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Silver enhanced nano-gold dot blot immunoassay for leptospirosis

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ABSTRACT

Leptospirosis is a widespread zoonotic disease and lacks in efficient diagnostic tools. In the present study, a nanogold based dot blot immunoassay was developed and evaluated for the detection of leptospirosis in human urine samples. This method was found to be rapid (< 4 h) with higher sensitivity (> 4.2–14.6%) than horse radish peroxidase (HRP) conjugated dot blot assay.

Leptospirosis is a re-emerging public health problem that is generally under diagnosed. The early and accurate diagnosis is still challenging and often leads to a fatal outcome (Bharti et al., 2003; Pappas et al., 2008). Annually, 500,000 cases of severe leptospirosis are reported, with fatality rates < 5 to 30% (WHO, 2011). The misdiagnosis of the disease can damage multiple organs and hence the development of a sensitive and reliable diagnostics is important. The gold standard diagnosis of leptospirosis is the microscopic agglutination test (MAT) that is often used for detection of leptospires. The other serological techniques including enzyme linked immunosorbent assay (ELISA), lepto dipstick, leptodridot and latex agglutination test are used but their sensitivity is dubitable during the acute illness. Advanced diagnostic formats including quantitative PCR and recombinant protein based ELISA have been developed (Raja and Natarajaseenivasan, 2015) but they have several drawbacks, including restricted availability; requiring skilled technicians and lack of uniformity. Moreover, seroconversion must be demonstrated with paired sera to confirm diagnosis. To overcome these limitations, there is a stringent demand for the development of new diagnostic formats for a simple and rapid method to perform in any diagnostic laboratory. Recently, the nanogold based diagnostic format has been evolving and showed significant outputs to resolve the above routes. Chirathaworn et al., 2011 reported detection of *Leptospira* in urine using anti-*Leptospira*-coated gold nanoparticles with high sensitivity. Furthermore, highly sensitive markers can be identified for rapid and prompt diagnostics and these lacunas will be empowered by nanogold based dot blot assay. The present study demonstrated the detection of human leptospirosis using a nanogold conjugate based dot blot immunoassay.

A total of 112 human urine and blood samples was collected from patients with febrile illness and suspected for leptospirosis at Government Hospital of Tiruchirappalli, Tamil Nadu, India between November 2010 and April 2012. Patients fulfilling any of the following criteria were considered as clinically suspected and laboratory confirmed cases of leptospirosis: (1) positive isolation of leptospires from blood/urine, (2) seroconversion or a 4-fold rise in titre in paired serum samples (collected with a mean interval of 23 days) by MAT; and/or (3) a seropositivity (> 1:400) in a crude leptospiral antigen based IgM ELISA. Diagnosis of leptospirosis was confirmed in 48 samples (group I) and 64 did not meet the diagnostic criteria and hence considered as discarded cases of leptospirosis (group II). Similarly, 45 seronegative healthy individuals matching for age (± 5 years) were recruited from patients attending the same hospital for complaints other than febrile illness (group III). Individual urine samples were centrifuged at 12,000 ×*g* for 10 min at room temperature; the supernatant was discarded leaving an aliquot of 50 µl, boiled for 30 min and centrifuged at 1000 ×*g* for 10 min. The supernatant obtained was subjected for protein estimation by bicinchoninic acid (BCA) method (Sigma-Aldrich, St. Louis, MO) and desired concentration was dotted individually onto NC strips (Kanagavel et al., 2017). Individual informed written consent was obtained from both cases and controls. This study was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University, India (Reference No.: DM/2010/101/14).

Several leptospiral proteins are reported to be used for diagnosis and LipL32 and LigA are being widely used for diagnostics. But the

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Table 1
Comparative analysis of Nanogold and HRP conjugated dot blot assay.

Antigen	LipL32		LigA		ArgC		RecA		LruC		LruD	
Conjugates	HRP	NG	HRP	NG	HRP	NG	HRP	NG	HRP	NG	HRP	NG
Sensitivity (%)	91.6	95.8	91.6	95.8	87.5	93.7	91.6	93.7	60.4	75	91.6	93.7
Specificity (%)	92.1	93.7	92.1	95.3	92.1	92.1	92.1	92.1	89	89	85.9	85.9
PPV[a] (%)	89.8	92	89.8	93.8	89.3	90	89.8	90	80.5	83.7	83	83.3
NPV[b] (%)	93.6	96.7	93.6	96.8	90.7	95.1	93.6	95.1	75	82.6	93.2	94.8

HRP: Horseradish Peroxidase; NG: Nanogold.
[a] Positive Predictive Value.
[b] Negative Predictive Value.

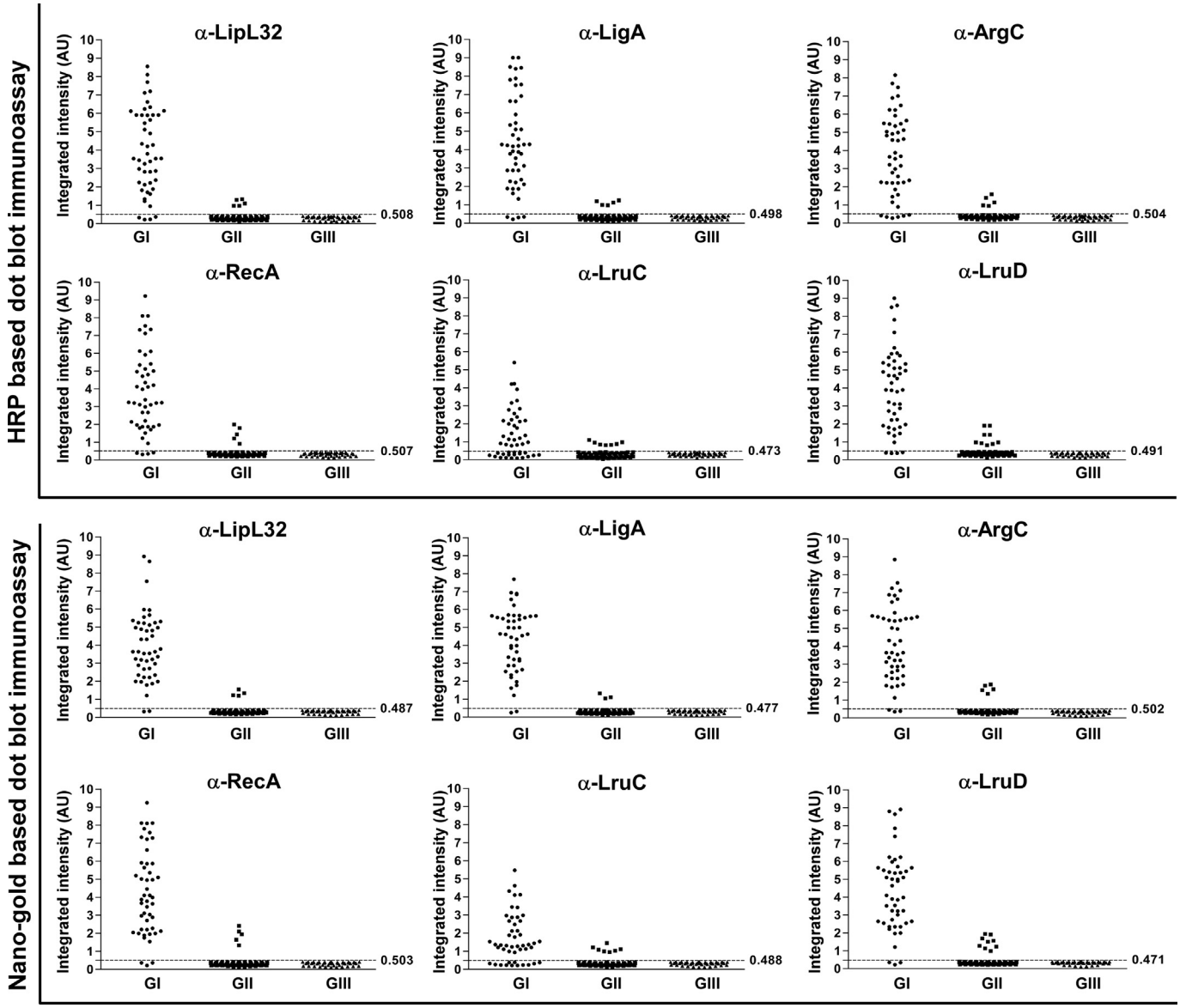

Fig. 1. HRP/nanogold conjugate based dot blot immunoassay graphs showing intensity of dots, GI- Laboratory confirmed cases (Group I), GII- Clinically suspected laboratory negative cases (Group II), GIII- Seronegative healthy controls (Group III). Recombinant proteins LipL32, LigA, ArgC, RecA, LruC, LruD were used for evaluation. A line at all graphs shows cut-off value for healthy control samples (Mean ± 2 SD).

present study has demonstrated yet other less studied proteins such as ArgC and RecA (Raja et al., 2015) along with the leptospiral recombinant proteins, LigA-C (Kanagavel et al., 2014), LipL32 (Vedhagiri et al., 2013), LruC and LruD (Verma et al., 2013) for the diagnostic evaluation. Polyclonal antisera were raised in ~2.5 kg 10–16 weeks of old female New Zealand White rabbits (National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Hyderabad) by subcutaneous administration of 1 µl of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Sigma, St. Louis, MO) and 100 µg of recombinant proteins adsorbed to aluminum hydroxide (Alhydrogel; Accurate Chemical & Scientific Corp., Westbury, NY) in a total volume of 200 µl. Booster injections containing 100 µg (subcutaneous) of the antigen were

administered 14 and 28 days after the primary immunization. 35 days after the primary immunization blood was taken by cardiac puncture and the clotted blood was centrifuged at 3000 ×*g* and serum was obtained (Raja et al., 2015). The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Bharathidasan University (BDU/IAEC/2011/29/29.03.2011).

Urinary shedding of leptospires is common during the early infection (Bal et al., 1994) and hence the present study was done to evaluate the nanogold based dot blot immunoassay with the urine samples. Individual urine samples (40 ml) was centrifuged at 12,000 ×*g* for 10 min at 4 °C and the supernatant was discarded leaving an aliquot of 50 µl, boiled for 10 mins and centrifuged at 1000 ×*g* for 10 min. The supernatant obtained was subjected for protein estimation by bicinchoninic acid (BCA) method (Sigma-Aldrich, St. Louis, MO) and ~1 µg concentration was dotted individually onto NC strips. The supernatant fractions, in 5 µl volumes (1 µg protein), were spotted on nitrocellulose (NC) membrane (0.22 µm) (Merck Millipore, USA) in triplicates and air-dried. Nonspecific binding was blocked by 1% blotting grade blocker (Bio-Rad, USA) in PBS with 0.05% Tween 20 (PBS-T) for 1 h. After washing with PBS-T, NC membranes were incubated with polyclonal sera of specific recombinant proteins in 1:200 dilutions with PBS-T for 1 h followed by PBS-T wash. Protein A-20 nm colloidal gold labeled (1:2000; Sigma-Aldrich, USA) or anti-rabbit IgG HRP conjugate (1:2000; Sigma-Aldrich, USA) was added and incubated for 1 h and washed with PBS-T. The membranes were developed with 4-chloro-1-naphthol and silver enhancer solution (50 mM hydroquinone in citrate buffer pH 3.5; 7.5 µM silver nitrate solution) for HRP and nanogold conjugate, respectively. The reaction was stopped with deionized water and membrane documented using XR + imaging system (Bio-Rad, USA) and the density of each spot was determined by Image J software (National Institute of Health, Bethesda, MD, USA). Densitometric values were obtained as the integrated intensity of all the pixels in a spot excluding the background and were expressed as arbitrary units (AU).

The overall sensitivity and specificity of nanogold and HRP based dot blot is shown in Table 1 and Fig. 1. Representative dot blots of HRP and nanogold conjugate based dot blot immunoassay is shown in Fig. 2. The mean ± 2 standard deviation of the AU values of seronegative healthy individuals was defined as the cutoff values to achieve diagnostic sensitivity and specificity. The nanogold based dot blot for different proteins demonstrated increased sensitivity (75–95.8%) than that of the HRP based dot blots (60.4–92.1%). Interestingly, nanogold based dot blots of proteins ArgC and RecA showed increased sensitivity of 93.7%, over the ArgC and RecA, HRP based dot blots. The widely used markers of leptospirosis, LipL32 and LigA also exhibit high sensitivity with nanogold conjugate immunoassay.

In conclusion, the present study has opened up a new avenue of nano-material based accurate diagnosis method. The nanogold based dot blot immunoassay demonstrated as a rapid, reliable, sensitive method to diagnose the early detection of leptospirosis.

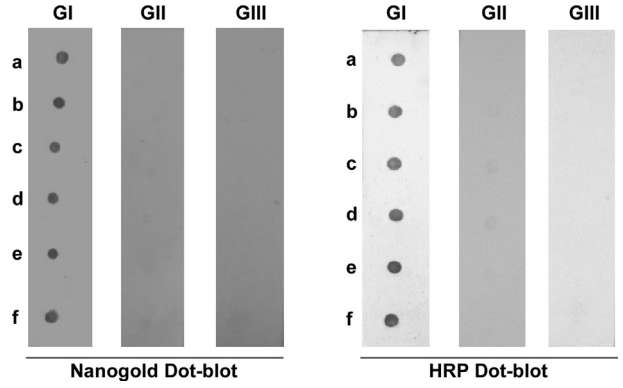

Fig. 2. HRP and nanogold conjugate based dot blot immunoassay.
GI: Laboratory confirmed cases (Group I); GII: Clinically suspected laboratory negative cases (Group II); GIII: Seronegative healthy controls (Group III). a) LipL32; b) LigA; c) ArgC; d) RecA; e) LruC and f) LruD.

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