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Development of rapid, inexpensive, sensitive, point of care (POC) antibody based diagnostic kit for canine *Leptospira serovars*

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Abstract
Leptospira, a zoonotic disease induces clinical as well as subclinical infections in animals, many times preceding in mortality. In the current study, using the nine canine serovars viz., Astratis, Ictohaemorrhagie, Canicola, Javanica, Grippotyphosa, Pomona, Pyrogenes, Potac and Banainang RDT (Rapid Diagnostic Test) kit developed to diagnose the leptospirosis disease. 190 MAT positive and negative samples were analyzed with RDT, ELISA (Enzyme-linked immunosorbent assay) kits when compared with MAT data applied as standard. 180 field samples were examined with ELISA kit and positive and negative samples were tested with RDT kit and ELISA has taken as standard. Based on the actual study the results disclosed that out of 190 samples, Positive (p) 114, Negative (n) 76 which were examined for leptospira sLPS based antigens with the gold standard method i.e. MAT were examined with RDT kit and ELISA kit. RDT showed a specificity and sensitivity of 94.73% and 93.85%, respectively, whereas ELISA showed a specificity and sensitivity of 98.63 % and 99.12 % respectively. 180 field ELISA Positive and Negative samples tested with RDT showed a specificity and sensitivity of 97.22% and 95.37% respectively. The sensitivity and specificity of developed lateral flow diagnostics for Canine Leptospira species were relatively similar to the existing gold standard method Microscopic Agglutination Test (MAT) and ELISA test kit accessible in the market.

Keywords: ELISA, Leptospira, microscopic agglutination test, validation, specificity, sensitivity

1. Introduction

Leptospirosis is a global zoonotic disease caused by spirochetes consisting to the genus Leptospira. There are more than 200 distinct leptospiral serovars noticed and these are arranged in 25 serogroups [1]. A zoonotic disease is a condition which can be shifted from an animal or insect to a human and the consequence will sicken the human. After intake of the *Leptospira* bacteria, the leptospires get into the bloodstream via the mucous membranes and followed by a fast replication in several tissues such as the kidney, liver, and spleen. Outbreaks have been associated with flooding and natural calamities. Leptospirosis is shifted by direct or indirect contact with urine of contaminated animals, indirectly through contaminated soil and water. Many studies have looked into the particular role of the dog as a zoonotic vector. To control the leptospirosis in preliminary phase is by way of antibiotics. The clinical signs of leptospirosis alter and are nonspecific. Some of the times pets do not have any symptoms. Basic clinical signs have been accounted in dogs. These include, fever, vomiting, abdominal pain, diarrhea, refusal to eat, severe weakness and depression, stiffness, severe muscle pain, inability to have puppies usually younger animals are further badly affected than older animals [2, 3, 4]. Several researchers have carried out investigations on the possibility of being weil's disease in the Andaman Islands during an early 20th century and the first report of leptospirosis was confirmed and published in 1931 [5].While leptospirosis is spread all over the world, Kerala, Gujarat, Maharashtra, Orissa, West Bengal, Uttar Pradesh and Delhi in India is covered as endemic areas [6]. There are different strains (known as hosts) that are responsible for the disease. Two strains (bivalent) vaccines provide protection against Icterohaemorrhagiae and Canicola servers, but recently a four strain (tetravalent) vaccine for leptospirosis has been certified and suggested for use in dogs within Europe. Previous estimates indicate that there are more than 500,000 cases of leptospirosis each year planetary. The majority of accounting cases have serious manifestations, for which death rate is greater then 10% [7].

The world burden of disease is being accounted by the leptospirosis burden epidemiology reference group (LERG), coordinated by the WHO and partners. The existing techniques to diagnose leptospirosis are sociological methods and culture of the organism in the laboratory. Leptospires grow late and the positivity rate in culture is very low. The utilise of molecular tools to detect and analyze pathogens in clinical samples would make it possible to diagnose the disease early and thus would help in decrease morbidity and death rate. Among the molecular instruments, PCR is the most popular method for diagnosis of the disease. This technique can find, even if only a minute number of organisms are present in the clinical samples. However, it requires advanced and pricey instruments, good laboratory facilities and skill, which may not be reachable in a common diagnostic laboratory. Total around twenty five serogroups of leptospira are available all over the world. Most of the existing Sero diagnostic kits in India are imported from abroad and they are poor in sensitivity and specificity because they are not planned for the Indian environment. By targeting all these issues into consideration in the current study, the Canine Leptospira Lateral flow diagnostics were developed that targets the nine Indian serovars of Canine Leptospira. To overcome these hurdles in diagnosing the disease developed an RDT kit which is very simple to execute, low cost, rapid to detect the antibodies in the sample [8]. The most commonly used laboratory assays are based on the detection of antibodies against the leptospires. Infective leptospirosis comes into the body through small cuts or abrasions, or via mucous membranes and possibly through wet skin. After infection, leptospires pass around in the bloodstream, with a bacteremic phase lasting for up to 10 days post onset of the disease [9]. These antibodies can be observed by a variety of laboratory assays such as the microscopic agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA). The MAT assay needs a extremely advanced microscope and tools and has been shown to be very complex linked to other methods. Therefore *Canine Leptospirosis* detection at clinical aspects suffering and demands novel tools for detection. Culture and the microscopic agglutination test (MAT) are gold standard methods for leptospirosis diagnosis, which requires the particular laboratory, facilities, finds agglutinating antibodies and paired serum samples with an interval of at least ten days are needed to diagnose acute infection which is not useful for early diagnosis [10]. So there is always a need for the development of simple, rapid diagnostic tests (RDTs) can play an important role in the immediate case detection and clinical management. Most generally used RDTs are based on the immunochroma to graphic lateral flow technology. The aim was to measure the diagnostic accuracy of RDTs, that are developed using particular sLPS antigen, applied on serum specimens from suspected leptospirosis.

2. Materials & Methods

2.1 Study site

The Development and the validation of canine Leptospira lateral flow diagnostics were executed at Genomix Molecular Diagnostics Pvt. Ltd, Hyderabad, India. A overall of 190 MAT control samples received from Translational research platform for veterinary Biologicals (TRPVB). The study group includes a total of 180 animal samples (26 German shepherd, 21 Doberman, 16 Boxer, 19 Bull Dog, and 98 street dogs) of all ages and sexes was chosen for the study purpose.

2.2 Microscopic agglutination test (MAT)

The Microscopic agglutination test is the serological gold standard assay for the diagnosis of *Canine Leptospira*. Laboratory diagnosis of *Leptospira* contagion is laborious, costly and time-consuming hence there is a need to develop simple, lowcost, well-characterized assays that are diagnostically informative. For detection of anti-leptospira antibodies, microscopic agglutination test (MAT) was conducted according to the recommendations of international standards. Bacteria were cultured in liquid Ellinghausen-McCullough-Johnson-Harris medium (EMJH) and the resulted bacteria were used for the preparation of antigens. Each serum sample was initially diluted 1:50 in buffered saline and 50 uL of this solution were channeled to vinyl micro plates containing 96 wells. Immediately after, an equal volume of each antigen was added to the corresponding well, with a last dilution of 1:100. The Microplates were incubated at 37°C for 90 minutes and checked under dark-field microscopy. Serum samples were initially analyzed at a dilution of 1:100, and those that showed agglutination level equal to or higher than 50% were then diluted once again in a ratio of 1:200, 1:400 and 1:800. Samples were considered as reactive when reached titers of 200 or 400 whereas reactions that presented titers ≥800 were considered as strongly reactive and considered as an indication of acute contagion.

2.3 Culture media

Difco™ Leptospira Medium Base EMJH is utilized in culturing of *Canine Leptospira* Species [11]. This specific culture medium was supplemented with ammonium chloride as a nitrogen source and thiamine as a growth factor. Sodium phosphate dibasic and potassium phosphate monobasic are buffering agents. Sodium chloride maintains the osmotic balance of this medium. Leptospira Enrichment EMJH contains albumin and polysorbate 80 are extra growth factors for Leptospira.

2.4 Culture Inoculation

A total of nine serovars of *Canine Leptospira* were used for the development of lateral flow diagnostics under the DBT Canine Network project. All these serovars were obtained from National leptospirosis reference center, Regional medical research center (ICMR), Andaman. From the stocks, a total of 10 mL of Leptospira culture was added to 1000 mL of EMJH medium. The nine strains of canine Leptospira (*Astratis*, *Ictohaemorrhagie*, *Canicola*, *Javanica*, *Grippotyphosa*, *Pomona*, *Pyrogenes*, *Potac,* and *Banainang*) selected were grown in liquid Difco™ Leptospira Medium Base EMJH culture medium specific for the canine species at 29 ± 1°C for 7 days [11].

2.5 Antigen preparation

sLPS is the major epitopes that can be targeted for seroprevalence diagnostic assays. The cultures of all the nine serovars of *canine Leptospira* were incubated 29 °C for a time period of one week and the grown cultures were centrifuged at 6000 g under disciplined temperature conditions. The pellet was collected and the supernatant was discarded. The collected pellet was washed three times by centrifugation at 6000 g with 1 x Phosphate buffer saline (pH 7.2) and the supernatant was discarded. After the process of washing, around 5.0 grams of the pellet were collected from one liter of culture. The collected pellet was resuspended in 10 mL of bicarbonate buffer with pH-9.6 containing 0.5% v/v formalin.

The resulted suspension was kept in a boiling water bath for 30 minutes followed by centrifugation at 6000 g for 30 minutes. The pellet was discarded and the supernatant was concentrated using the Amicon Ultra-15 (Millipore) centrifugal filter units 10Kda (15mL) and concentrate the final volume of sample to 5 mL [11]. The final volume was stored at -20^{0}C for further use.

2.6 Antigen Characterization

The purified fractions of all the nine serovars of canine stains were concentrated. The concentrated fractions were pooled in a single vial. The pooled sample was characterised on a 12% SDS gel. The size of the pooled sLPS of *Canine Leptospira* was about 42 kDa. After the process of verification on SDS gel the pooled sample was dialyzed with 13 kDa cut off dialysis membrane using 1 x phosphate buffer saline with pH 7.4 at 4ºC with continuous stirring for nightlong and finally, the concentration of antigen was adjusted to 1 mg/mL. The concentrations were evaluated using the NanoDrop 1000 spectrophotometer V3.8 (Thermo Fisher Scientific). Finally, 1 mL of antigen was applied with 1mg/mL concentration in the development of lateral flow diagnostics for Canine Leptospirosis [11].

2.7 Sample preparation

The whole blood samples were collected from veterinary health care centers and veterinary hospitals in and around Hyderabad in BD Vacutainer Lithium Heparin coated whole blood collection tubes (BD Vacutainer) for serological studies. Around 2 mL volume of whole blood was collected from the jugular vein of the animal aseptically with the consent of the animal owners under the supervision of the local veterinarians. The collected samples were applied directly for a microscopic agglutination test (MAT) and remaining volume was stored at -20ºC for further use.

2.8 Development of Lateral flow Diagnostics

Protein-G based Lateral flow diagnostics were used for the diagnosis of *canine leptospirosis* in this work, developed by applying the purified and highly concentrated lipopolysaccharide (sLPS) from the nine serovars of *canine leptospirosis*. The pooled antigen was surfaced on nitrocellulose membrane with 300mm length and 60mm width by applying BioDot Quanti-2000 Biojet apparatus. Concisely, one end of nitrocellulose membrane is flanked by conjugation pad possessing Protein-G conjugated to colloidal gold Nanoparticles (Arista Biologicals.Inc, USA) and the other end is flanked by the absorption pad (MDI membranes, Ambala). Sample pad is flanked by another end of conjugation pad. All the components were assembled on a laminated baking sheet. Approximately 3.8 mm width of strips was cut into pieces using a lateral flow strip cutting machine (Advance tooling systems, MDI, Ambala). The composite was backed by a support and was cut into test strips per cassette to fit a plastic housing with an oval sample application well positioned above the sample pad and a rectangular detection window positioned preceding the detection strip. Atlast, the assembled test devices were packed in a foil pouch with desiccant and sample holding dropper. The volume of antigen and the sample were optimized in a step-by-step procedure with a panel of positive and negative control sera. Sample diluent buffer carried 0.2% Tris, 2% casein, 1% Triton-X and pH was adjusted to 8.9. The lateral flow assay with an optimized protocol was performed adding 5 µl of the sample on the sample pad and following the addition of 120 µl of sample diluent buffer at the sample application place of the test device. The assay interpretation was done within 20 minutes. The assay is said to be positive when distinct colored lines of both test and control are observed. The assay is said to be negative when distinct staining of control line alone is observed. The test line may stain at different intensities depends upon the binding of particular antibody titer present in the clinical samples with the sLPS as well as protein-G gold colloidal particles [12] [Fig. 1].

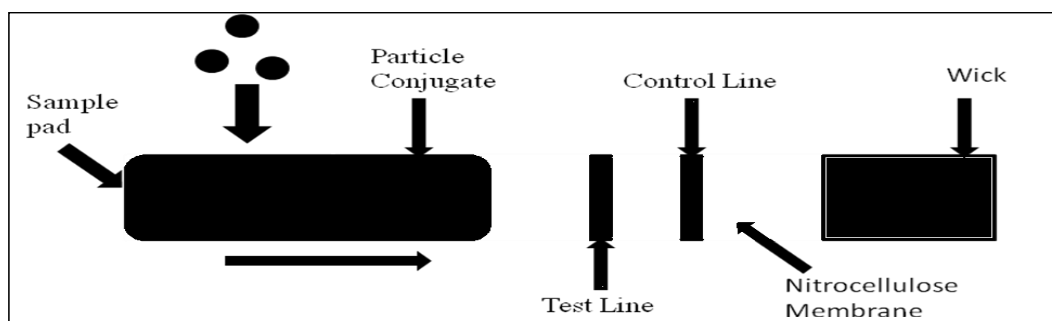

Fig 1: Diagrammatic representation of Canine Leptospira Lateral flow Device

2.9 Statistical Analysis and data Management

The collected canine all blood samples were assayed with the in-house developed lateral flow assay comparatively validated with gold standard serological assay. Microscopic agglutination Assay (MAT) and with the indirect antibody ELISA test. The acknowledged positive and negative control samples were used to calculate the sensitivity and specificity of the developed assay. Calculation of specificity, sensitivity, positive predictive (PPV), negative predictive values (NPV) and efficiency was carried out applying statistical analysis software SPSS20.0.

3. Results

The *canine Leptospira* serovars were grown in EMJH culture medium at 29±1 ºC for 7days. The sLPS antigen further purified in bicarbonate buffer containing formalin. Antigen concentrated using the 13KDa cut off dialysis membrane using 1 X PBS buffer. 5 mL of sLPS antigen found from one liter of culture with 1mg/mL concentration. The concentration of sLPS antigen was varying from 0.8 to 1.0 mg/mL from batch to batch. The purified antigens are pooled and additional characterized using SDS-PAGE. The pooled antigens showed a molecular weight of 42 kDa compared with standard protein marker [Fig. 2].

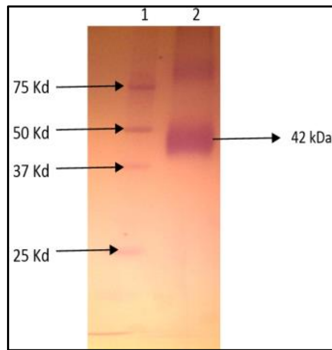

Fig 2: Purification of nine canine serovars by SDS-PAGE.
1. Standard marker, 2. Nine canine serovars bands with 42 kDa

190 MAT samples Positive (p) 114, Negative (n) 76 tested with RDT and ELISA. RDT showed a specificity and sensitivity of 94.73% and 93.85%, respectively, with the false negative rate of 6.0% and a false positive rate of 5.0% [Table 1 & Table 2]. The positive predictive value was 96.39% and a negative predictive value was 91.13%. ELISA showed a specificity and sensitivity of 98.63% and 99.12%, respectively, with the false negative rate of 0.88 % and false positive rate of 1.33%. The positive predictive value was 99.12% and a negative predictive value was 98.68 %. The statistical analysis showed p value 1, hence there was no much difference between two methods for the detection of canine leptospirosis [Table 1 & Table 2].

Table 1: Evaluation of the Test results of diagnostic assays used for Canine Leptospira diagnosis

S. No	Name of the Detection	Test type	Specimen Volume required	Specimen Type Serum/whole blood	Total no. of samples (n=190)		False Positive (FP)	False Negative (FN)
					True Positive (TP)	True Negative (TN)		
1.	Ab based	MAT	1 µl	Serum	TP- 114	TN- 76	-	-
2.	Ab based	ELISA	1 µl	Whole blood/serum	TP- 113	TN- 75	1	1
3.	Ab based	Lateral flow	5 µl	Whole blood/Plasma	TP- 107	TN- 72	4	7

Table 2: Comparison of parameters between diagnostic assays

S. No.	Name of the method	Sensitivity %	Specificity %	Positive predictive value (PPV)	Negative predictive value (NPV)	Efficiency
1.	ELISA	99.12	98.63	99.12	98.68	98.9
2.	Lateral flow	93.85	94.7	96.39	91.13	94.2
McNemars Chi-square (X^2)= 0 and p= 1						

180 Field samples tested by ELISA as standard and found Positive (p) 108, Negative (n) 72. Positive and Negative samples tested with RDT showed a specificity and sensitivity of 97.22% and 95.37%, respectively, with the false negative rate of 5.0% and a false positive rate of 3.0%. The positive predictive value was 98.0 % and a negative predictive value was 93.3%. The validation data in terms of Specificity, Sensitivity, Positive predictive Value (PPV) and Negative predictive values (NPV) were tabulated [Table 3 & Table 4].

Table 3: Comparative parameters of 180 No's field samples by ELISA and lateral flow assay

S. No	Name of the Detection	Test type	Specimen Volume required	Specimen Type Serum/whole blood	Total no. of samples (n=180)		FP	FN
					Total Positive	Total Negative		
1.	Ab based	ELISA	1 µl	Wb/serum	TP- 108	TN- 72	-	-
2.	Ab based	Lateral flow	5 µl	Wb/Plasma	TP- 103	TN- 70	2	5

Table 4: Evaluation data with field collected samples

S. No.	Name of the method	Sensitivity %	Specificity %	PPV	NPV	Efficiency
1.	Lateral flow	95.37	97.22	98.00	93.3	96.11

TP: True positive; TN: True negative; FP: False positive; FN: False negative; PPV: Positive predictive value; NPV: Negative predictive valve, Wb: Whole blood

4. Discussion

Canine Leptospirosis is a widely accepted acute febrile disease and is endlessly emerging or reappearing in tropical and sub-tropical regions. Most transmissions currently documented in dogs are from other (incidental) serovars, with differences between geographic areas [13]. European studies describe mostly the serovars Australis, Grippotyphosa, and occasionally Pomona [14]. Leptospirosis is often under-diagnosed, also challenging as the culture of *Leptospira* and seroconversion require weeks to grow and maintain [15]. Isolation of *Leptospira* from the clinical specimen is difficult because leptospires are fastidious, slow growing that requires special growth culture media like and it is time-consuming and laborious [16]. It is important for an early and definitive detection of leptospirosis among the animals so that the clinician can start appropriate treatment as soon as possible and minimize the associated complications. Fewer studies have analyzed the presence of infective Leptospira in the daily human environment where regular contact with contaminated water sources may occur [17]. Since diagnosis of leptospirosis, MAT has been a gold standard with high sensitivity and specificity. However, it does not represent per-fect sensitivity due to the requirement of all of each live serovar existing in each local region. Since a better diagnostic method is not available at present, and leptospira is distributed as pathogens having over 200 serovars, species or genus specific, a simple, rapid and accurate diagnostic method is necessary [12]. Lepto lateral flow reaches the highest sensitivity during the third and fourth weeks of illness. The diagnosis of leptospirosis in dog veterinarians often dictates broad-spectrum antimicrobial agents to treat not only the possible leptospiral infection, but also for other potential

infectious diseases considered as differential diagnosis. The potential of leptospires as well as other pathogens to develop resistance to antimicrobial agents can consequently not be over-emphasized. Reports exist on the in vitro antimicrobial susceptibilities of Leptospira spp. This is also a reason to develop a new diagnosis technique to detect leptospires. The detection limits for lateral flow test kits for the detection of Leptospira antibodies alters based on the detection methods. If the Leptospira antibodies below the detection limits there might be a change in the sensitivity of the assay. In some cases because of the more number of antibodies in the patient that may lead to cross-reactivity with the RDT kit. Even in some other cases where there is a high titer of antibodies raised with some other diseases that may cause cross-react with leptospira antibodies resulting in false positivity. While MAT is considered to be a gold standard method, it is not a rapid test and is used mainly in the research laboratories. Therefore we have developed an RDT kit which is comparatively faster. Several commercial kits are available for the diagnosis of systemic leptospiral infection using broadly reactive Leptospira antigen [18, 19].

5. Conclusion
In this work, the results concluded that the RDT kit is used for the diagnosis of *canine leptospirosis*. The additional methods are expensive and time-consuming, however to perform molecular diagnostic tests require skilled technicians because of these reasons developed RDT kit and it is very simple, lowcost, quick result and highly sensitive method to detect the antibodies in the sample. We can strongly recommend that the developed RDT kit is very useful and easy to perform.

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