml should be present in the urine for detection by Fontana staining when examining the whole smear. It is recommended to observe the whole smear in order to improve the sensitivity of the method.

Table 1: Results-of Fontana silver staining for leptospire in PBS and urine (Uncentrifuged and sediment)

	Serovar	Concentration of each dilution (organisms/ml)					
		6×10^6	6×10^5	6×10^4	6×10^3	6×10^2	6×10^1
PBS	Icterohaemorrhagiae	40-50*	10-20*	3*	3***	Negative	Negative
	Canicola	25-30*	2-3*	3*	3***	Negative	Negative
Urine Uncentrifuged	Icterohaemorrhagiae	30	5*	1**	3***	Negative	Negative
	Canicola	40*	15-20*	5*	2**	1***	Negative
Urine Sediment	Icterohaemorrhagiae	40*	5*	3**	3***	Negative	Negative
	Canicola	25-30*	6-7*	3**	1***	Negative	Negative

* - Organisms / Field

** - Organism / 30 Fields

*** - Organism / Smear

Discussion

Rapid, simple to perform and inexpensive diagnostic tests which can be carried out in a resource poor setting will be of great value to support the clinical diagnosis in developing countries, enabling initiation of antimicrobial therapy in the early disease stages. The Microscopic Agglutination Test is considered as the gold standard for diagnosis of leptospirosis.⁹ This test is currently available only at the national leptospirosis reference center (MRI, Colombo). MAT requires culture and maintenance of live leptospira strains and is a technically demanding procedure. PCR which can detect *Leptospira* specific DNA and isolation of leptospira by culture are also technically demanding and expensive. Furthermore, culture may take up to one month to become positive,⁶ thereby limiting its usefulness in clinical practice.

Direct demonstration of leptospira in clinical specimens can be useful in early diagnosis. Light microscopy for detection of *Leptospira* has been reported by Babudieri (1965) using Giemsa stain and silver stain.¹⁴ Silver stain impregnation staining methods can be applied to body fluids for detection of leptospire. In this technique, chemically reducing surface properties of leptospire reduce silver ion to its metallic state,¹² where the deposition appear as black or brown in colour. This enables visualization of the slender spiral bacteria using a conventional light microscope. However the stain will also interact with protein fibrils which can be misinterpreted as leptospire during microscopy. In addition, in protein rich specimens (serum) or in the presence of cells (urine sediment), the strong colour of silver stained deposits will mask the fine slender stained leptospire. Therefore observation of leptospire in such smears will be limited to areas containing thin smears with low background staining.

In this study, depending on the concentration of organisms spiked to the test sample, it was necessary to observe the whole smear in samples containing 6000 organisms/ml and at least an average of 30 fields when the specimen contained 6×10^4 organisms/ml for identification. The stain enabled observation of the typical hooked ends of *Leptospira interrogans* which supported the microscopic identification of the organism. Using the modified Fontana staining of the two serotypes, Canicola and Icterohaemorrhagiae, visualization of the latter was clear compared to Canicola which may be due to the difference in the staining properties and size variation.

Quantitative PCR has demonstrated between 10^2 to 10^6 leptospira/ml in blood⁸ and between 10^1 to 10^6 leptospira/ml in urine¹⁵. The bacteria are known to colonize the proximal renal tubules of kidneys and are excreted intermittently in the urine from the second week onwards up to a month or even upto one year.¹⁶ In our study, the ability to detect above 6000 organisms/ml in urine using the modified Fontana staining suggests its possible diagnostic utility although having a low sensitivity. A study by Rodriguez et al (2013) is the only recent study reporting the usefulness of the modified Fontana method developed by Gangadhar and Rajsekhar (1998). In their study, serial dilutions of *Leptospira* were spiked into urine and results of modified Fontana staining was compared with culture and dark field microscopy. Similar to our study they were able to detect presence of leptospire by staining in smears containing $>10^3$ organisms/ml and found 100% sensitivity at and above the concentration of $(1-10) \times 10^5$ organisms/ml in culture, natural urine (uncentrifuged and sediment) and alkalized urine (uncentrifuged and sediment).¹²

Live leptospire are generally visualized by dark ground microscopy, while phase contrast microscopy is also a possible means of visualization.⁶ Dark ground microscopy may be used for detection of live motile leptospire in body fluids. However the presence of lysed red blood cells (RBC), fibrils and other bacteria may give false positive results by this method, thereby limiting its usefulness in diagnosis. Further, dark ground microscopy is unavailable in most laboratories due to the high cost, expense and limited utility. The diagnostic utility of dark ground microscopy is therefore low. The results of the present study suggest that the modified Fontana stain can be useful in resource poor settings as a rapid and low cost preliminary test for diagnosis of leptospirosis.

However the silver impregnation staining method cannot be used for quantitative enumeration as the smear may vary in thickness giving high background in different areas limiting the observation of leptospire. Serum or blood specimens cannot be used for modified Fontana staining as it is high in protein content. Serum specimens gave a high

background by modified Fontana stain and therefore investigators were unable to observe the leptospire in smears of spiked serum in comparison with smears made from leptospira spiked urine specimens. On the other hand urine specimens also contain some proteins and there is a possibility of false positive results. The intermittent secretion of leptospire in urine is another limiting factor which may give rise to false negative results. However it should be considered that in the absence of the availability of rapid diagnostic assays, modified Fontana stain may offer supportive evidence for diagnosis in patients with high leptospiraemia.

In conclusion, the modified Fontana staining method is a simple and rapid test for detection of leptospira in urine. This method enabled microscopic observation of 6000 organisms/ml in PBS, and urine (uncentrifuged specimen and centrifuged sediment).

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Conflicts of Interest:

The authors declare no conflicts of interest

Ethical statements:

Ethical approval was obtained from the Ethics Review Committee of Faculty of Medical Sciences, University of Sri Jaywardenepura, Nugegoda (MLS 10/2015).

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