

Original Research Article

Proteinuria in early detection of human leptospirosis

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ABSTRACT

Background: Leptospirosis is an infectious disease caused by spirochetes bacteria *Leptospira* spp. and is reported from all over the world. As the clinical signs and symptoms of Leptospirosis often are nonspecific and the disease is early mistaken for other major infectious febrile illness, laboratory test to confirm the clinical diagnosis thus is essential for optimal treatment and patient management.

Methods: Serum and urine samples were collected from patients clinically suspected cases of Leptospirosis. Preparations of urine concentrate by precipitation and centrifugation.

Results: It was interesting to note that immunoglobulins are present in the urine protein concentrate of patients with Leptospirosis on the day of admission in the hospital, with urine albumin reports either positive or negative. By ELISA test it was noted that antibodies present in urine and serum were of both IgM and IgG class against the Leptospiral antigens from three pathogenic serovars and one non-pathogenic serovars. In the immunospot test which was done and compared with standard ELISA test for serum antibodies using same antigen showed that antibodies present in urine protein concentrate, which was collected on the day of admission when patients come with suspecting symptoms of Leptospirosis.

Conclusions: Proteinuria is the most frequent abnormality noted in all patients at some stage of illness. This is the first report on the presence of immunoglobulins in urine samples, which were found to be of IgM and IgG classes. These findings are of significant diagnostic potential as a simple immune-spot test can be done for detecting anti-leptospiral antibodies in urine samples of suspected cases. The present attempt was aimed at developing an immunospot test, a simple and rapid diagnostic test to detect Leptospirosis using urine samples of clinically suspected patients of the infection at the earliest. It was found to be in good correlation with standard ELISA method which is being used to detect serum antibodies in *Leptospira* infected patients using the same antigen.

Keywords: Immunospot, Leptospirosis, Urine IgM and IgG

INTRODUCTION

Leptospirosis can be endemic in countries with wet and warm climates. It is an important infectious disease of humans and animals worldwide, caused by the

pathogenic serovars of genus *Leptospira*. The symptoms and severity of Leptospirosis vary greatly from mild flu-like illness to the often fatal hemorrhagic form, called Weil's disease characterized by Jaundice, pulmonary haemorrhage, renal damage due to involvement of vital

organs such as liver, lung and kidney.¹ Early and accurate diagnosis of Leptospirosis is important for proper and prompt treatment, which is life-saving for patients with severe illness. Leptospirosis may be confused with Malaria, viral hepatitis, influenza, dengue fever, rickettsial infections, typhoid fever, melioidosis and others.²

Human are accidental host and acquire the infection from contaminated environments. Rodents and farm animals contribute to the transmission of the disease by contaminating the environment by shedding the leptospires in their urine. Hence, the disease transmission is enhanced due to poor hygienic conditions and is an occupational. In humans, while the disease is often self-limiting, the pathogen may cause serious damage when it colonises in the kidneys, where it evades the host immune response.³ Due to the strong humoral immune response, several serological assays have been developed for detecting the rise of serum antibodies such as Flow-through, Ig dipstick immunofluorescence, latex agglutination, microcapsule agglutination test, the direct enzyme linked immune sorbent assay (ELISA) for immunoglobulin IgM antibodies, and dot ELISA for IgM.⁴⁻¹¹

These methods are being questioned, thinking that appropriate immune response might not yet have been elicited by the time of specimen collection. Immunochromatography based methods for detection of Leptospiral lipopolysaccharide antigen in urine have also been developed.¹² The sensitivity and specificity of these methods are often debatable due to prior exposure of the individuals in endemic areas and due to the contaminating nonpathogenic leptospires in the environment.

Leptospires are shed in the urine of patients with the disease. However, it has not been practical to detect them either by culture or molecular methods. Detection of urine leptospiral LPS, though the sensitive and specific, suffers from the disadvantage of laborious sample preparation before analysis. In this study we report for the first time the presence of antibodies of IgM and IgG classes that are specific for leptospiral antigens in the urine of patients with leptospirosis.¹³ This has significant diagnostic implications as a simple, rapid, cost effective test for early diagnosis by detection of anti-leptospiral antibodies in urine of patients with the disease.

METHODS

The sample collection was coordinated by department of Biochemistry in collaboration with Department of Medicine. The antigen preparation, ELISA test and MAT analysis were shared by Microbiology of MOSC Medical College, Kolenchery and Department of Animal Biology, University of Hyderabad, India. Tests including immuno-electrophoresis and immune spot test were done in Biochemistry Department, MOSC Medical College. The

study was approved by institutional ethics committee. Urine and serum samples were collected from patients admitted in our hospital with various febrile illnesses, with their consent. Preparation of antigen as per world health organization guide lines.^{2,10}

Leptospiral serovars used in this study: *L. interrogans* serovars Lai and Pomona; *L. borgpetersenii* serovar Hardjobovis and the non-pathogenic *L. biflexa* serovar Andamana for the detection of IgM and IgG –specific antibodies in serum and urine samples. *L. biflexa* Patoc I for routine ELISA test for Diagnosis of Leptospira infection in patients was used for immune-spot test.

Preparation of whole cell sonicates of the leptospiral serovars

All the serovars were grown till log phase in EMJH-BSA liquid media. The cultures were induced with 120mM NaCl for 4h at 30°C (this was to induce the expression of sphingo-myelinases). The cells were harvested and washed twice with 0.1M PBS. Whole cell sonicate of each serovars were prepared by sonication at 40Hz amplitude, 20s pulses for 5 min with cooling for 20s in Vibra Cell sonicator, (Sonics, Newtown, CT, USA). Protein was estimated by BCA method .

Clinical samples

Serum and urine samples were collected from clinically suspected cases of Leptospirosis.

Preparation of urine concentrate by precipitation and centrifugation-5ml urine collected from normal and clinically suspected cases of *Leptospira* infection, mixed with 5ml of saturated ammonium sulphate (AR) solution and kept for 10 min. The mixture was centrifuged at 12,000rpm for 10 min. The supernatant was decanted and the precipitate was re-suspended in 200µl distilled water. Protein concentration was estimated by Qubit Fluorometer along with normal urine protein concentrate.

Electrophoresis of Urine protein concentrate was done along with serum protein to detect the presence of gamma globulin fraction. Amido-black staining solution was used.¹⁴ Immuno-electrophoresis for detecting the presence of anti leptospiral antibodies using Patoc antigen was done on Whatman paper - IV.¹⁵

Microscopic agglutination test (MAT)

The leptospiral reference strains used for the study were obtained from the Regional Medical Research Centre and WHO Collaborating Centre for Diagnosis, Reference, Research and Training in Leptospirosis (ICMR), Port Blair, Andaman and Nicobar Islands. The following *Leptospira* spp. were used: *L. Interrogans* (Australis strain Ballico; Bankinang strain B. Bankinang I; Canicola strain Hond Utrecht IV; Hebdomadis strain Hebdomadis; Lai strain 56601; Pomana strain Pomana; Hardjo strain

Hardjoprajitno), *L. weilii* (Celedoni strain Celedoni), *L. borgpetersenii* (Hardjo-bovis strain L550, Tarassovi strain Perepelicin), *L. biflexa* (Andamana strain CH11; Patoc strain Patoc I). The organisms were grown in liquid EMJH medium with 10% enrichment medium (Difco Laboratories, USA) and maintained at 30°C.

MAT was done with the above live serovars on a total of 12 serum samples from patients and 5 normal controls. The end point was taken as the highest dilution of the serum in which 50% of the organisms were agglutinated or there was a 50% reduction in the number of organisms as compared to control. Titres ≥ 100 were considered as positive.

Enzyme-linked immunosorbent assay (ELISA)

This was done as reported earlier. Three pathogenic serovars, namely *L. interrogans* serovars Lai and Pomona, *L. borgpetersenii* serovar Hardjobovis and one non-pathogenic spp. *L. biflexa* serovar Andamana were used as antigens for the detection of IgM and IgG specific antibodies in serum and urine samples. The organisms were grown to a cell density of $1-2 \times 10^8$ cells / mL in EMJH – enrichment medium and the incubated for 4 hr after adding 120 mM NaCl. The cells were harvested at $12,000 \times g$ for 20 min, washed twice with 0.1M phosphate-buffered saline PBS, sonicated for 5 min (20 second pulses at 40 Hz in a Vibra Cell sonicator, USA) and centrifuged to obtain the cell-free whole cell sonicate, in which the total protein was estimated by the commercial BCA kit (Sigma Aldrich, St. Louis, MO, USA).

ELISA was done with the serum and urine samples using the above three antigens for the detection of IgM and IgG antibodies. Briefly, 500 ng antigens was used and serum and urine were added at 1:200 dilution and the secondary antibody-enzyme conjugate included goat anti-human IgG (Fc specific)-peroxidase conjugate and goat anti-human IgM (μ -chain specific)-peroxidase conjugate (1:5000 dilution) respectively for the detection of IgG and IgM-specific antibodies. The substrate was 3, 3', 5, 5'-tetramethylbenzidine (Sigma Aldrich, St. Louis, MO, USA) and 3 μ l of H₂O₂. The colour was allowed to develop for 20 min, followed by addition of 1.25 M sulphuric acid as stop solution. The absorbance was measured at 450 nm with ELISA reader (Model 680XR, Bio-Rad, CA, USA). Experiments were performed in duplicate and repeated twice. The controls included antigen and antibody blanks, known positive and negative serum samples. Statistical analysis of the data was done with Graph Pad Prism software (Version 6, Graph Pad Software, Inc., San Diego, CA) and graph was generated using Sigma Plot 10 software.

The results were analysed by Mann Whitney test considered significant when $P < 0.05$. The cut-off value for IgM and IgG ELISA was calculated by adding two

times standard deviation (SD) to the mean value of normal endemic controls (2SD + Mean).

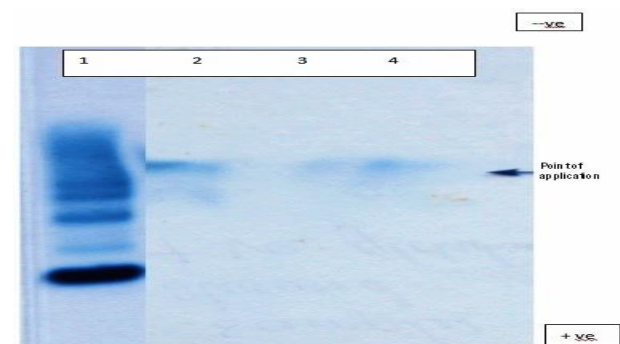
Immunospot test

According to the above observations tried to develop Immunospot test. Two sets of clinical specimens were used in this study. In the first set, 192 urine samples were collected from patients with different febrile illness with history of 3-8 days of fever. WHO criteria used for the diagnosis of Leptospirosis and compared with the Enzyme linked immune-sorbent assay for IgG antibodies, which is the diagnostic test performed in our Microbiology lab. In second set, serum and urine samples were collected on the first day of admission and after 5 or 6 days hospitalization from clinically suspected cases of Leptospirosis. In blood samples, the presence IgM antibodies were detected using ELISA assay. In urine samples, protein was concentrated by precipitation and centrifugation. 3 μ l of antigen (*L. biflexa* Patoc I) spotted on Whatman paper IV and allowed to dry for 3 min. Applied 5 μ l of urine concentrate on the same spot and kept 3min. for antigen-antibody reaction, the excess protein concentrate was removed by washing with distilled water, stained with diluted (1:9 with distilled water) amido-black protein stain for 3min. and destained with destaining solution¹⁵ for 10 min. Deeply stained spot could be observed in samples with *Leptospira* infection compared to less or no staining for normal and differently infected samples. Healthy inhabitants of the same geographical area were considered as normal.

RESULTS

Detection of anti-leptospiral antibodies in the urine of leptospirosis patients

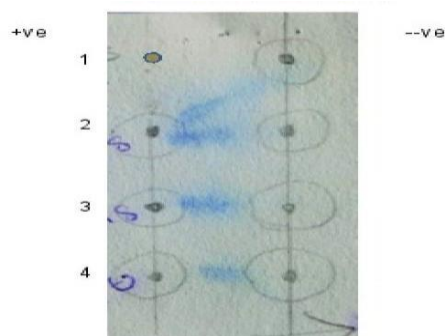
The preliminary study of urine protein concentrate showed that the protein concentration was increased in infected samples compared to normal samples. The electrophoresis of urine protein concentrate along with serum showed the presence of Immunoglobulins in infected samples with urine albumin positive or negative (Figure 1).



Sample no.1-serum sample; 2, 3, and 4-Urine protein concentrate of *Leptospira* infected patients

Figure 1: Agar gel-electrophoresis.

The immune-electrophoresis showed the presence of immuno-precipitin formation, indicated that the antibodies of *Leptospira* is present in the urine concentrate. There is an interesting finding that Leptospiral antibodies are moving towards the nearby antigen spot when the corresponding antigen spot is kept as blank (Figure 2).



Sample: 1- Blank; 2, 3, 4 Urine protein Concentrate, Leptospiral antigen move towards anode and form precipitin line with antibodies. Antigen opposite to blank move towards sample no.2

Figure 2: Immunoelectrophoresis on Whatman IV, paper.

Anti-leptospiral antibodies are of IgM and IgG classes

A selective panel of 12 serum and urine samples were tested by MAT and ELISA to identify if the antibodies were against the leptospiral antigens. The serovar Lai was identified as the predominant serovar by MAT analysis. MAT identified 10 of the 12 serum samples as positive, with titres ranging from 100 to 3200, with mixed infection also seen. Serovar Lai was predominant (41%), with the others including Hardjobovis (18%), Pomona and Celledoni (6%) (Table 1). All the healthy controls and two patients' samples tested negative by MAT.

IgM and IgG ELISA done with were serum and urine samples are represented in Figure 3 and 4. The two MAT negative samples were not included for analysis. The serum showed high titres of both IgG and IgM classes were observed against the pathogenic serovars as compared to the non-pathogenic serovar Andamana.

Interestingly, the urine samples showed antibodies of the IgG class. Notable is the unusually high titre (with OD_{450 nm} being 1.953) in one of the samples and it must also be noted that antibodies were seen against the non-pathogenic serovar Andamana. As this reflects cross-reacting antigens, pathogen-specific antigens may be preferred.

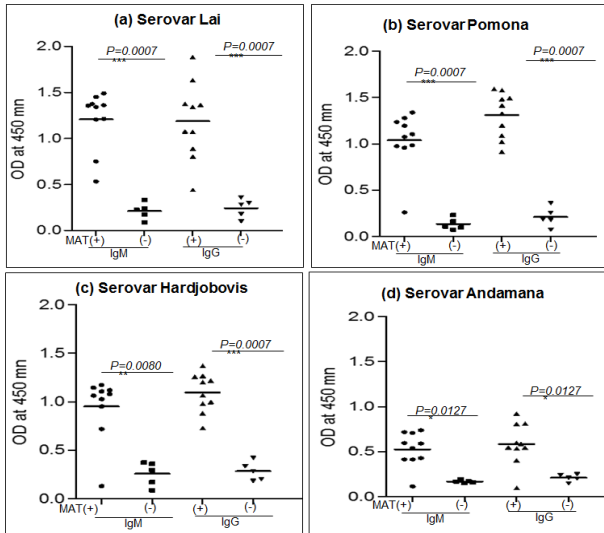
Table 1: Serotyping of patient's serum samples by gold standard MAT.

Sample ID	MAT Result	Titre
NS	P	Lai and Ponona (1:3200), Hardjobovis (1:400), Tarassovi (1:200), Celledoni (1:100)
JH	P	Lai (1:3200), Ponona (1:1600), Hardjobovis (1:400), Celledoni (1:200)
Sample 1	P	Lai (1:3200), Hardjobovis (1:1600), Tarassovi (1:200), Pomona (1:100)
Sample 2	P	Hardjobovis (1:3200), Pomona (1:400), Bankinang (1:200), Lai (1:100)
Sample 3	P	Hardjobovis (1:800), Lai (1:400), Pomona (1:200), Celledoni and Tarassovi (1:100)
Sample 4	N	MAT negative
Sample 5	P	Pomona (1:1600), Lai (1:800), Hardjobovis (1:400), Tarassovi (1:100)
Sample 6	N	MAT negative
Sample 7	P	Lai (1:800), Pomona, Hardjobovis and Tarassovi (1:100)
Sample 8	P	Hardjobovis (1:800), Pomona and Celledoni (1:400), Lai (1:200)
Sample 9	P	Lai (1:800), Pomona, Hardjobois and Celledoni (1:100)
Sample 10	P	Celledoni (1:3200), Lai (1:200), Pomona, Hardjobovis and Tarassovi (1:100)
HC1*	N	MAT negative
HC2	N	MAT negative
HC3	N	MAT negative
HC4	N	MAT negative
HC5	N	MAT negative

*HC refers to healthy controls.

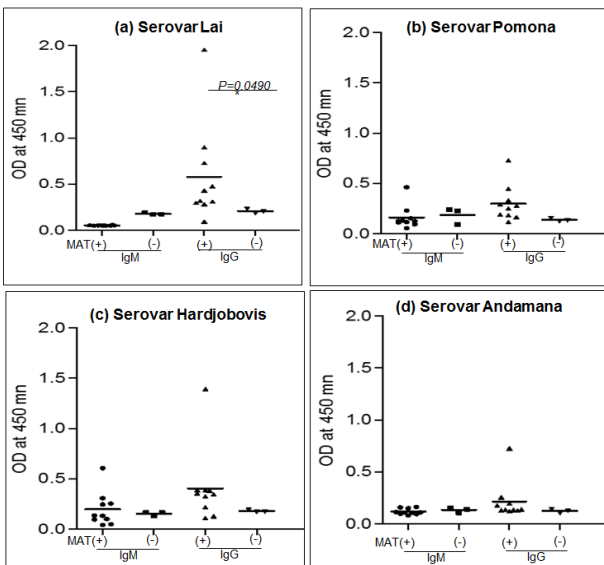
The IgM class of antibodies was low in the urine, with only one sample showing the presence of antibodies against the serovars Pomona and Hardjobovis. It is highly likely the larger size of this class does not leak through

unless the renal damage is severe. Urine, usually free of any proteins in normal healthy individuals showed the presence of protein, especially immunoglobulins under disease conditions may be indicating the involvement of the kidneys.



All the MAT positive (+) represent the patient samples with the MAT (-) representing the controls. The figure was generated using Graph Pad Prism. The values are the mean of four values obtained from duplicates done from two independent experiments.

Figure 3: IgG and IgM-specific anti-leptospiral antibodies in the serum of leptospirosis patients. Panels (a), (b) and (c) represent the serum antibodies against the cell-free whole cell sonicates of pathogenic serovars Lai, Pomona and Hardjovobis and the panel (d) represents the non-pathogenic serovar Andamana.

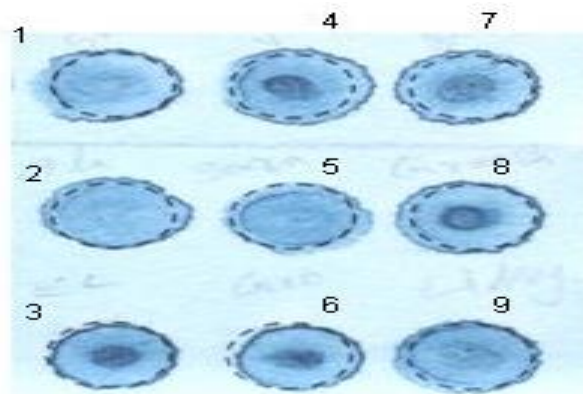


All the MAT positive (+) represent the patient samples with the MAT (-) representing the controls. The figure was generated using Graph Pad Prism. The values are the mean of four values obtained from duplicates done from two independent experiments

Figure 4: Urinary immunoglobulins: IgM and IgG-specific anti-leptospiral antibodies in patients with leptospirosis. Panels (a) to (d) represent the levels of IgM and an IgG-specific antibody against whole cell sonicates of serovars Lai, Pomona, Hardjovobis and Andamana.

Potential of the immune spot test for the diagnosis of Leptospirosis

The immune-spot test was read in comparison with standard ELISA test using the same antigen (Figure 5). In the first set of the study it was observed that out of 192 samples 130 (68%) showed immunospot positive for Leptospira antibodies in urine. Among this 130 samples, 60% were positive for both ELISA and immunospot. 30% of immunospot positive samples have not been requested for ELISA. The 10% immune spot positive showed IgM titre in ELISA is <20, below the cut off value (<80). It can be noted that even at low titre value of serum antibodies, if the clinical findings are supportive, urine concentrate can give a positive result with immunospot test.



Samples 1 and 2 are Negative controls, Sample 3 positive control; Samples 4, 5, 6,7,8,9, are test samples. 4, 6, 8 were found to be positive and 5, 7, 9 found to be negative.

Figure 5: Immunospot test was done with urine samples of clinically suspected Leptospirosis infection.

In the second set, out of 21 samples collected from clinically suspected cases of Leptospirosis, 19 urine samples collected on the day of hospitalization and its corresponding serum samples collected during 5-8 days of hospitalization for ELISA test (WHO guide lines) showed significant correlation. Sensitivity found to be 100% and specificity 25% (Table 2) Though antibody titre was less in serum samples collected on the day of hospitalisation it was found to be raised after 5-8 days. The second urine samples which collected during 5-8 days of hospitalization also showed positive correlation with first urine samples and the corresponding serum samples.

DISCUSSION

The infectious disease Leptospirosis is found to be life threatening due to its increase incidence in developing countries, increased severity of the disease, delayed diagnosis and/or misdiagnosis. Leptospirosis is not readily distinguishable based on the clinical presentation and epidemiological background of the patients from other infectious fevers which share the same geographical

areas of endemicity. Laboratory confirmation plays an important supplementary role to the clinical findings and helps in sorting out the early differential diagnosis. There are existing methodologies for Leptospirosis like MAT

assay, which are laborious and time consuming, Flow through IgM dipstick, Immuno-fluorescence and latex agglutination, which are with low sensitivity and specificity during early stage of infection.⁷

Table 2: Comparative study of Leptospira infected urine samples using Immuno-spot test and ELISA Test.

ELISA IgM Titre 1 st sample	ELISA IgM Titre 2 nd sample	Immuno spot for 1 st sample	Immuno spot for 2 nd sample	Febrile days prior to hospitalisation
80	320	+ ve	+ve	5
20	20	+ ve	+ve	2-3
20	20	+ ve	+ve	3
20	640	+ ve	+ve	3
80	320	+ ve	+ve	5
20	80	+ ve	+ve	2
20	20	+ ve	+ve	2
80	80	+ ve	+ve	4
160	320	+ ve	+ve	3
160	160	+ ve	+ve	3
20	20	-ve	-ve	7
20	20	-ve	-ve	2-3
40	80	+ ve	+ve	4
20	20	+ ve	+ve	2
20	20	+ ve	+ve	4-5
20	80	+ ve	+ve	3
160	640	+ ve	+ve	5
40	320	+ ve	+ve	3
80	40	+ ve	+ve	4
80	80	+ ve	+ve	5
20	160	+ ve	+ve	2 weeks

PCR which is expensive and development of immunochromatography for urine Leptospiral antigens needed to have laborious pre-treatment of samples. In this situation there is in need to develop a simple, early, rapid and cost effective method to diagnose Leptospirosis in endemic areas.

The preliminary studies showed the presence of Leptospiral antibodies in urine of patients when they come with the clinical symptoms including 3-5 days of fever.¹⁹ This is also been supported that Leptospire colonize host renal tubules, and *Leptospira* persists despite active immune process, such as interstitial nephritis characterized by lymphocyte infiltration, anti-leptospiral immunoglobulin production, MHC-II expression and TLR (toll-like receptors) activation.^{16,17}

In this study presence of proteins in the urine of patients with renal damage indicating pathology. In leptospiral infected renal injury, albumin is often analyzed in urine samples for assessing renal damage. This is found to be first global report that reports the presence of immunoglobulins in the urine, immediately upon clinical

diagnosis. Antibodies, predominantly of the IgG class are seen in the urine, compared to high levels of IgM and IgG in serum samples. The larger IgM antibodies were seen only in one patient urine sample. Though the titre of antibodies against the pathogenic serovars in urine is much lower than that seen in serum, the presence of these antibodies reflects the involvement of the kidney associated with leakage of the serum proteins in to the urine. The high levels of antibodies against the antigens from pathogenic serovars are of clinical relevance.

In the immunospot test, performed well when compared with serum ELISA with same antigen and with clinical symptoms. The test showed the presence of antibodies in the urine that was collected on the day of admission when patients come with suspecting symptoms of the disease. The sero conversion with increase in serum titres and the correlation of the results with urine samples collected on the subsequent days is further evidence of the usefulness of this test. The false positive results in comparison to ELISA Test may be due to the renal involvement during Leptospirosis.¹⁸ Screening of a large sample size and inclusion of other cases with fever, including viral flu,

dengue and malaria will help to validate this test as a useful diagnostic tool.

CONCLUSION

Anti-leptospiral antibodies are present in the urine of leptospirosis patients. The immune-spot assay that is developed in this study can be used for the early, rapid, cost effective diagnosis of the disease in resource poor areas, where leptospirosis is usually endemic.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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