



Laboratory Training Manual

CDC & ASM Sponsored

Workshop

on

**Laboratory Capacity Building for
Leptospirosis**

11th to 15th September, 2017

Organized by

**ICAR-National Institute of Veterinary Epidemiology and
Disease Informatics (NIVEDI), Bengaluru, India**

and

Centers for Disease Control and Prevention (CDC), Atlanta, USA

2017



Workshop on Laboratory Capacity Building for

Leptospirosis

11th -15th September, 2017

Laboratory Training Manual

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Sponsored by :

Centers for Disease Control and Prevention (CDC),

and

American Society for Microbiology (ASM)

Organized by :

**ICAR-National Institute of Veterinary Epidemiology and Disease Informatics,
Bengaluru, India**

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Centers for Disease Control and Prevention (CDC), Atlanta, USA

Published by :

Director

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Acknowledgement

The constant support, encouragement and financial assistance benevolently from Indian Council of Agricultural Research, Krishi Bhawan, New Delhi for ICAR-NIVEDI. Centers for Disease Control and Prevention (CDC), Atlanta, USA and American Society for Microbiology (ASM) for sponsoring capacity building programme on “Leptospirosis” using Global Health Security Funds (GHSF) and providing opportunity to ICAR-NIVEDI for conducting such a workshop and stakeholder meeting in the field of “Leptospirosis” are gratefully acknowledged.





Indian Council of Agricultural Research
Krishi Bhawan :New Delhi-110001

Dr. Joykrushna Jena,
Deputy Director General
(Animal Science and Fisheries)



MESSAGE

I am happy to note that the ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Bengaluru is organizing “Laboratory capacity building workshop cum training programme on Leptospirosis” during 11th to 15th September 2017 with financial support from the Centers for Disease Control and Prevention (CDC), Atlanta, and American Society for Microbiology (ASM), USA.

This programme is indented to build capacity for laboratory-based Leptospirosis surveillance in the endemic states of India in co-ordination with NCDC’s surveillance network on leptospirosis in order to facilitate and strengthen inter-sectoral (animal and human sector) leptospirosis mitigation efforts in ‘One Health’ approach.

I congratulate the organizers for taking the initiative for conducting workshop-training programme for the benefit of scientists/officers from different scientific institutions in India.

I wish the Workshop a grand success.



(J. K. Jena)

Place : New Delhi
Dated : 4th September, 2017



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डा० अशोक कुमार

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सहायक महानिदेशक (पशु स्वास्थ्य)

Assistant Director General (Animal Health)



MESSAGE

It is heartening to know that National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Bengaluru is organizing “Laboratory capacity building workshop cum training programme on Leptospirosis” during September 11 to 15, 2017 in collaboration with Centers for Disease Control and Prevention (CDC), Atlanta and American Society for Microbiology (ASM), USA.

Leptospirosis is highly important diseases affecting both animals and human beings. The efforts to build capacity for laboratory based leptospirosis surveillance are essentially required in the country and thereby the right approach has been taken by ICAR-NIVEDI. Stakeholder interactive meeting during the workshop will provide a platform to layout a road map using “one health” approach with mutual coordination and contributions from both the animal and human health sectors.

I wish the workshop all the success and compliment the organizers for selecting the appropriate theme for deliberation.

(Ashok Kumar)



भाकृअनुप—राष्ट्रीय पशुरोगजान पदिकएवंसूचना विज्ञान संस्थान
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(ISO 9001-2008 certified)



Dr. Parimal Roy
Director & Programme Director



From the Director Desk...

I am pleased that ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bengaluru is organising stakeholder meeting and laboratory capacity building workshop on “Leptospirosis” during 11th to 15th September २०१७ jointly with Centers for Disease Control and Prevention (CDC), Atlanta, with financial support from American Society for Microbiology (ASM), USA.

As technology dissemination and HRD process, the institute has been conducting plethora of training programmes related to epidemiology, economic impact, development of economic analysis modules, research methodologies, sampling frame, laboratory disease diagnosis, biosafety practices and zoonotic diseases. Besides, this Institute also conduct training on application of epidemiological software for various stakeholders associated with animal health especially scientists/officers and veterinary professionals drawn from various parts of the country.

The plan of this workshop is to organize a stakeholder meeting of apex laboratories to draw blueprint for capacity building for leptospirosis within the surveillance network involving both human and veterinary sector under one health approach and to train the scientists/officers working in various laboratories on diagnosis of leptospirosis.

Leptospirosis is considered to be the most widespread emerging infectious zoonosis, affecting livestock by its different manifestations and causing considerable economic losses. Surveillance and early diagnosis of the disease will help in control of the disease without much loss. I hope this workshop will help the participants from different institutions/organization to strengthen their capabilities in leptospirosis laboratory diagnosis and surveillance.

I wish the participants a pleasant stay in green city and wish the workshop a great success.

(Parimal Roy)



INDIA Country Office - U.S. Centers for Disease Control and Prevention (CDC), New Delhi

Dr. Kayla Laserson

Country Director, CDC-India



Leptospirosis is a global problem and considered one of the most common zoonotic infections in the world. In India, CDC-supported Acute Febrile Illness (AFI) and Acute Encephalitis Syndrome (AES) surveillance sites from different states have revealed that leptospirosis is a significant cause of undifferentiated fever, including fever characterized as acute encephalitis, and is not limited to only a few endemic states of the country.

Early diagnosis of leptospirosis is challenging but crucial as appropriate treatment with antimicrobial drugs can reduce illness and death. Clinical diagnosis is difficult as the clinical signs and symptoms range from a self-limiting mild influenza-like illness to a much more serious disease with multi-organ failure and the potential for death. Further these signs and symptoms are characteristic of numerous fever-causing pathogens. Laboratory diagnosis of leptospirosis is also challenging; most of the laboratories are dependent upon serologic tests. The capacity for performing the gold standard serologic test (Microscopic Agglutination Test, MAT) is generally lacking as it requires maintenance of live serotypes of leptospira. Rapid serologic tests are non-reliable because of the high false positivity and cross reactivity with other pathogens, e.g. dengue. Moreover, serologic testing for leptospirosis detects infections only after the second week of illness. Molecular testing using PCR allows the identification of acute infection as it provides rapid and reliable diagnosis during the bacteremic phase. But, the capacity for molecular and the gold standard serologic testing is limited to only a few laboratories. More regional laboratory capacity is needed to combat the nationwide problem.

Control and prevention activities of leptospirosis are often limited due to the diagnostic challenges identifying leptospirosis, wide variation in the host's symptomatology, multiple serotypes of the agent and complexity of interactions among humans and animals enabling the disease transmission in local settings. The Government of India, through the National Program on Prevention and Control of Leptospirosis spearhead by NCDC, is trying to build surveillance capacity in several endemic states of India e.g. Kerala, Karnataka, Tamil Nadu, Gujarat, Maharashtra and Andaman & Nicobar. With respect to the Veterinary sector, NIVEDI is a leading Government institution with state of the art laboratory capacity and highly experienced scientists involved in leptospirosis surveillance and is the most appropriate organization to work as a collaborative partner with NCDC for inter-sectoral coordination. Through this workshop and additional activities comprising partnership with India on Global Health Security, CDC is supporting NCDC and NIVEDI to build capacity for lab based leptospirosis surveillance within the national surveillance network of leptospirosis, using a One Health approach.

I strongly believe that this workshop will be useful for all the participants from both the human health and Veterinary sectors. This will give them an opportunity to learn standard testing protocols for leptospirosis as well as exchange ideas and discuss challenges with national and international experts.

(Kayla Laserson)



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Dr. V.Balamurugan,
Principal Scientist & Programme Coordinator



Greetings from ICAR-NIVEDI

As you are aware, leptospirosis is one of the emerging zoonosis leading to significant morbidity and mortality in human and livestock causing financial, and psychological burden on the family. It is known to be endemic in several states of India primarily Andaman & Nicobar, Gujarat, Kerala, Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra, etc., Hence, it is necessary to improve capacity of personnel on leptospirosis diagnosis and surveillance to combat the disease threat. The design of workshop is to organize a stakeholder meeting of apex laboratories to layout the roadmap for capacity building for leptospirosis within the surveillance network under National program on Prevention and Control of Leptospirosis involving veterinary sector in one health approach; to conduct wet laboratory training for different tiers of veterinary and public health laboratories and link the district and apex laboratories and develop the sample transportation system within the leptospirosis network. To achieve the aforementioned objectives, CDC has planned this stakeholder meeting and laboratory training in ICAR-NIVEDI with technical supports from NCDC, NIVEDI, Manipal and BSBP, CDC, Atlanta, for networking of veterinary and public health laboratories within the NCDCs network. I hope that, this training course will refresh and strengthen the personnel / participant's skills in the fields of leptospirosis surveillance and diagnosis. I extend warm welcome to resource persons and trainee and wish them a memorable stay in this garden city, Bengaluru and enrich their knowledge and skills and leave beside difficulties experienced, if, any.

With warm regards,

(V.Balamurugan)



About ICAR-NIVEDI

Convergence of Animal Health and Research Par Excellence



Historical Background

ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), (Formerly, Project Directorate on Animal Disease Monitoring and Surveillance, PD_ADMAS) under the Indian Council of Agricultural Research (ICAR), a pioneer research institute in veterinary epidemiology is carrying out disease surveillance, monitoring and analysis of livestock diseases in India through 32 collaborative centers of AICRP_ADMAS located in different states of the country.

The AICRP on animal disease monitoring and surveillance (AICRP-ADMAS) initiated by the ICAR, made a humble beginning during the VII five-year plan and became fully functional in 1987 with establishment of four Regional Research Units (RRUs) at Bengaluru, Hyderabad, Pune and Ludhiana. The Central Coordinating Unit (CCU) was established at the Institute of Animal Health and Veterinary Biologicals, Bengaluru to co-ordinate research activities of the regional units. In the VIII plan, the institute was strengthened with support of ICAR and European Union by taking up the major responsibility under National Project on Rinderpest Eradication (NPRE) involving 32 state level diagnostic/disease investigation laboratories in the country. On 1st April 2000 (during the IX plan), the CCU was given the status of Project Directorate and named as 'Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS)' with ten collaborating units under AICRP_ADMAS component. In the X and XI five year plan period, five more collaborating units were added for providing impetus to a nationwide animal disease monitoring and surveillance.

Appreciating the contributions made by the Directorate to country's livestock health sector and the need to strengthen the effort, the council rechristened PD_ADMAS as 'National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI)' on 25th October 2013 (XII plan period) with its exclusive campus at Bengaluru. Further, during the same plan period, 17 additional collaborating units covering other states were added under AICRP_ADMAS component totaling to 32 collaborating units for providing the needed impetus to a strong nationwide animal disease monitoring and surveillance network.

On 9th January, 2015, NIVEDI's newly constructed administrative building and Biosafety Laboratory (BSL-2) was dedicated to the nation by Shri Radha Mohan Singh, Hon'ble Union Minister



for Agriculture, New Delhi in the presence of Shri D.V. Sadananda Gowda, Hon'ble Minister of Law and Justice, GOI and Shri T. B. Jayachandra, Hon'ble Minister for Law, Justice & Human Rights, Parliamentary Affairs and Legislation and Animal Husbandry, Govt. of Karnataka and Dr. S. Ayyappan, Secretary DARE and Director General, ICAR. The centralized administrative and laboratory complex of the institute is located in a sprawling campus at Yelahanka, Bengaluru.

ICAR - National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), a pioneer research institute under Indian Council of Agricultural Research (ICAR) has been entrusted to conduct R&D in the field of veterinary epidemiology and surveillance of economically important livestock diseases in the entire country, its role is extremely pivotal for developing models for animal disease forewarning, forecasting, economic impact, risk assessment, and need based animal disease diagnostics. The institute has developed various technologies covering both products and processes and some of them are marketed and/or patented/copyright protected, which are being utilized by various institutes/organizations and different stakeholders in the country. The role of this institute in the eradication of Rinderpest disease in India and development of National Animal Disease Referral Expert System (NADRES) - interactive software for forecasting are noteworthy. The institute conducts various training programmes related to basic epidemiology, sampling frame and sampling techniques, outbreak investigation, research methodologies, disease diagnosis protocols for various stakeholders associated with animal healthcare. Overall, NIVEDI has been proving its worthiness to the Indian animal health sub-sector covering critical gaps in diagnostic techniques, animal disease modelling, economic impact assessment and analysis of animal diseases, human resource development in the form of skill development and empowerment, capacity building programmes etc. Further, NIVEDI envisions to provide newer direction to undertake in-depth R & D activities on epidemiology of emerging and re-emerging, transboundary animal diseases to others involved in the sub-sector in the country, leading finally to prevention, control and eradication of the diseases for achieving animal welfare and safer animal - human interface under one health approach.

(i) Vision

Achieving freedom from animal diseases, animal welfare, food and nutritional security through healthy foods of animal origin, poverty alleviation and economic growth of rural India.

(ii) Mission

Capacity building in frontier areas of Veterinary Epidemiology: dynamics of animal diseases including zoonosis and animal healthcare intelligence.

(iii) Focus

- Improving disease monitoring and surveillance through development of population assays and pen side diagnostics
- Risk assessment for occurrence of economically important animal diseases
- Adapting strategies to improve animal disease data quality
- Understanding the threat from animal diseases in the background of climate change and globalization



- Developing early warning system and disease modeling/ forecasting
- Understanding economic impacts of animal diseases and the management strategies
- Promoting innovations and improving human resource capacity
- Fostering linkages and collaborations with public and private, national and international organizations
- Improving knowledge management system

(iv) Mandate of Institute

- Epidemiology, informatics and economics of animal diseases including zoonosis
- Surveillance, forecasting and forewarning for management of animal diseases including Zoonosis
- Repository and Capacity Development

(v) AICRP on ADMAS

All AICRP collaborating units are extensively working on animal disease diagnosis, outbreak investigation, disease reporting, pathogen characterization and mapping etc., with major focus on bacterial (Brucellosis, Leptospirosis, Mastitis, Haemorrhagic Septicaemia, Anthrax, Black Quarter, Enterotoxaemia,), viral (Infectious Bovine Rhinotracheitis, Bluetongue, Classical Swine Fever, *Peste des Petits Ruminants* and Sheep and Goat Pox) and parasitic (Trypanosomosis, Theileriosis, Babesiosis, Fascioliosis and Amphistomiosis) diseases of economic importance with the following mandates.

Mandates of AICRP on ADMAS

- Sero-monitoring of animal diseases based on sample frame,
- Investigation of endemic, emerging and re-emerging animal disease outbreaks using innovative technologies,
- Strengthening of National Livestock Serum Repository,
- Effective updating of NADRES with active disease data and climatic and non-climatic risk-factors,
- Utilization of forecasting models through NADRES for forecasting and forewarning of animal diseases,
- Analysis on economic losses due to animal diseases and the control measures adopted for their management, and
- Surveillance of diseases/pathogens of companion, laboratory and wild animals.

AICRP - ADMAS Centres, ICAR-NIVEDI, Bengaluru



Leptospirosis is one of the emerging zoonosis leading to significant morbidity and mortality in human as well huge loss in livestock. It is known to be endemic in several states primarily Andaman & Nicobar, Gujarat, Kerala, Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra. Hence, it is necessary to improve capacity of personnel on leptospirosis diagnosis and surveillance to combat the disease threat. The plan is to train district level veterinary and public health laboratories along with Apex laboratories to perform tests at different levels /tier (peripheral and reference level), link the district and apex labs and develop the sample transportation system within the network. To achieve the afore mentioned objective, CDC is planning the following activities with ICAR-NIVEDI and the laboratory trainings will be conducted with technical supports from NCDC, NIVEDI, Manipal and BSBP, CDC, Atlanta.

- To organise a stakeholder meeting of Apex laboratories to layout the roadmap for laboratory capacity building for Leptospirosis within the GOI’s surveillance network of Leptospirosis (spearheaded by NCDC) involving veterinary sector under one health approach.
- To conduct wet laboratory training for different levels/tiers of laboratories:
 - a. Training of Apex/reference labs on Microscopic Agglutination Test- MAT (including maintenance of live strains of leptospira) and Molecular tests (PCR).



- b. Training of district level veterinary and public health labs on ELISA and rapid diagnostic tests (Lateral Flow Assays).

To reduce the time and expenditure on logistics, the stakeholder meeting and training of Apex/ Reference laboratories organized back to back in this period at ICAR-NIVEDI, Bengaluru and the scientific personnel from the different laboratories in endemic states working on leptospirosis invited to participate in the above said programme as per decision with CDC officials.

Agenda of Workshop

Stakeholder meeting (11th September 2017).

Stakeholder interactive meeting with resource persons to blueprint a road map using “one health” approach with mutual coordination and contributions from both the animal and human health sectors.

Laboratory Capacity Building Training (12-15th September 2017)

Topics of Practical / Hands on training /Lecture

- Laboratory Biosafety: Principles and Practices
- Preparation of culture media (EMJH), culturing and sub culturing of leptospira.
- Live leptospira culture antigen preparation for microscopic agglutination test (MAT) and its maintenance in liquid and semi solid media.
- Dark field examination and staining of leptospira.
- Isolation and maintenance of the leptospira *in vitro*.
- Sero-screening of the samples by microscopic agglutination test (MAT)-Determinant of the frequency distribution of the prevalent serovars and its prevalence.
- Hands on training on conventional and point of care diagnostics for leptospirosis rapid diagnostic tests /lateral flow assay. Interpretation of diagnostic methods
- Hands on training on Diagnosis of human leptospirosis by human IgM based ELISA and diagnosis of bovine leptospirosis by Linnodae Hardjo ELISA kit
- Hands on training on Molecular diagnosis: Extraction of DNA from Leptospira cultural or clinical samples (Blood/Plasma/ serum/urine)
- Hands on training on Diagnosis of leptospirosis by PCR techniques and differentiation of pathogenic and non-pathogenic leptospira species.
- Surveillance, Epidemiological Mapping, Making Point Maps of outbreaks locations, choropleth Maps, economic impact of leptospirosis in animals and human and KAP studies including sampling techniques, etc.,



Organizing Committee for Stakeholder meeting and Laboratory Capacity Building workshop on “Leptospirosis”

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Programme Coordinator: Dr.V.Balamurugan, Principal Scientist, ICAR-NIVEDI, Post Box No. 6450, Yelahanka, Bengaluru-560064, Karnataka, India. Phone: +91-80-23093100 / 23093111(O) Mobile: 9481807438; E-Mail: balavirol@gmail.com; b.vinayagamurthy@icar.gov.in

Stakeholder meeting Co-coordinators

Dr .G.Govindaraj, Senior Scientist, ICAR-NIVEDI, Bengaluru

Dr. G.B.ManjunathaReddy, Scientist, ICAR-NIVEDI, Bengaluru

Laboratory training Co-coordinators

Dr. M.Nagalingam, Scientist, ICAR-NIVEDI, Bengaluru

Dr. R. Sridevi, Scientist, ICAR-NIVEDI, Bengaluru

Technical Assistants

Mrs. Anusha Alamuri,

Ms. S. Sowjanya Kumari

Mrs. L. Linshamol

Committee for Stakeholder meeting and Laboratory Capacity Building workshop on “Leptospirosis”

Committee	Members
Registration, Inauguration and valedictory committee	Dr. R. Sridevi Dr. G.B Manjunatha Reddy Dr. Siju Susan Jacob
Technical Course Manual Publication committee	Dr. V.Balamurugan Dr. M.Nagalingam Dr. G.Govindaraj, Dr. R. Sridevi Dr. G.B.Manjunatha Reddy
Transportation and Accommodation committee	Dr.V.Balamurugan Dr. G.B.Manjunatha Reddy Dr. Md.MudassarChanda Dr. G.Govindaraj Mr. Rajeevalochana
Food and tea committee	Mr. R.K.Babu Dr. Jagadish Hiremath Dr. P.Krishnamoorthy Dr. G.B.Manjunatha Reddy
Financial Committee	Dr. V.Balamurugan Mr. Rajeevalochana Mr. Babu R.K

(Parimal Roy), Director



Programme Schedule (11th to 15th September, 2017)

Day 1	Time	Topic	
11.9.2017	8.30-10 AM	Registration	
	10-11 AM	Inauguration of Programme	Details in Leaflet
	11 to 11.15 AM	Tea Break	
	11.45 to 5 PM	Stakeholder Meetings	Presentation and Discussion by Experts - Details in Leaflet

Laboratory Capacity Building for Leptospirosis (12th to 15th September, 2017)

Day	Time	Topic	SPEAKER / Resource persons
12.09.17	Introduction, Leptospira Culture and Characters		
	9-10 AM	Glimpse of NIVEDI, Overview of the course and Pre-training evaluation	Dr. Parimal Roy Dr. V. Balamurugan Dr. M. Nagalingam
	10-12 PM	Laboratory Biosafety: Principles and Practices; BSL 2+ visit	Dr. Jagadish Hiremath Dr. R.Sridevi Dr. Yogish Aradhya
	12-1 PM (Practical)	Preparation of culture media (EMJH) Culturing, and examination of Leptospira. Maintenance in liquid and semi-solid media.	Dr. V. Balamurugan Dr. M. Nagalingam Dr. R. Sridevi
	1-2 PM	Lunch	
	2- 5 PM (Practical)	Dark field examination and staining of leptospira, Live leptospira culture antigen preparation for microscopic agglutination test (MAT)	
13.09.17	Diagnosis of Leptospirosis-MAT and LFA		
	9.30-11 AM (Practical)	Live leptospira culture antigen preparation for microscopic agglutination test (MAT).	Dr. V. Balamurugan Dr. M. Nagalingam Dr. R. Sridevi
	11-1 PM (Practical)	Screening of the human and animals serum samples for leptospira antibodies by MAT.	
	1-2 PM	Lunch	
	2-5 PM (Practical)	Diagnosis of human and animal leptospirosis by Lateral flow assay Sero-screening of the samples by MAT	

14.09.17 Diagnosis of Leptospirosis-PCR and ELISA			
	9.30-1 PM (Practical)	Molecular diagnosis: Extraction of DNA from Leptospira cultural or clinical samples (Blood/Plasma/ serum/urine). Diagnosis of leptospirosis by PCR techniques namely Duplex PCR, SYBR green RT-PCR, etc.,	Dr. V. Balamurugan Dr. M. Nagalingam Dr. R. Sridevi
	1-2 PM	Lunch	
	2-5 PM (Practical)	Diagnosis of human leptospirosis by human IgM based ELISA (Pan Bio kit) and diagnosis of bovine leptospirosis by Linnodae Hardjo ELISA kit	
15.09.17 Surveillance, GIS, Economic impact of leptospirosis			
	9.30-12 PM (Theory and Practical)	Surveillance, Epidemiological Mapping, Making Point Maps of outbreaks locations, choropleth Maps	Dr. Md. Mudassar Chanda
	12-1.30 PM (Theory)	Economic impact of leptospirosis in animals and human and KAP studies including sampling designs	Dr. G. Govindaraj
	1.30-2.30 PM	Lunch	Dr. V. Balamurugan Dr. M. Nagalingam Dr. R. Sridevi Dr. ManjunathaReddy GB Dr. G. Govindaraj
	2.30-3.30 PM	Discussion with Participants	
	3.30-4.00 PM	Post-training evaluation and Feedback	
	4.00-5.00 PM	Valedictory and Certificate Distribution	

List of Abbreviations

5-FU	- 5-Fluoro Uracil
Abs	- Antibodies
Ag	- Antigen
CAAT	- Cross Agglutination Absorption Test
DFM	- Dark Field Microscopy
DNA	- Deoxyribonucleic Acid
ELISA	- Enzyme-Linked Immuno Sorbent Assay
EMJH	- Ellinghausen McCullough Johnson Harris Medium
IFA	- Immuno Fluorescent Antibody Test
IHA	- Indirect Haemagglutination Test
ILS	- International Leptospirosis Society
LAF	- Lateral Flow assay
LAT	- Latex Agglutination Test
MAbs	-Monoclonal Antibodies
MAbs	- Monoclonal Antibodies
MAT	- Microscopic Agglutination Test
MCAT	-Microcapsule agglutination test
MSAT	-Macroscopic slide agglutination test
PAbs	-Polyclonal antibodies
PCR	- Polymerase Chain Reaction
POCD	-Point of care diagnostics for leptospirosis
QPCR	-Quantitative Polymerase chain reaction
RT-PCR	- Real-time Polymerase Chain Reaction

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Manual

This training manual is developed as a concise bench side protocol that would suit the need of a basic leptospira laboratory. It is designed for the laboratory with detailed experimental protocols and comprehensive list of materials required for such experiments. Since it is not possible to include all techniques, the selected experiments are those that are representative and best illustrate key points in basic leptospira laboratory. The emphasis has been given to develop a sequential treatise of commonly encountered practical experimentation protocols that are relevant to the field of leptospira laboratory.

The sections in this manual are basic Leptospira laboratory techniques, Laboratory Biosafety: Principles and Practices; Preparation of culture media; Culturing, and examination of Leptospira, Maintenance of leptospira culture in liquid and semi-solid media. Dark field examination and staining of leptospira, Live leptospira culture antigen preparation for microscopic agglutination test (MAT), Screening of the human and animals serum samples for leptospira antibodies by MAT. Diagnosis of human and animal leptospirosis by immunoassays including ELISA / lateral flow assay, etc., surveillance, preparation of epidemiological mapping, making Point Maps of outbreaks locations, choropleth Maps using GIS techniques, economic impact of leptospirosis in animals and human, KAP studies including sampling designs, etc., Molecular biology techniques including extraction of DNA from Leptospira cultural or clinical samples (blood/plasma/ serum/urine), diagnosis of leptospirosis by PCR techniques such as Duplex PCR, multiplex PCR, SYBR green RT-PCR, etc.

The major thrust is at developing working skills in the form of a bench side protocol and all these techniques can be performed in a basic leptospira laboratory with basic facilities.



Good Laboratory Practices (GLPs)

- ✓ Use laboratory clothing's (apron, head cover, face masks, gloves) while working in the laboratory. Give preference to operating room gowns that fasten in back. Wearing shoe cover minimizes dust and resultant contamination in the laboratory.
- ✓ Wash hands thoroughly with soap and finally disinfect with 70% alcohol before actual working.
- ✓ Keep hands away from your mouth, nose, face, eyes and ears. This will prevent self-inoculation and process contamination.
- ✓ Evaluate the extent to which the hands may become contaminated with some agents and operations. Use forceps/scissors and wear rubber gloves.
- ✓ Disinfect the working surface/area by swabbing with disinfectant wet cotton before and after work.
- ✓ Use automatic liquid handlers for pipetting infectious or toxic fluids. Must avoid mouth pipetting.
- ✓ Handle all the tissues/serum in a biosafety laminar flow cabinet.
- ✓ Periodically clean out refrigerators, deep-freezers and dry ice chests in which cultures are stored to remove broken ampoules or tubes. Use rubber gloves and respiratory protection during the cleaning.
- ✓ Avoid eating, drinking and smoking in the laboratory.
- ✓ Make special precautionary arrangements for respiratory, oral, intranasal and intratracheal inoculation of infectious material.
- ✓ Avoid blowing infectious materials out of pipettes as it generates aerosols.
- ✓ Plug glass pipettes with cotton or use plastic pipettes (already plugged) available commercially.
- ✓ Avoid preparing mixtures of infectious materials by bubbling expiratory air through the liquid with a pipette.
- ✓ Follow biosafety norms in disposing off the biomedical waste.
- ✓ Sterilize all contaminated discarded material before disposing off. Never leave a discard tray containing infected material unattended.
- ✓ Discard broken glass, needles and other sharp objects separately.
- ✓ Wrap a lyophilized glass ampoule with disinfectant wetted cotton before breaking. Wear gloves and protect face from glass splinters by wearing acrylic face mask/cover.
- ✓ Use an alcohol moistened pledget around the stopper and needle when removing a syringe and



needle from a rubber stoppered vaccine bottle or infectious material container.

- ✓ Use only needle locking hypodermic syringes. However, avoid using syringes where possible.
- ✓ Expel excess fluid and bubbles from a syringe vertically into cotton pledget moistened with disinfectant, or into a small bottle containing cotton.
- ✓ Swab the site of injection (both before and after injecting an animal) of animal using a disinfectant swab. After use, sterilize discarded pipettes and syringes before disposing them off.



Introduction

Leptospirosis is a transmissible disease of animals and humans caused by spirochaete bacteria belonging to the genus *Leptospira*. The disease was first described by physician Adolf Weil in 1886 in Germany. Twenty one species of *Leptospira* have been identified. Thirteen species cause disease or have been detected in human cases. It is estimated that seven to ten million people are infected by leptospirosis a year. Leptospirosis is transmitted directly or indirectly from animals to humans. Leptospirosis is a major direct zoonosis. Human to human transmission occurs only very rarely. Leptospire bacteria are corkscrew-shaped bacteria which can be either pathogenic (i.e. having the potential to cause disease in animals and humans) or saprophytic (i.e. free living and generally considered not to cause disease). Pathogenic leptospire are maintained in nature in the renal tubules and genital tracts of certain animals. Leptospirae belong to the order *Spirochaetales*, family *Leptospiraceae*, and genus *Leptospira*. *Leptospira* are also classified based on their serovar. Recently twenty species are traditionally classified into 30 serogroup and over 300 serovars with about 250 pathogenic serovars of *Leptospira* are recognized.

Virtually all mammalian species can harbour leptospire in their kidneys and act as source of infection to human beings and other animals. However, cattle, buffaloes, horses, sheep, goat, pigs, dogs and rodents are common reservoirs of leptospire. Rodents were the first recognized carriers of leptospirosis. They are the only major animal species that can shed leptospire throughout their lifespan without clinical manifestations, i.e. prolonged carrier state. The most often associated serovars are Icterohaemorrhagiae, Copenhageni, Grippotyphosa and Ballum. Pig and cattle are associated with serovars Pomona, and Hardjo (Hardjo Bovis, Hardjo Prajitino). They are incriminated as a primary source of infection to human beings. Pigs and cattle can excrete very large amounts of leptospire in the carrier state (i.e., chronic leptospiral colonization of the renal tubules) and can be an important source of human infection. Animals that are natural hosts to a particular serovar usually show no or comparatively few ill effects after infection with that serovar. However, they may develop illness after infection with another serovar.

Leptospirosis is transmitted by both wild and domestic animals. The most common animals that spread the disease are rodents. Humans become infected through contact with water, food, or soil that contains urine from infected animals. This may happen by swallowing contaminated food or water or through coming into contact with breaks in the skin, eyes, mouth, or nose. The disease is not known to spread between humans, and bacterial dissemination in convalescence is extremely rare in humans. Leptospirosis is common among water-sport enthusiasts in specific areas, as prolonged immersion in water promotes the entry of this bacterium. Surfers and white-water paddlers are at especially high risk in areas that have been shown to contain these bacteria, and can contract the disease by swallowing contaminated water, splashing contaminated water into their eyes or nose, or

exposing open wounds to infected water. In the developing world the disease most commonly occurs in farmers and poor people who live in cities. In the developed world it most commonly occurs in those involved in outdoor activities in warm and wet areas of the world.

The leptospirosis situation in India is a cause of concern and it is endemic in all Southern states as well as in coastal states like Gujarat, Maharashtra, including Andaman and Nicobar Islands of India, where high prevalence was recorded both in animals and humans. The local abundance of several species of pathogenic leptospirae may be a useful indicator to assess the potential for transmission of leptospira to humans and livestock and it may help policy makers to implement or take appropriate control measures to reduce the impact of leptospirosis. Moreover, knowledge of the prevalent serovar (s) and their maintenance host (s) in any particular geographical location (s) is essential to understand the epidemiology of leptospirosis.

Chronic infections in animals may lead to reproductive problems, such as abortion and low fertility in cattle or pigs. Occasionally, calves and piglets may suffer from an icterohaemorrhagic syndrome with potentially fatal outcome. Dogs may suffer from a chronic disease leading to kidney damage, but may also suffer from an acute Weil's-like disease syndrome after infection with certain serovars. Leptospiral infection in humans causes a range of symptoms, and some infected persons may have no symptoms at all. Signs and symptoms can range from none to mild such as headaches, muscle pains, and fever to severe with bleeding from the lungs or meningitis. If the infection causes the person to turn yellow, have kidney failure and bleeding, it is then known as Weil's disease. If it causes a lot of bleeding into the lungs then it is known as severe pulmonary haemorrhage syndrome. Leptospirosis is a biphasic disease that begins suddenly with fever accompanied by chills, intense headache, severe myalgia (muscle ache), abdominal pain, conjunctival suffusion (red eye), and occasionally a skin rash. The symptoms appear after an incubation period of 7–12 days. The first phase (acute or septic phase) ends after 3–7 days of illness. The disappearance of symptoms coincides with the appearance of antibodies against *Leptospira* and the disappearance of all the bacteria from the bloodstream. The patient is asymptomatic for 3–4 days until the second phase begins with another episode of fever.

Diagnosis is typically by looking for antibodies against the bacterium or finding its DNA in the blood. Efforts to prevent the disease include protective equipment to prevent contact when working with potentially infected animals, washing after this contact, and reducing rodents in areas people live and work. The antibiotic doxycycline, when used in an effort to prevent infection among travellers, is of unclear benefit. Vaccines for animals exist for certain type of *Leptospira* which may decrease the risk of spread to humans. Treatment if infected is with antibiotics such as: doxycycline, penicillin, or ceftriaxone.

Antibiotic therapy should be started as soon as the diagnosis of leptospirosis is suspected regardless of the phase of the disease or duration of symptoms. Treatment is effective within 7 to



10 days of infection and it should be given immediately on diagnosis or suspicion. The antibiotic of choice is benzyl penicillin by injection in doses of 5 million units per day for five days. Patients who are hypersensitive to penicillin may be given erythromycin 250 mg 4 times daily for 5 days. Doxycycline 100 mg twice daily for 10 days is also recommended. For animals infections, tetracycline and oxytetracycline, erythromycin, enrofloxacin, tiamulin, and tylosin have been reported to be successful in acute cases. Oxytetracycline, amoxicillin, and enrofloxacin may be useful to treat chronic infections. Effective prevention and control measures can be achieved through proper diagnostic and prophylactic aids to curtail further spread as in most of the zoonotic diseases. We can minimize the risk to humans by avoiding contact of water with animal urine, control of rodents and ensuring the proper vaccination of pets. Annual vaccinations, confinement rearing, and chemoprophylaxis are to be employed for animals. Selecting replacement stock from herds that are seronegative for leptospirosis, chemoprophylaxis and vaccination of replacement stock are important.

The most effective preventive measure is avoidance of high-risk exposure (i.e. wading in floods and contaminated water, contact with animal's body fluid). If high risk exposure is unavoidable, appropriate personal protective measures include wearing boots, goggles, overalls and rubber gloves. Pre-exposure antibiotic prophylaxis is not routinely recommended. However, in those individuals who intend to visit highly endemic areas and are likely to get exposed (e.g. travelers, soldiers, those engaged in water-related recreational and occupational activities), pre-exposure prophylaxis may be considered for short-term exposures.

Various factors influencing the animal activity, suitability of the environment for the survival of the organism and behavioral and occupational habits of human beings can be the determinants of incidence and prevalence of the disease. The disease was considered inconsequential till recently, but it is emerging as an important public health problem during the last decade or so due to sudden upsurge in the number of reported cases and outbreaks. A clear understanding of epidemiology of the diseases with wildlife as reservoir namely the virulence and transmissibility of many diseases could help in understanding the severity and thereby to take appropriate measures in control of diseases.

To safeguard the public health from pathogens of zoonotic infections, application of skills, knowledge and resources of veterinary public health is essential. Further, the control measures for emerging and re-emerging pathogens are demanding, as there is population explosion. Novel, highly sensitive and specific techniques comprising genomics and proteomics along with conventional methods would be useful in the identification of emerging and re-emerging pathogen or microorganisms, thereby therapeutic/prophylactic/preventive measures would be applied on time. The first line of measure to control any disease is the surveillance. Control and prevention strategies should be designed based on transmission pattern and characteristics of microbes, involvement of intermediate host, environment and epidemiology of the disease.

Laboratory Biosafety: Principles and Practices

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General Biosafety Principles and Practices

Laboratory biosafety refers to all the measures to protect self, people around and environment against the biological risk posed by handling of various biohazard materials and agents in laboratory conditions (Fig.1). Laboratory biosafety involves the containment principles, technologies and practices that are implemented to prevent unintentional exposure to pathogens and toxins, or their accidental release. Depending upon the nature of agent handled and risk group categorization, the containment requirements in the form of laboratory practices, safety equipment, facility design and laboratory biosafety levels for safe handling of the agent is developed.

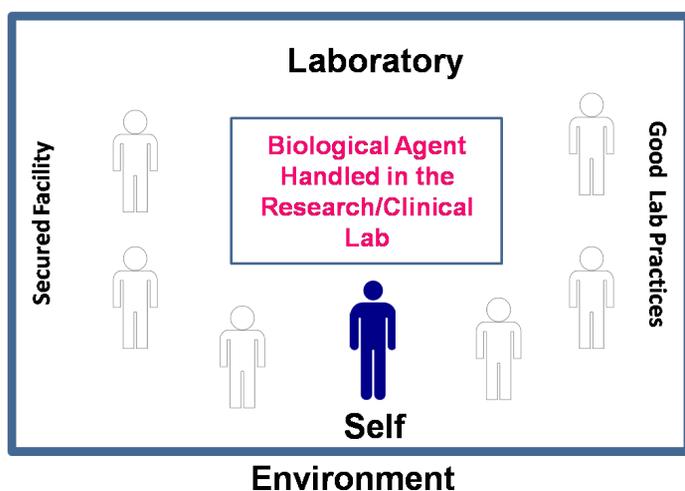


Fig. The biosafety is aimed at keeping laboratory workers, other persons and the environment safe from hazardous agent by following good laboratory practices and working in secured facility

The most important element of containment is strict adherence to standard microbiological practices and techniques. Persons working with infectious agents or potentially infected materials must be aware of potential hazards, and unless proficient in the practices and techniques required for handling such material safely one should not indulge in to such works.

Hygiene and Housekeeping: Keeping work areas clean and uncluttered reduces the chance for cross-contamination and inadvertent exposure to biohazards. To avoid ingestion of contaminated material, use a mechanical pipetting device, keep food out of refrigerators and microwaves in work areas, eat, drink, or apply cosmetics only in designated “clean” areas.

Other standard practices include: (1) Wear a lab coat and tie back long hair, (2) Wash hands after removing gloves, before leaving the lab, and when handling materials known or suspected to be contaminated, (3) Perform procedures in a manner that minimizes the creation of aerosols.

(4) Clean work surfaces and decontaminate with a suitable disinfectant at the end of the day and after any spill of potentially hazardous materials, (5) Bench tops and floors should be impervious to water



and easy to clean (6) Remove gloves before leaving the lab, touching the face, keyboards, or control panels, and before using the elevator

Personal Protective Equipment: (1) Wear gloves if skin on the hand is broken, if a rash is present, and when handling biological waste. (2) Remove rings or other jewelry that could puncture gloves.

(3) Wear the appropriate glove for the hazard. Usually a type of latex glove is recommended for working with biological material. (4) Avoid reusing gloves unless they can be decontaminated.

(5) Wear eyewear approved for UV light or other rays that could damage eyes. (6) Wear protective eyewear during procedures in which splashes of microorganisms or other hazardous materials is anticipated (Fig.2). (Laboratory biosafety manual, Third edition, WHO)

Procedures: 1) Pipetting by mouth must be strictly forbidden, 2) Materials must not be placed in the mouth. Labels must not be licked, 3). All technical procedures should be performed in a way that minimizes the formation of aerosols and droplets, 4) The use of hypodermic needles and syringes should be limited. They must not be used as substitutes for pipetting devices or for any purpose other than parenteral injection or aspiration of fluids from laboratory animals, 5) A written procedure for the clean-up of all spills must be developed and followed, 6) Contaminated liquids must be decontaminated (chemically or physically) before discharge to the sanitary sewer. An effluent treatment system may be required, depending on the risk assessment for the agent(s) being handled, 7) Written documents that are expected to be removed from the laboratory need to be protected from contamination while in the laboratory (Laboratory biosafety manual, Third edition, WHO).

Laboratory working areas: 1) The laboratory should be kept neat, clean and free of materials that are not pertinent to the work, 2) Work surfaces must be decontaminated after any spill of potentially dangerous material and at the end of the working day, 3) All contaminated materials, specimens and cultures must be decontaminated before disposal or cleaning for reuse, 4) Packing and transportation must follow applicable national and/or international regulations. When windows can be opened, they should be fitted with arthropod-proof screens (Laboratory biosafety manual, Third edition, WHO)

Security and Access: Access to the laboratory should be restricted at the discretion of the laboratory manager when experiments or work with cultures and specimens are in progress. Bio hazardous material must be clearly marked with a biohazard symbol.

Safety Equipment: Safety equipment includes biosafety cabinets (BSCs), enclosed containers, and other engineering controls designed to remove or minimize exposures to hazardous biological materials. The BSC is the principal device used to provide containment of infectious droplets or aerosols generated by many microbiological procedures. Three types of BSCs (Class I, II, III) used in microbiological laboratories. Open-fronted Class I and Class II BSCs are primary barriers that offer significant levels of protection to laboratory personnel and to the environment when used with good microbiological techniques. The Class II biological safety cabinet also provides protection from external contamination of the materials (e.g., cell cultures, microbiological stocks) being manipulated inside the cabinet. The gas-tight Class III biological safety cabinet provides the highest attainable level of protection to personnel and the environment. An example of another primary barrier is the safety centrifuge cup, an enclosed container designed to prevent aerosols from being released during

centrifugation. To minimize aerosol hazards, containment controls such as BSCs or centrifuge cups must be used when handling infectious agents. Safety equipment also may include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles (Fig.2). Personal protective equipment is often used in combination with BSCs and other devices that contain the agents, animals, or materials being handled. In some situations, in which it is impractical to work in BSCs, personal protective equipment may form the primary barrier between personnel and the infectious materials.



Fig. Personal Protective Equipment: 1. Gloves, 2. Goggles, 3. Gown, 4. Shoe cover, 5. Mask & Head cover, 6. Coverall (https://cpraedcourse.com/course/hc_fa_bp).

Biosafety practices in handling suspected clinical materials and cultures of *Leptospira*

Leptospirosis, the most widespread zoonosis in the world, is an emerging public health problem, particularly in large urban centers of developing countries. The disease is caused by spirochetes belonging to the genus *Leptospira*. The pathogen enters the body via cuts or abrasions in the skin or through mucous membranes of the eyes, nose or throat. Ingestion, accidental parenteral inoculation, and direct and indirect contact of skin or mucous membranes, particularly the conjunctiva, with cultures or infected tissues (liver, kidney, spleen) or body fluids (blood, urine, CSF) are the primary laboratory hazards. The importance of aerosol exposure is not known.

Biosafety practices and Containment facility recommended:

BSL-2 practices, containment equipment (BSC-Class II, B2), and facilities (Effluent treatment plant, double doored autoclave for decontamination of biohazard waste) are recommended for all activities involving the use or manipulation of known or potentially infective tissues, body fluids, and cultures. The housing and manipulation of infected animals should be performed at ABSL-2. Gloves should be worn to handle and necropsy infected animals and to handle infectious materials and cultures in the laboratory

Disinfection

Leptospira can be inactivated by 1% sodium hypochlorite, 70% ethanol, iodine-based disinfectants, quaternary ammonium disinfectants, accelerated hydrogen peroxide, glutaraldehyde, formaldehyde, detergents and acid. This organism is sensitive to moist heat (121°C [249.8°F] for a minimum of 15 min). It is also killed by pasteurization.



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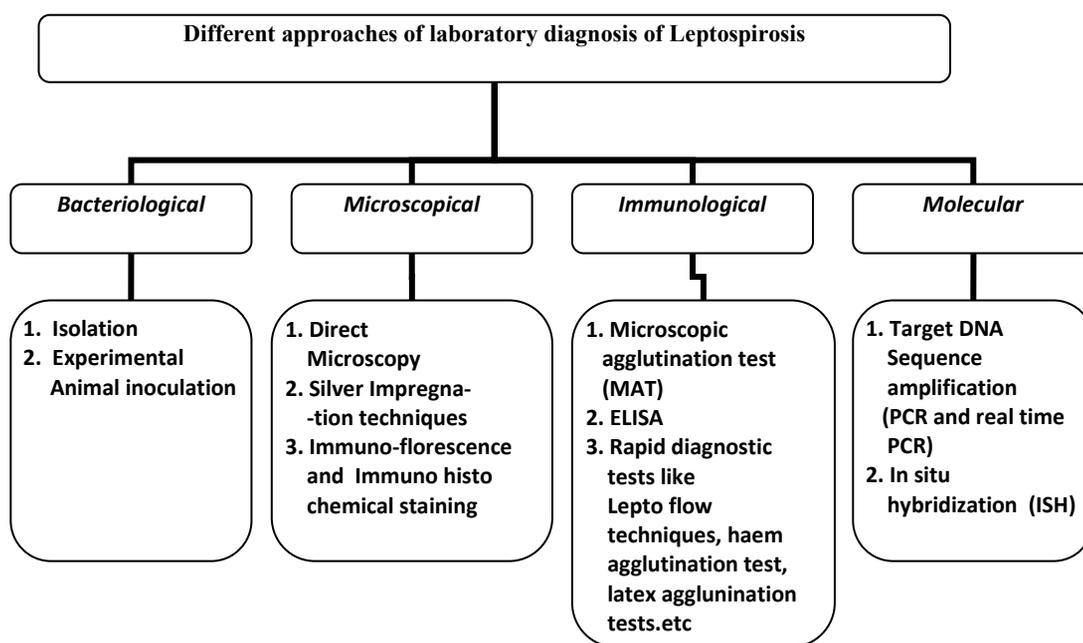
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Diagnosis of Leptospirosis

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Laboratory diagnosis of leptospirosis remains a challenge. An array of laboratory tests is available for diagnosing leptospirosis. These can be broadly divided according to their methodology into: a) methods demonstrating the organism in culture or clinical specimens, b) immunological methods, and c) genomic methods. However selection of the right specimens and tests and correct interpretation of test results are important. Further, laboratory diagnosis is broadly divided into two categories- those that give direct evidences and those that give indirect evidences. The direct evidences include either demonstration of leptospire or its DNA and isolation of organism from clinical specimens. Detection of specific antibodies to leptospire (serological diagnosis) is indirect evidence.



Direct Diagnosis include, demonstration of the organism in body fluids by DFM, Culture and isolation of the bacteria from blood, urine or tissues, direct staining of the organisms, Immuno fluorescence, polymerase chain reaction, animal Inoculation studies etc., where as in Indirect Diagnosis, in which demonstration of antibodies to leptospire by various serological primary, secondary and tertiary immune assays or testes, namely, Microscopic agglutination test (MAT), ELISA, Macroscopic slide agglutination test (SAT), Lateral Flow assays, Indirect Haemagglutination test (IHA), Counter



immuno electrophoresis (CIE), Complement –fixation test (CFT), Latex agglutination tests (LA), Indirect fluorescent antibody test(IFAT), etc., however, ELISA and MAT are the commonly used serodiagnostic techniques.

Criteria for diagnosis of leptospirosis in Presumptive diagnosis are -a positive result in IgM based immune- assays, slide agglutination test or latex agglutination test or immunochromatographic test. A Microscopic Agglutination Test (MAT) titre of 100/200/400 or above in single sample based on endemicity. Demonstration of leptospire directly or by staining methods. Where as in Confirmatory diagnosis- Isolation of leptospire from clinical specimen, Four fold or greater rise in the MAT titer between acute and convalescent phase serum specimens run in parallel, Positive by any two different type of rapid test, Sero-conversion and PCR assay. To say the infection as definite /confirmed one, serological data, clinical data and epidemiological data (history of possible exposure, presence of risk factors) all should be considered.

Dark field Microscope Examination

Leptospire may be visualized in clinical specimens by dark field microscopy (DFM) or by immunofluorescence or light microscopy after appropriate staining like silver impregnation technique. Approximately 10^4 leptospire / ml are necessary for one cell per field to be visible by DFM. The quantitative buffy coat method has a sensitivity of approximately 10^3 leptospire/ml. Microscopy of blood is of value only during the first 7-10 days of the acute illness during leptospiremia. DFM examination of body fluids such as blood, urine, CSF, and dialysate fluid has been used but is both insensitive and lacks specificity. False positive and false negative results are easily made even in technically expertise hands. Immunofluorescence staining of bovine urine, water, and soil and immunoperoxidase staining of blood and urine has been applied to increase the sensitivity of direct microscopic examination. Histopathological stains and immunohistochemical methods have been applied for the detection of leptospire in tissues. This method is simple and rapid, but, it has low sensitivity and specificity. Further, it requires technical expertise and necessary to differentiate serum proteins and fibrin strands in blood from actual leptospirae.

Microscopy is a very simple yet effective technique and well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual water-borne single-celled organisms. Considering the simplicity of the setup, the quality of images obtained from this technique is impressive. Microscope is ideal for viewing objects that are unstained, transparent and absorb little or no light. It is more useful in examining external details, such as outlines, edges, grain boundaries and surface defects than internal structure. Effectively, the research microscopes

can yield high magnifications of living bacteria and low magnifications of the tissues and cells of certain organisms. In bacteriology laboratory, certain bacteria and fungi can be studied with the use of research microscopes. The color, composition and movement are studied for identification.

Dark-field microscopic (DFM) examination is insensitive and requires a skilled observer to differentiate leptospire from artifacts. Leptospira are present in blood early in course of disease. After 10-14 days, they may be found in the urine. DFM for the field collected samples is difficult. Hence it has to be isolated and then observed under DFM. Cultures should be examined by dark-field microscopy every 1–2 weeks. It is important to use a 100 watt light source and a good quality dark-field microscope.

Principle:

To view a specimen in dark field, an opaque disc is placed underneath the condenser lens, so that only light that is scattered by objects on the slide can reach the eye. Instead of coming up through the specimen, the light is reflected by particles on the slide. Everything is visible regardless of color, usually bright white against a dark background. Pigmented objects are often seen in “false colors,” that is, the reflected light is of a color different than the color of the object. Better resolution can be obtained using dark field as opposed to bright field viewing.

Materials:

Glass slides (0.1mm thickness); Dark Field Microscope (Nikon Japan Trinocular Microscope Model NI – U) (equipped with objective 10x, 20x and 40X dark field condenser and 10x eyepieces); Micro pipettes (10 μ l); Centrifuge; Cover slip and Sample to be screened.

Procedure:

- Take a clean grease free glass slide and wipe it thoroughly.
- Place 5 μ l of the sample on the slide and place a round cover slip without air bubbles.
- Observe under the 20X and 40X
- To observe under oil immersions place a drop of oil on the oil condenser and then place the slide. Place another drop of the oil on slide and observe under the 100x.

(Note:

- 1. The condenser and 100X lens should touch the slide while viewing**
- 2. Leptospira are mistaken for rods and other spiral like Trypanosome. It can also be mistaken with artifacts. Hence careful observation needs to be done for cork screw motility.)**



Live Leptospira Culture Under DFM:

Liquid Media culture is preferred as the semisolid media culture will have agarose which will tamper the visibility.

Serum Sample:

Serum is separated from the whole blood and observed under 20X, 40X and 100X. Careful observation is required as it's not easy to detect Leptospira in field collected serum.

Plasma Sample:

Blood is spun at the 5000 rpm for 5 mins. The separated plasma on the top is placed on the slide and viewed at 20X, 40X and 100X.

Urine Sample:

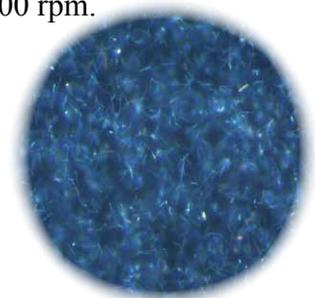
Spin the urine sample at 2000 rpm for 10 mins. The sediment is placed on the slide and observed under 20X, 40X and 100X.

Motility of the Organism:

The cork screw motility has to be observed carefully for leptospira organism. If the culture is auto agglutinating then observe the culture after the 3-5 mins of spin at 1000-2000 rpm.

Result:

Leptospira organism is thin spirals with cork screw motility. These are highly motile in nature. Observed under 200X and 400X in Dark Field Microscope (Nikon Japan Trinocular Microscope Model NI- U)



Advantage: Dark-field microscopy is useful for observing leptospire in culture, particularly when they are present in large numbers, and for observing agglutination in MAT.

Disadvantages: Dark-field microscopy is technically demanding. Recognizing leptospire becomes difficult, particularly when they are present in less numbers. Artifacts, such as fibrin threads in blood, are easily mistaken for leptospire. False-positive or false-negative results i.e. misdiagnosis is very much possible. Approximately 10^4 leptospire/ml are necessary for one cell per field to be visible under DFM

Dark-field microscopy is therefore useful only to those with considerable experience in observing leptospire. The results of dark-field microscopy of clinical material should always be confirmed by other tests.

Silver impregnation technique

The special stains that utilize silver include various staining procedures and these procedures are based on different principles. Silver impregnation staining techniques are used for the leptospiral staining in patients samples (body fluids and tissues) since the organism is difficult to stain by other methods. These techniques lack sensitivity and specificity, so they can be used as an adjunct for histopathological diagnosis but not for direct diagnosis of leptospirosis. The morphological feature of the bacteria *Leptospira* is extremely thin, elongated and spiral. It is about 0.1 to 20 μ in size. Hence basic aniline dyes fail to stain the bacteria. To overcome such disadvantage, Fontana developed silver impregnation technique (Fontana Staining). This technique was more cumbersome hence a modified silver staining was developed using mordant as an additional reagent. Thus *Leptospira* staining kit contains the following reagents:

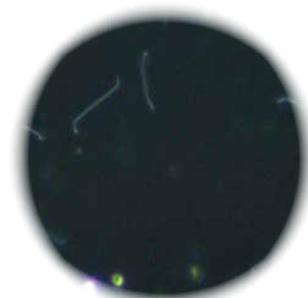
- **Reagent 1:** Fixative
- **Reagent 2:** Absolute Alcohol
- **Reagent 3:** Mordant
- **Reagent 4:** Silver Stain



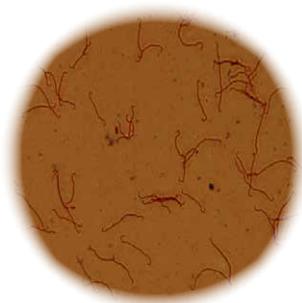
Principle: The impregnation of the silver salts on the soma of the organism in order to increase its thickness, and to render its visibility by light microscope.

Staining Protocol

- Take a clean grease free glass slide. Wipe it with the alcohol and allow it to dry.
- Label the slide towards one corner and cello tape it to avoid the wetting of the Label.
- Take 25 - 30 μ l of the culture to be stained in the middle of the slide and make a smear. Allow it to air dry completely.
- Pour the fixative in a coupling jar and place the smeared slide. Allow it to get fixed for 2 minutes. Care should be taken to immerse the slide completely. Wipe the underneath of the slide and allow it to air dry completely.
- Place the smeared slide in the coupling jar containing absolute alcohol for 3 minutes. Dip it in the same jar for 2 to 3 times and remove the slide. Wipe the underneath of the slide and allow it to air dry.
- Immerse the smeared slide for 1 minute in the preheated mordant (at 80 $^{\circ}$ C for 1 m) wipe the underneath of the slide and dip it in the distilled water for 3 to 5 times. Allow the smear to air dry completely.



- Finally place the smear in the silver stain which is preheated for 1 minute at 80°C. Allow the slide to get stained for 1 minute. Wash the slide in the fresh distilled water and air dry it completely and mount in Canada balsam.
- It is essential that the specimen be mounted in balsam under a cover slip before examination, as some immersion oils cause the film to fade at once. The spirochetes are stained brownish black on a brownish-yellow background.
- Observe under light microscope at 400x and oil immersion for brownish pink colored, both ends hooked like spirochete with coiled morphology with a yellowish brown background. Note the size and the shape of the microorganism. Stained debris or fibrin materials or artifacts will look fibrous and can be differentiated.



Note: Serum/Urine/CSF can also be stained. If the sample is blood, it is necessary to spin it for 20 minutes at 2000 rpm. Clarity of the stained slide depends on how clear is fluid sample and how thin is the smear

Isolation and maintenance of the leptospira

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Isolation of leptospire from clinical specimens is the strongest evidence for confirmatory diagnosis. Leptospiremia occurs during the first stage of the disease, beginning before the onset of symptoms, and ends by the first week of the illness. Thus blood cultures should be taken as soon as possible after the patient's presentation and before antibiotics treatment. Leptospire survive in conventional blood culture media for a number of days. CSF and dialysate fluid can also be cultured during the first week of illness. Urine can be cultured from the second week of symptomatic illness. Even from aborted fetused heart blood samples can be used for isolation and confirmation due to abortion in animals (Fig) Collection of urine following treatment of the animals with a diuretic enhances the chances of detecting the organism. The duration of urinary excretion varies but may last for several weeks. In important cases involving individual animals (e.g. clearing an infected stallion to return to breeding), negative tests on three consecutive weekly urine samples has been considered to be a good evidence that an animal is not shedding leptospire in the urine.



A modified Ellinghausen Mecullough Johnson and Harris (EMJH) medium supplemented with 300 μg / ml of 5- Fluoro uracil 8 is ideally suited for isolation and maintenance of pathogenic leptospire. If tissues or fluids cannot be transported promptly to the laboratory for leptospiral culture, the sample should be kept at 2–5°C to prevent overgrowth with other bacteria and autolysis of tissue samples. Liquid culture medium with 1% bovine serum albumin (BSA) solution containing 5-fluorouracil at 100–200 μg /ml should be used as transport medium for the submission of samples. Addition of 0.4–1% rabbit serum to semisolid culture medium enhances the chances of isolating fastidious leptospiral serovars.

Cultures should be incubated at $29 \pm 1^\circ\text{C}$ for at least 16 weeks, and preferably for 26 weeks. The time required for detection of a positive culture varies with the leptospiral serovar and the numbers of organisms present in the sample. Less fastidious serovars (e.g. Pomona and Grippotyphosa) may result in positive cultures as soon as 7–10 days after inoculation; other serovars (e.g. Hardjo and Bratislava) may take much longer. Cultures should be examined by dark-field microscopy every 1–2 weeks for identifying the leptospira. It is important to use a 100 watt light source and a good quality dark-field microscope.

The test is a definite proof of confirmatory diagnosis. Isolation and identification is the method of choice to identify circulating serovars in a particular geographical region. In addition, locally isolated and identified strains will be more useful to be used as antigens in MAT, as local strains were found to be more sensitive and



strongly reactive than reference strains. However, the test has the following demerits 1. Culture methods are very tedious, complicated, expensive, technically demanding, time consuming, requiring prolonged incubation before declaring a sample negative and may not be successful (low sensitivity) always. 2. Leptospirae are highly infectious organisms requiring 'Biosafety level II' facilities. 3. Prior treatment of antibiotics to the patients /animals greatly reduces the chances of successful isolation.

Leptospira are aerobes and long chain fatty acids are their carbon and energy sources. In addition to long chain fatty acids, vitamin B1, B12 and ammonium salts are also required for their growth. Leptospirae utilize purine bases but not pyrimidine bases and hence they are resistant to the anti-bacterial activity of pyrimidine analogue 5-fluoro uracil. This compound is used in selective media for the isolation of leptospirae from contaminated sources. Because of the inherent toxicity of free fatty acids, these must be supplied either bound to albumin or in non-toxic esterified form. Pyruvate enhances the initiation of growth of parasitic Leptospirae.

The isolation of leptospirae depends on the choice of the material and the stage of infection. During the leptospiraemic phase (days 1 to 10 or so from the onset of illness) the materials are blood, vascular organs (liver, spleen and kidneys) and CSF.

Blood: Blood cultures should be done in the first 10 days of the illness and before antibiotics are given. Venous blood is collected by aseptic technique and inoculated at the bedside. Small inocula of one or more drops of blood are inoculated into several tubes, each containing 5 ml of suitable medium. Large inocula will inhibit the growth of leptospirae.

CSF: Leptospirae may be observed and isolated from cerebrospinal fluid during the end of the first week of illness. However, this procedure is not usually performed.

During the phase of leptospiruria and increasing concentrations of antibodies (after about 1 week from onset) the renal cortex and the urine are the most suitable inocula for isolation of leptospirae from man but during the carrier state also to wild and domestic animals.

Urine: Fresh midstream urine is collected and inoculated immediately. One drop of undiluted urine is inoculated into the first tube containing 5 ml of medium. Some more tubes are similarly inoculated with urine in increasing 10-fold dilution. Urine should be examined within 2 hours after voiding, using culture or animal inoculation. Media containing antibiotics are essential to reduce contamination of urine cultures.

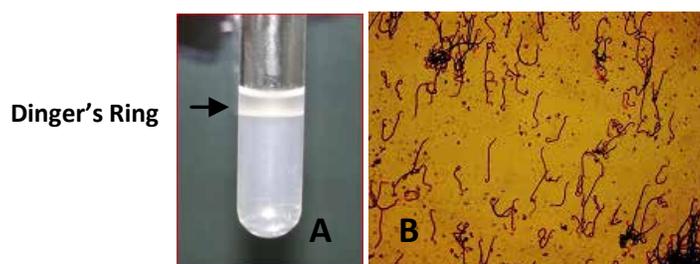
Post Mortem Material: In fatal cases of human and animal leptospirosis, the organisms may be seen and cultured from ground post-mortem specimens of tissues: liver, kidneys and brain are the tissues most suitable. Leptospirae may also be successfully isolated from aborted animal foetuses in this manner; the tissues should not be frozen.

The isolation of leptospirae is not only dependent on number of organisms but also on number of viable organisms. For instance, post mortem changes can rapidly reduce the number of viable organisms. This reduction in numbers is also temperature dependent: leptospirae survive well at 4°C but are rapidly killed in tissues held at 20°C never mind the 30 to 40°C which dead foetuses are held at before being expelled by their dams.

Culturing is very time consuming and labour intensive: isolation can take up to six months to achieve and involves the use of multiple tubes of media containing 2-4 different dilutions of tissue and

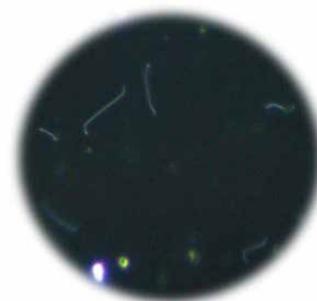
several different media for each dilution, all of which have to be examined by dark field microscopy at least every two weeks with perhaps the additional problem of passages having to be made before success is achieved.

The degree of success achieved in isolating leptospire is also dependent on how the culture tubes inoculated with tissue are manipulated. It is not sufficient to merely leave them and read at regular intervals. The length of time for which cultures are kept also effect the degree of success achieved in isolating leptospire. Some investigators investigate their cultures for a period of 26 weeks. Culture tubes are inoculated from 1:10, 1:100 and 1:1000 dilution. The amount of inoculum given to each tube ranges from 100 to 250 μ l.



Isolation and identification is the method of choice to identify circulating *Leptospira* species in a particular geographical region. In addition locally isolated and identified strains will be more useful to be used as antigens in MAT as local strains were found to be more sensitive and strongly reactive than reference strains. Leptospire is a slow growing organism and require several days or weeks to yield cultures and weeks to months for identification. Prior administration of antibiotics to the patients or animals will reduce the chances of successful isolation from them. A modified Ellinghausen McCullough Johnson and Harris (EMJH) liquid medium containing bovine serum albumin fraction V (Ellis 1986) was prepared as per standard bacteriological procedure with 200 μ g of 5-fluorouracil per mL of medium.

For isolation of leptospira, the serum/blood/urine samples that were collected from the suspected animal were inoculated (1–2 drops) directly to the transport EMJH medium containing 500 μ g of 5-fluorouracil per mL in the field level laboratory or veterinary dispensary or on the site of collection and transported to the laboratory at room temperature. After incubation at 30°C in the laboratory for 4–5 days, the culture were filtered through 0.2 μ m membrane filter and sub-cultured periodically from 8 to 10 weeks. On successful isolation the cultures were stored in the semisolid EMJH medium for long-term storage as archived culture with periodical sub-culturing every 2 to 3 months for the viability of the organism. Some of these samples were subjected for isolation in the EMJH medium and observed periodically for detection or observation of the organisms by DFM as spirochetes. The cultures were subjected to minimum of five screenings up to 45 days to rule out negative (either with top up of media or subculture in fresh media).





Guidelines for collection and transportation of samples

The Guidelines on specimen collection for the diagnosis of leptospirosis are presented in Table.

Sample type	Ideal time for collection	Comments
Blood with heparin (to prevent clotting)	First 10 days	Blood culture more than 10 days after disease onset is not worthwhile as leptospire have mostly disappeared from the blood and antibodies will have become detectable in the serum allowing serodiagnosis. One or two drops of blood are inoculated into 10 ml of semisolid medium containing 5-fluorouracil at bedside/penside. For the greatest recovery rate, multiple cultures should be performed, but this is rarely possible. Inoculation of media with dilutions of blood samples may increase recovery. Samples for culture should be stored and transported at ambient temperatures, since low temperatures are detrimental to pathogenic leptospire.
Clotted blood or serum for serology	Collected twice at an interval of several days	The testing of paired sera is necessary to detect a rise in titers between the two samples or seroconversion to confirm the diagnosis of leptospirosis. A negative serological result in the early phase of the disease does not exclude leptospirosis.
Urine for culture	Inoculated into an appropriate culture medium not more than 2 hours after voiding	Leptospire die quickly in urine. Survival of leptospire in acid urine may be increased by making it neutral. Urine should be processed immediately not more than 2 hours after voiding by centrifugation, followed by resuspending the sediment in phosphate buffered saline (to neutralize the pH) and inoculating into semisolid medium containing 5-fluorouracil. Cultures are incubated at 28 to 30°C and examined weekly by DFM for up to 13 weeks before being discarded. Contaminated cultures may be passed through a 0.2-µm or 0.45-µm filter before subculture into fresh medium.
Cerebrospinal fluid and dialysate for culture	First week of illness	Leptospire may be observed by DFM and culture isolation by inoculating 0.5 ml cerebrospinal fluid into 5 ml semi-solid culture medium during the first weeks of illness.
Postmortem samples	As soon as possible after death	The specimens collected will depend on the resources available and cultural restrictions. Postmortem samples should be collected aseptically and as soon as possible after death; they should also be inoculated into culture medium. The samples should be stored and transported at +4 °C.

Source: World Health Organization (2003)

Sample collection for Leptospira culture

Sample should be collected from suspected leptospirosis cases from endemic areas. 1-2 drops of blood should be aseptically transferred into EMJH (Ellinghausen and McCullough modified Johnson and Harris) transport media during the acute septicemia phase (ie, within 10 days of fever). During immune phase (ie, after 10 days) 2-3 drops of Urine can be transferred into EMJH transport media containing tubes.

Labeling of Specimen:

Specimen should be labeled with Name & Age of Patient, Sample type, Date & time of collection and completely filled Laboratory request form (annexure) should be submitted along with the specimen. Keep the photo copy of the Laboratory request form for your reference.

Sample Transportation:

Sample inoculated in the transport media can be transported at normal room temperature by courier or speed post to National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI) (Address mentioned below) within 2-3 days of collection.

Sample to be sent:

The Director,
ICAR - National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI),
Ramagondanahalli, Post Box No. 6450; Yelahanka, Bengaluru, PIN- 560064

Note: EMJH transport media can be stored at room temperature

Preparation of EMJH culture media

I. Basal media preparation

Difco EMJH Media Base- (Catalogue no.279410 BD, Difco)

Media type per 100ml	Difco EMJH Media Base	Millipore autoclaved H ₂ O	Agarose
Liquid media	0.23g	95ml	
Semi Solid media	0.23g	95ml	0.1g

- Autoclave at 121°C for 30 mins
- After cooling add filtered 5 Fluro Uracil @ 1.25ml (1% stock solution filtered)



- Aliquot 4.5 ml of this media into sterile 15 ml centrifuge alcon tubes (Tarsons, India).
- Sterility check at 37°C for 2 days and store at 30°C till use

II. Difco Enrichment Media:

Difco Enrichment Media (catalogue no. 279510, BD, Difco) ready to use-available

Before use aliquot 10 ml of this media into sterile 15 ml centrifuge tubes (Tarsons, India).

Sterility check at 37°C for 2 days and store at 4°C till use

III. Complete media preparation:

To the sterility checked aliquoted 4.5 ml of this media, 0.5 ml of ready to use Difco Enrichment Media (Sterility check) to be added.

Sterility check at 37°C for 3 days and store at 30°C till use for culture.

Now Media is ready for use for leptospira culture subculture or isolation study.

Preparation of 5-Fluro Uracil (5-FU)

- Add 1g of 5-FU in 50ml Millipore autoclaved H₂O.
- Add 1 ml 2N NaOH gently with stirring in the 56°C hot water bath for 2 hrs.
- Cool to room temperature.
- Adjust pH to 7.4 with 1N HCl
- Make up the volume to 100ml
- Filter with the Millipore 0.22µm filter.
- Make aliquots and Store at 4°C.
- Allow it to come to room temperature when it is at use.

{5 –FU is added at the rate of 1g in 100ml Millipore autoclaved H₂O } Since **125 to 250µg/ml** of the media is the required}

It is necessary to add this 5-FU stock solution at the following rate:

Total volume of the EMJH media	Quantity of the stock solution to be added
100ml	1.25ml
250ml	3.125ml
500ml	6.25 ml
1000ml	12.50ml

For EMJH Transport Media

Total volume of the EMJH media	Quantity of the stock solution to be added
100ml	2.5ml
250ml	6.25ml
500ml	12.5 ml
1000ml	25.0ml



Culturing of Leptospira

- Leptospira reference cultures received from the referral laboratory regional medical research centre, WHO reference laboratory (ICMR, RMRC, Portblair) are maintained in liquid and semisolid medium at ICAR-NIVEDI, Bengaluru.
- The medium used in current practice is Difco EMJH Media base, Difco Leptospira Enrichment media and Agarose for semisolid media (as described earlier). Growth of other bacteria contaminants can be inhibited by the addition of 5-fluorouracil in the media.
- The media is prepared in sterile condition and semisolid media is aliquoted into screw cap borosilicate bottles and liquid media into plastic falcon tubes/ vials of 15 ml for subsequent use. The media is kept for sterility check for three days at 37°C and then store at 30°C until use.
- All cultures received from reference laboratory from semisolid stock are revived and maintained in liquid media. Weekly subculture is done for revived cultures from stock and maintained in liquid medium for regular use in MAT. Liquid media is used for the regular weekly subculture maintenance.
- 0.6 ml of the original 5 -7 days grown culture (2×10^8 /ml) is taken in plastic dropper (Pasteur Pipette, Tarson products) and inoculated in to the 6 ml of liquid media and culture is allowed to grow at 30°C for 5- 7 days. These grown 5 to 7 days cultures were used as live leptospira antigen in MAT.
- Semi solid media is used for the backup storage of culture and sub cultured once in every 3 months for revival in liquid medium and subsequent storage in semi-solid medium. This preservation method is labour-intensive and at high risk for contamination.
- 1 ml of the of the original culture (2×10^8 /ml) is taken and inoculated in to the 10 ml of semi solid media and culture is allowed to grow at 30°C for one week and later on store at room temperature for long term storage.

Microscopic Agglutination Test

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The MAT using live antigens is the most widely used serological test. For optimum sensitivity, it should use antigens representative of all the serogroups known to exist in a particular region. The specificity of the MAT is good; antibodies against other bacteria usually do not cross-react with *Leptospira* to a significant extent. However, there is significant serological cross-reactivity between serovars and serogroups of *Leptospira* and an animal infected with one serovar is likely to have antibodies against the infecting serovar that cross-react with other serovars (usually at a lower level) in the MAT.

The strains selected should be grown in liquid leptospiral culture medium (at least 4 days old culture, but no more than 8 days). Live cultures with densities of approximately 2×10^8 leptospores per ml are to be used as the antigens. The number of antigens to be used is determined and a screening test may be performed with a 1/50 serum dilution (or a different starting dilution based on the purpose). A volume of each antigen, equal to the diluted serum volume, is added to each well, making the final serum dilution 1/100 in the screening test. The endpoint is defined as that dilution of serum that shows 50% agglutination, leaving 50% free cells compared with a control culture diluted 1/2 in phosphate buffered saline, after incubation at $29 \pm 1^\circ\text{C}$ for 2–4 hours. The result of the test may be reported as the endpoint dilution of serum (e.g. 1/100 or 1/400) or as a titre that is the reciprocal of the endpoint serum dilution (e.g. 100 or 400). Many laboratories perform a screening test at a final serum dilution of 1/100 and then retest sera with titres of ≥ 100 to determine an endpoint using doubling dilutions of sera beginning at 1/100 through to 1/12,800 or higher.

As an individual test, the MAT is very useful in diagnosing acute infection; the demonstration of a four-fold change in antibody titres in paired acute and convalescent serum samples is diagnostic value. In addition, a diagnosis of leptospirosis is likely based on the finding of very high titres with a consistent clinical picture in case of single serum test. In order to achieve maximum reliability and standardization, laboratories are encouraged to obtain a collection of serovars from a certified reference laboratory and to participate in quality assurance programs such as the International Leptospirosis MAT proficiency test scheme organized by the International Leptospirosis Society. MAT is relatively serovar / serogroup specific and test of choice for sero-epidemiologic studies. However, it has following demerits like 1.Representative serovars of all serogroups have to be maintained 2.Complex and time consuming procedure 3.Reading results requires experienced personnel 4.Not possible to distinguish between IgM and IgG antibodies 5.Second serum sample essential 6.False



negativity in the early course of the disease 7 Selection of battery of serovars is not easy.8 The age and density of antigens 9.Reproducibility

Antibody levels drop in a week or month time, they may persist for about 2 – 10 years in human sometimes. In animals they may persist in animals for their life time as they can be frequently exposed. The same is seen in the endemic region. These antibodies any have low titers. Hence the antibodies against several *Leptospira* serovar detected in the early sera may not be serovar specific. Hence the later sera are more sero specific.

Serial dilution of serum kept in contact with an equal volume of a well grown suspension of *Leptospira* at a certain temperature for a certain period of time and read microscopically by estimating 50% agglutination as the end point titer of the reaction mixture.

The Microscopic agglutination test (MAT) is the basis of serological diagnosis and classification. In 1926 Schuffner and Mochtar described the phenomenon of agglutination and lysis with human and animal sera and made their dilution series with drops from one and the same pipette. The reaction was read after 16-20 hours.

Since then the method was improved by others (Borg-Petersen and Fagroeus, 1949; Wolff, 1954; Carbrey, 1960 and Cole et al., 1973). They tried to standardize factors like incubation time, incubation temperature, reading of the end point titer and density and age of the culture. To discuss these variables it is necessary to define the microscopic agglutination test which is still used today for different purposes.

Microscopic agglutination test (MAT) for Animal serum samples

Principle:

Antibodies in the test serum react with antigens on the surface of the bacteria and agglutinate them.

Materials:

Micro test plates U – Bottom (TARSONS – 941296); Universal reagent reservoir (TARSONS - T524091); Incubator (30°C); Glass slides (0.1mm thickness); Dark Field Microscope (Nikon Japan Trinocular Microscope Model NI – U) (equipped with objective 20x and 40X dark field condenser and 10x eyepieces); Micro pipettes (1000µl, 100µl and 10µl); Multichannel pipettes; Marker Pens.

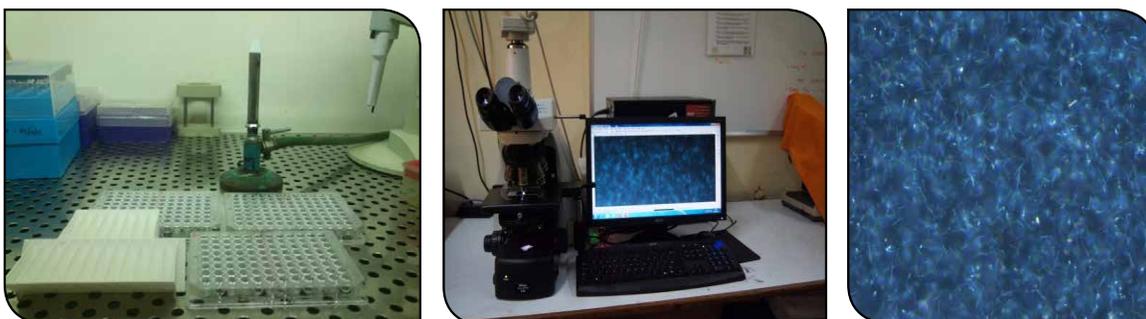
Reagents: Pure live reference *Leptospira* cultures (5 – 10 days old); Sterile PBS (pH7.2)

Procedure:

Sero-screening of the samples by microscopic agglutination test (MAT)-Determinant of the frequency distribution of the prevalent serovars and its prevalence.

Live *Leptospira* Culture antigen Preparation:

Different serovars received from the referral laboratory regional Medical research center (Port Blair, Andaman & Nicobar Islands) were maintained in the EMJH liquid and semisolid media. For MAT cultures of 5 – 8 days old when the motility is good. As the culture get old there will be auto agglutination and hence would be difficult in taking the MAT reading. Good growth culture taken for MAT is approximately 2×10^8 /ml bacterial culture. This can be counted using the Cell counter with the grid or can be simply assumed the approximate when seen under the DFM. The field focused showed be full of motile organism. (Ref. picture below)



1. EMJH Liquid and semi solid media are prepared using Difco (279410) EMJH Media Base (0.23g/100ml media) + 10ml of Difco Leptospira Enrichment media (279510) + 125mg/ml 5 Fluoro Uracil, (Sigma - F6627) (optional 0.2 % Agarose for the Semi solid media).
 2. The media is kept for sterility check for one week and then used for sub –culturing.
 3. The media is prepared in sterile condition and is aliquoted into corning glass tube or screw cap borosilicate bottles (Tensil Glass works - 7010014) or the plastic vials (Tarsons - T500030) of 15 ml in sterile condition.
 4. Semi solid media is used for the backup culture where as the liquid media is used for the regular weekly culture maintains.
 5. 0.5 ml of the of the original culture is taken and inoculated in to the 5 ml liquid or semi solid media and the culture is allowed to grow at 30°C for 5 days and the used for further procedure of MAT. Semisolid media is used for back up and is revived and sub cultured once in every 3 months.
1. Take 490µl of the sterile PBS (pH 7.2) to 12 rows of the Universal reagent reservoir.
 2. Add 10µl of the serum to be tested to each of the 11 rows and 10µl PBS for the 12th row (i.e., for antigen control). (i.e., 1:50 dilution of serum for animal samples; for human serum samples 1:10 dilution of serum has to be made (50µl of serum in 450µl of the PBS).



3. Mix thoroughly-Universal Reagent reservoir and U- Bottomed Micro Test Plates.
4. Take 50µl of each sample and add to the Micro U bottom test plates (Tarson) in such a way that each row has one sample and the last row is the control. i.e., A row from 1 to 11 diluted 1:50 serum samples, and 12th column is antigen control. Similarly, add diluted serum in the row B, C,..... H as stated above.
5. Add 50 µl of the well grown live Leptospira antigen (8 different serovars) culture of 5 – 8 days old antigen row-wise. Eg. A row for antigen Australis, B row for Autumnalis, C row for Canicola and another serovars , like that etc.,
(ie., each plate can accommodate 8 serovars antigen only).

Similarly for other serovars, another plate can be used.

{Finally one serum sample to be tested for “n” of live leptospira serovars antigen for determining the specific reactivity with different serovars}

Plate Layout	1	2	3	4	5	6	7	8	9	10	11	12 **
A	*	*	*	*	*	*	*	*	*	*	*	*
B												
C												
D												
E												
F												
G												
H												

Note: A Row-*50 µl of diluted serum plus 50 µl of live culture antigen of one serovar--- Similarly B row another serovar-----like that H row another serovar ** Antigen Control column (12). 1- 11 column – test sera (1 to 11) samples.

6. Mix thoroughly by tapping for few seconds and cover the plates with the aluminum foil.
7. Keep the plate at 30°C in an incubator for 2 – 4 hours.

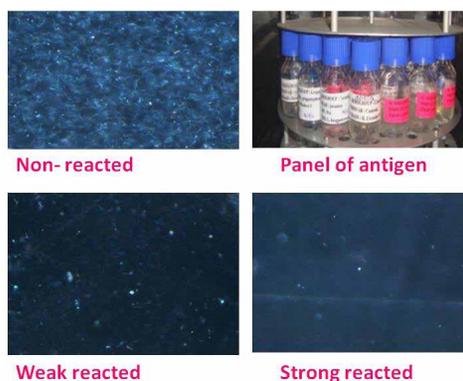
Reading: After incubation of 2 hours, the serum-antigen mixtures are examined under a dark field microscope for agglutination. This can be done by transferring one drop of mixture to a microscope slide. The endpoint (titer) is taken as that dilution which gives 50% agglutination, leaving 50% of the cells free when compared with a control suspension of Leptospira is considered positive at 1:100 dilutions. No agglutination should be seen in the control row.

Microscopic agglutination test (MAT) for human serum samples

Dilution should start from 1:10 so that the positive reactive serum sample result will be at 1:20.

For eg., Take 450µl of the sterile PBS (pH 7.2) to 12 rows of the Universal reagent reservoir.

Add 50µl of the serum to be tested to each of the 11 rows and 10µl PBS for the 12th row (i.e., for antigen control). (i.e., 1:10 dilution of serum).



Reference

- H. Korver; Microscopic Agglutination Test (MAT) for the diagnosis of Leptospirosis and serotyping of Leptospire; Royal Tropical Institute The Netherlands
- Leptospira Manual, Regional Medical Research Center; Port Blair, Andaman And Nicobar Islands.
- Levett PN (2001). Leptospirosis. *Clini Microbiol Rev.* 14:296-326.
- World Organisation for Animal Health (Office International des Épizooties -OIE). (2013) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 2013. OIE. Paris. Chapter 2.1.9. Pp. 251-264.



Maintaining the Panel of *Leptospira* reference Serovars at ICAR, NIVEDI, Bengaluru

Species	Serovar	Strain	Serogroup
<i>L. interrogans</i>	Australis	Ballico	Australis
<i>L. interrogans</i>	Bankinang	Bankinang 1	Autumnalis
<i>L. interrogans</i>	Canicola	HondUtrech IV	Canicola
<i>L. interrogans</i>	Hardjo	Hardjoprajitno	Sejroe
<i>L. interrogans</i>	Hebdomadis	Hebdomadis	Hebdomadi6
<i>L. interrogans</i>	Pyrogenes	Salinem	Pyrogenes7
<i>L. borgpetersenii</i>	Tarassovi	Perepelicin	Tarassovi8
<i>L. interrogans</i>	Icterohaemorrhagiae	RGA(ATCC443642)	Icterohae9orrhagiae
<i>L. interrogans</i>	Pomona	Pomona	Pomona
<i>L. Santarosai</i>	Shermani	1342 K	Shermani
<i>L. inadai</i>	Kaup	LT 64 - 68	Tarassovi
<i>L. kirschneri</i>	Grippotyphosa	MoskvaV	Grippotyphosa
<i>L. fainei</i>	Hurstbridge	BUT 6	Hurstbridge
<i>L. borgpetersenii</i>	Javanica	Poi	Javanica
<i>L. noguchii</i>	Panama	CZ 214 K	Panama
<i>L. interrogan</i>	Djasiman	Djasiman	Djasiman
<i>L. interrogan</i>	Copenhageni	M 20	Icterohaemorrhagiae
<i>L. interrogan</i>	Bataviae	Swart	Bataviae

Original Source: WHO reference Laboratory-ICMR-RMRC, Port Blair, Andaman & Nicobar Island, India

Enzyme Linked Immuno-Sorbent Assay

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ICAR-NIVEDI, Yelahanka, Bengaluru

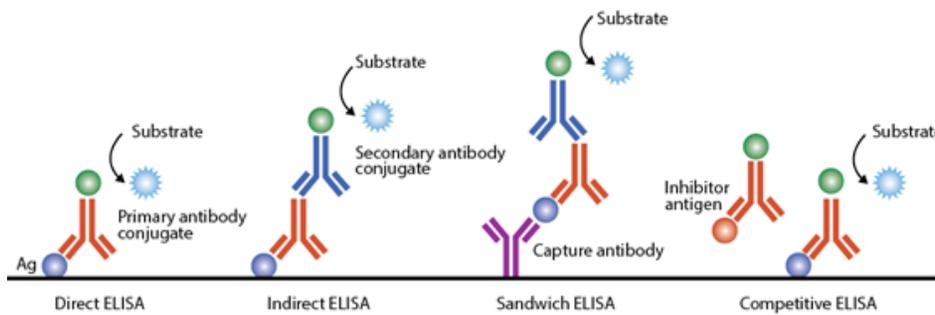
The immunological gold standard for diagnosis of leptospirosis is the MAT. However, this test involves the cumbersome procedure of reacting serum with different panels of live leptospira antigens. MAT is not specific for IgM, and detects both IgM and IgG, and may not be able to differentiate acute from previous infection. Thus ideally, the MAT requires two samples (acute and convalescent) for confirmation. In a clinical setting where rapid decision making is necessary, MAT is not the ideal test to go for diagnostic confirmation. Other immunological tests available include IgM ELISA, agglutination test, Lepto-dipstick, Lepto Dri Dot, and Leptocheck-WB test. These allow rapid diagnosis, and are simpler to perform than MAT. Still, all these tests can be negative in early leptospirosis as it takes time for antibodies to form. The sensitivities and specificities of the tests vary depending on the antibodies present and the leptospira antigen used.

ELISA is the most commonly used diagnostic technique for detecting specific antibodies against *Leptospira*. ELISA have been developed using a wide variety of antigen preparations, from leptospiral sonicates to recombinant lipoproteins, immunoglobulin, outer membrane proteins, surface antigens etc., The antigen antibody reaction is visualized or measured by spectrophotometer/ ELISA reader using a conjugate (enzyme conjugated to anti-IgM or IgG) and a colour reagent. The assay obviates the need for maintenance of live cultures and is amenable to automation. However, sensitivity and specificity do not match those of the MAT, and reliance on ELISA alone is not recommended. ELISA can detect IgM antibodies earlier than MAT using single antigenic preparation. Heat stable antigens which are stable at room temperature for long periods can be used and it allows rapid processing of large number of samples. However, the test has some demerits like inability to assess infecting serovar, necessity of cut off value calibration, and significant titre requirement and comparatively less specific.

ELISA methodology has travelled a long path and undergone various changes such as different types of antigen preparations (starting from whole cell preparations to recombinant outer membrane proteins), assay protocols (direct, indirect, sandwich), assay platforms (plate tests, dipstick, dot strips tests). The specificity of the ELISA depends on the antigen used for the assay. IgM ELISAs are useful in the diagnosis of acute infection. The available recombinant OMP-based ELISAs are broadly reactive to antibodies to all pathogenic leptospire and so are of no value in epidemiological investigations. In contrast, lipopolysaccharide antigen-based ELISAs are serogroup specific and have value in epidemiological investigations and control schemes. In case of animals, ELISAs have also been developed for use in milk from individual cows or in bulk tank milk for the detection of serovar Hardjo antibodies. These tests have been helpful in identifying Hardjo infected herds and in serovar Hardjo control/eradication programmes (Pritchard, 2001). However, herds that are vaccinated against serovar Hardjo will also be positive in these various ELISAs, decreasing their usefulness in regions where vaccination is a routine practice. OMP based tests are not yet widely available and may have a role in the diagnosis of incidental infections but for host maintained infections, such as serovar



Hardjo, outer envelope lipopolysaccharide antigens produce strong immune response (Ellis et al., 2000). ELISA can detect 10^5 leptospire/ml.

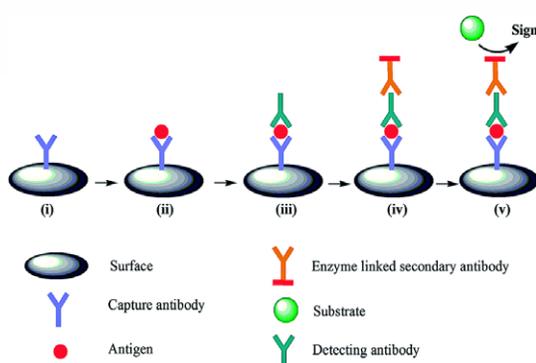


Diagnosis of Bovine leptospirosis by *Linnodee Leptospira Hardjo* ELISA

An animal may undergo two types of Leptospiral infection in its life either host-adapted infection or incidental infection. In case of host-adapted serovar infection, clinical manifestation is less severe than incidental infections and the manifestations varies with the age of the animal also. Cattle are the maintenance hosts for *Leptospira Hardjo*. *L. Hardjo-bovis*, infection will often produce a carrier state in the kidneys associated with long-term urinary shedding. Lactating cows produce less milk, and, for a week or more, the milk they produce is thick and yellow. Low antibody titers are typically associated with hardjo-bovis infections, making detection and diagnosis difficult. Linnodee Bovine *Leptospira* ELISA kit is designed to detect the antibody response to a lipopolysaccharide (LPS) outer envelope epitope common to both *L.borgpetersenii* serovar Hardjo (HB) and *L.interrogans* serovar Hardjo (HP) in either sera or milk (bulk tank or individual animals).

Principle

This is a Double antibody Sandwich ELISA, in which ELISA plates are pre coated with specific MAb against Hardjo LPS. Hardjo antigen is bound to the wells which reacts with the Hardjo specific antibodies in the bovine milk/sera. Immobilised Hardjo antibodies are then detected by a monoclonal antibody linked to enzyme (HRP). This is quantified by measuring the amount of labelled detection antibody bound to the matrix using a chromogenic substrate (TMB-E) in spectrophotometer at an wavelength (450nm) with or without optical correction (620-700nm).



Materials required but not provided in the Kit

- Distilled water
- Adjustable single and multichannel micropipettes
- Single-use micropipette tips
- Incubator at 37°C, with or without shaker
- ELISA 96-well microplate reader equipped with 450nm filter (preferably with 630nm reference filter).
- Container for dilution of wash buffer solution
- Microtitre plate
- Plate sealers, universals, reservoirs, foil.

Materials provided in the kit

- Pre-coated ELISA plates
- Wash Buffer (20 X concentrated)
- Sample Diluent *
- Positive Milk control*
- Negative Milk control*
- Positive Sera control*
- Negative Sera control*
- Substrate (TMB)*
- Stop reagent (1M H₂SO₄)*
- Peroxidase conjugated antibody (1000 x concentrate) Ready to use



Preparation

- Determine the total number of samples and controls to be tested. **At least 3 replicates of each control (milk and/or sera) are recommended for each test run.** For the strip kit, remove any strips not required from the frame and store in the resealable bag provided at 4°C.
- Crystals in the concentrated wash buffer can be dissolved by heating to 37°C. Dilute the wash buffer 1:20 in distilled water.
- Dilute the concentrated conjugate 1:1000 in diluted wash buffer just before use. The diluted conjugate is light sensitive and should be protected from exposure to light.

Procedure

- Dilute sera sample and control sera samples in sample diluents in the ratio of 1:50 in a dummy plate. / Take undiluted 150µl milk sample in dummy plate.



- Mix the contents thoroughly in dummy plates and transfer 100µl of samples to test wells.
- Incubate the wells at 37°C for 40 minutes with shaking (1 hour without shaking). Cover the wells with a plate sealer.
- Wash the test wells with diluted wash buffer at least 200µl/well for four times. Following final wash, remove residual wash buffer by inverting the plate and blotting firmly on absorbent paper.
- Add 100ul of the diluted conjugate to each well.
- Incubate in the dark at 37°C for 30 min. with shaking (40 min. without shaking). Cover the wells with a plate sealer.
- Wash the wells with wash buffer four times. (Follow the step 4)
- Add 100ul of substrate to each well.
- Incubate the plate at room temperature in the dark for 10 min.
- Add 50ul of stop solution to each well at the same order as the substrate was added.
- Read at a wavelength of 450nm or with a corrected OD using a reference filter (630nm).

Calculations for Milk & Sera samples

S/P ratio (Sample value related to Positive Control value) can be calculated using the following formula

The S/P ratio must be calculated using the respective controls for the sample type.

Interpretation of results

$\frac{\text{Mean Sample OD} - \text{Mean Negative control OD}}{\text{Mean Positive control OD} - \text{Mean Negative control OD}}$

A. In case of Bulk milk samples

Result	If the ratio is:	Interpretation
Negative	≤ 0.03	Naive and/or unvaccinated
Low Positive	$\geq 0.04 \leq 0.10$	A few seropositive cows
Medium Positive	$>0.10 \leq 0.50$	Mix of infected and susceptible animals
High Positive	>0.50	Heavily infected/vaccinated

B. In case of individual animal milk / sera

Result	If the ratio is:	Interpretation
Negative	Sera ≤ 0.05 Milk ≤ 0.03	Naive and/or unvaccinated
Inconclusive	Sera $\geq 0.06 \leq 0.12$ Milk $\geq 0.04 \leq 0.10$	A retest is recommended after a few weeks. If the sample is inconclusive after retest, the animal can be considered negative depending on the herd history
Positive	Sera > 0.12 Milk > 0.10	Exposed to infection/vaccinated

Human is the accidental host in leptospirosis and gets infection through contact with infected urine or from infected animals or water and soils contaminated with infected animal urine. The routes of entry are cuts or abrasions of the skin, or through mucosal membranes. Leptospire will not get transmitted from human to human. IgM antibodies appear as early as 3 days after infection and may persist for up to 5 months, may persist for years or life term. Detection of specific antibody of the IgM class to leptospira genus specific antigen by ELISA is a valuable screening procedure for the diagnosis of acute infection. It is an acceptable alternative to genus specific complement fixation and agglutination tests. The presence of a significant or rising level of IgM is considered presumptive evidence of active leptospira infection.

The Panbio Leptospira IgM ELISA has been demonstrated to detect infections caused by a number of *L. interrogans* serovars including: Hardjo, Pomona, Copenhageni, Australis, Madanesis, Kremastos, Nokolaevo, Celledoni, Canicola, Grippotyphosa, Szwajizak, Djasiman and Tarassovi. This ELISA is for the qualitative detection of IgM antibodies to leptospira in serum as an aid in the clinical laboratory diagnosis of patients with clinical symptoms consistent with leptospirosis.

Principle

Serum containing antibodies to leptospira antigen, when present, combine with leptospira antigen attached to the polystyrene surface of the microwells. Residual serum is removed by washing and peroxidase conjugated anti-human IgM is added. The microwells are washed and a colourless substrate system, tetramethylbenzidine / hydrogen peroxide (TMB Chromogen) is added. The substrate is hydrolysed by the enzyme and the chromogen changes to a blue colour. After stopping the reaction with acid, the TMB becomes yellow. Colour development is indicative of the presence of IgM antibodies to leptospira in the test sample.

Specimen Collection and Preparation

Blood obtained by venipuncture should be allowed to clot at room temperature (20-25°C) and then centrifuged according to the Clinical and Laboratory Standards Institute (CLSI) (Approved Standard - Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture, H3).



The serum should be separated as soon as possible and refrigerated (2-8°C) or stored frozen ($\leq -20^{\circ}\text{C}$) if not tested within two days. Self- defrosting freezers are not recommended for storage. The use of icteric sera or sera exhibiting haemolysis, lipaemia or microbial growth is not recommended. The CLSI provides recommendations for storing blood specimens, (Approved Standard - Procedures for the Handling and Processing of Blood Specimens, H18).

Procedure

- First dilute 10 μL serum in 90 μL of Sample diluents.
- Take 20 μL of the diluted serum and add 180 μL Sample Diluent. Mix well. (Alternatively, 10 μL serum added to 1000 μL of Sample Diluent. Mix well,)
- In the same way, dilute Negative control (N), Reactive control (R), and Calibrator (CAL) (in triplicates). Mix well.
- Load 100 μL of diluted patient samples, Controls and calibrator into their respective microwells. (Microwells are in the form of removal strips)
- Cover plate and incubate for 30 minutes at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- Wash the wells with diluted Wash Buffer for six (6) times (refer to
- Pipette 100 μL HRP Conjugated Anti-human IgM into the wells.
- Cover plate and incubate for 30 minutes at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- Wash the wells with diluted Wash Buffer for six (6) times
- Pipette 100 μL TMB into each well.
- Incubate for 10 minutes at RT ($20-25^{\circ}\text{C}$), from the time of addition
- Wait for the colour development. A blue colour will develop.
- Add 100ul of Stop solution to all the wells in the same sequence as TMB addition .Mix well .The blue colour changes to yellow
- Take the absorbance of each well within 30 min. Within 30 minutes read the absorbance of each well at a wavelength of 450 nm with a reference filter of 600-650 nm.

Interpretation of Results

Index	Panbio units	Result
<0.9	<9	Negative
0.9 – 1.1	9 – 11	Equivocal
>1.1	>11	Positive

Note: If a dual wavelength spectrophotometer is available, set the reference filter between 600-650 nm. Reading the microwells at 450 nm without a reference filter may result in higher absorbance values due to background.

Calculations

Index Value = Sample Absorbance/Cut-off Value

Example: Sample A Absorbance = 0.94

Sample B Absorbance = 0.070

Mean absorbance of Calibrator = 0.802

Calibration Factor = 0.62

Cut-off Value = $0.802 \times 0.62 = 0.497$

Sample A $(0.949/0.497) = 1.91$ index value

Sample B $(0.070/0.497) = 0.14$ index value

Panbio Units = Index Value X 10

Sample A $1.91 \times 10 = 19.1$ Panbio Units

Sample B $0.14 \times 10 = 1.4$ Panbio Units

Index Value	= Sample Absorbance
	Cut-off Value
Example:	Sample A Absorbance = 0.949
	Sample B Absorbance = 0.070

Mean absorbance of Calibrator = 0.802

Calibration Factor = 0.62

Cut-off Value = $0.802 \times 0.62 = 0.497$

Sample A $(0.949/0.497) = 1.91$ Index value

Sample B $(0.070/0.497) = 0.14$ Index value

Panbio Units = Index Value X 10

Sample A $1.91 \times 10 = 19.1$ Panbio Units

Sample B $0.14 \times 10 = 1.4$ Panbio Units

Note: The Calibration factor is batch specific calibration factor value before commencing calculations

- Calculate the average absorbance of the triplicates of the Calibrator and multiply by the calibration factor. This is the Cut-off Value.
- An index value can be calculated by dividing the samples absorbance by the Cut-Off Value (calculated in step (1) above.)
- Alternatively, Panbio Units can be calculated by multiplying the index value (Calculated in step (2) above) by 10.



Interpretation of diagnostic tests

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Diagnosis plays a vital role in establishing decisions such as whether to treat (or implement a program) or to do nothing or to wait in addition to the importance of the disease. The tools for the veterinarian in arriving diagnosis include experience, factual knowledge, intuition as well as diagnostic tests. The outcome of the diagnostic process is a statement as to whether an animal is considered normal or not normal. This could relate to disease or infection status as well as to productive performance or quality of life from an animal welfare perspective. In this chapter we will see how a diagnostic test is evaluated and how it can be used to interpret in field situations.

Diagnostic tests

A diagnostic test is any device or process designed to detect, or quantify a sign, substance, tissue change, or body response in an animal or human being. There is one more category called screening test which are used on clinically healthy animals. The diagnostic test is a more or less objective method for reducing diagnostic uncertainty. The outcome of the diagnostic process often is interpreted as a dichotomous variable as well, such as the animal having or not having the disease. But if the diagnostic device measures on a continuous scale, such as serum antibody levels or somatic cell counts, a cut-off value has to be determined so that the result can be condensed into a dichotomous scale. The problem with any cut-off point is that it is likely to result in overlap between healthy and diseased individuals with regard to test results. It is desirable to quantify the relationship between diagnostic test result and “true” disease status so that the clinician can take account of this uncertainty when interpreting test results. The usefulness of diagnostic tests, that is their ability to detect an animal with disease or exclude an animal without disease, is usually described by terms such as sensitivity, specificity, positive predictive value and negative predictive value (NPV).

To work out how well a diagnostic test performs, we need to compare it with a ‘gold standard.’ A gold standard is a test or procedure that is absolutely accurate. It diagnoses all diseased animals that are tested and misdiagnoses none. Once samples are tested using the gold standard and the test to be evaluated, a 2×2 table can be constructed, allowing test performance to be quantified.

Table 1 Contingency table showing disease and test details for evaluation of the test

Test	Disease		Total
	Positive	Negative	
Positive	(a)	(b)	(a+b)
Negative	(c)	(d)	(c+d)
Total	(a+c)	(b+d)	(a+b+c+d)

Sensitivity

The sensitivity of a test is the probability of the test to generate positive results among animals that actually possess the disease.

$$\begin{aligned}\text{Sensitivity} &= \text{No. of true positives} / (\text{No. of true positives} + \text{No. of false negatives}) \\ &= a / (a + c)\end{aligned}$$

A test with a high sensitivity is useful for ‘ruling out’ a disease if an animal tests negative. The mnemonic SnNout (highSensitivity, Negative test=rule out) is a useful way of remembering this principle.

Specificity

The specificity of the test is the probability of a test to generate negative results among animals that are genuinely free of the disease.

$$\begin{aligned}\text{Specificity} &= \text{No. of true negatives} / (\text{No. of true negatives} + \text{No. of false positives}) \\ &= d / (b + d)\end{aligned}$$

A test with a high specificity is useful for ‘ruling in’ a disease if an animal tests positive. The mnemonic for remembering this is SpPin (high Specificity, Positive test, rule in)

Positive predictive value

It refers to the proportion of animals actually with the disease among all of the animals with positive test results. It answers the question: “If the test result is positive what is the probability that the animal actually has the disease?”

$$\begin{aligned}\text{Positive predictive value} &= \text{No. of true positives} / (\text{No. of true positives} + \text{No. of false positives}) \\ &= a / (a + b)\end{aligned}$$

If the prevalence of the disease is high, the predictive value of a positive test will also be high, but a good test should have a high predictive value even though the prevalence of the disease is low. Also a large difference in sensitivity makes a small difference in the predictive value of a positive test and that a small difference in specificity makes a big difference in the predictive value of a positive test. This means that the characteristic of a screening test described by specificity is more important in determining the predictive value of a positive test than is sensitivity.

Negative predictive value

It refers to the proportion of animals free of the disease among all of the animals with negative test results. It answers the question: “If the test result is negative what is the probability that the animal does not have disease?”

$$\begin{aligned}\text{Negative predictive value} &= \text{No. of true negatives} / (\text{No. of false negatives} + \text{No. of true negatives}) \\ &= d / (c + d)\end{aligned}$$



Likelihood ratios

The dependence of predictive values on prevalence is a major disadvantage when a summary measure of a test's performance, when the test is applied in a population, is required. The likelihood ratio provides a suitable summary measure, which is independent of prevalence. It compares the proportion of animals with and without disease, in relation to their test results. The likelihood ratio of a positive test result (LR+) is the ratio of the proportion of affected individuals that test positive, and the proportion of healthy individuals that test positive.

$$LR+ = [a / (a + c)] / [b / (b + d)]$$

The LR+ is therefore a quantitative indication of the strength of a positive result. The perfect diagnostic test would have an LR+ equal to infinity (detecting all true positives, and generating no false positives), and the best test for ruling in a disease is therefore the one with the highest LR+

The likelihood ratio of a negative test result (LR-) is the ratio of the proportion of affected individuals that test negative, and healthy individuals that test negative; that is:

$$LR- = [c / (a + c)] / [d / (b + d)]$$

The perfect diagnostic test would have an LR- equal to zero (producing no false negatives, but detecting all true negatives), and the best test for ruling out a disease is therefore the one with the lowest LR-.

Likelihood ratios if used in combination with the initial expectation of the probability that an animal has a certain condition (= pre-test probability), a revised estimate of the overall probability of the condition (= post-test probability) can be calculated.

General rules

- Sensitivity and specificity are generally independent of prevalence.
- If the prevalence increases, positive predictive value increases and negative predictive value decreases.
- If the prevalence decreases, positive predictive value decreases and negative predictive value increases.
- The more sensitive a test, the better its negative predictive value.
- The more specific a test, the better its positive predictive value.

Prevalence estimation

Tests produce false negatives and false positives, therefore any diagnostic test can only produce an estimate of the apparent prevalence. The apparent prevalence is the proportion of all animals that give a positive test result. It can be more than, less than or equal to the true prevalence. Estimates of the true prevalence can be obtained taking account of test sensitivity and specificity using the formula

$$\text{True prevalence} = [\text{Apparent prevalence} + (\text{Specificity}-1)] / [\text{Specificity} + (\text{Sensitivity}-1)]$$

Diagnostic strategies

Clinicians commonly perform multiple tests to increase their confidence. Multiple test results can be interpreted in parallel or series.

1. Parallel – the tests are performed at the same time and interpreted together.

Table 2. Parallel testing

Test A	Test B	Diagnosis
(-)	(-)	Negative
(+)	(-)	Positive
(-)	(+)	Positive

When two tests are used simultaneously, disease positives are defined as those who test positive by either one test or by both tests and disease negatives are defined as those who test negative by both tests. When two (or more) tests are conducted in parallel, the goal is to maximize the probability that subjects with the disease (true positives) are identified (increase sensitivity). Consequently, more false positives are also identified (decrease specificity).

2. Serial – the tests are performed sequentially. The results of the first test usually determine whether the second test is still necessary or not. Only the positive cases are retested.

Table 3. Serial testing

Test A	Test B	Diagnosis
(+)	(-)	Negative
(-)	(+)	Negative
(+)	(+)	Positive

In parallel testing, there is a net gain in sensitivity but a net loss in specificity, when compared to either of the tests used but in serial testing, there is a net loss in sensitivity but a net gain in specificity, compared to either of the tests used.

Screening and confirmatory testing

With a strategy of screening and confirmatory testing, as it is often used in a disease control scheme, the screening test is applied to every animal in the population to search for test-positives. This test should be easy to apply at a low cost. It has to be a highly sensitive test so that it misses only a small number of diseased or infected animals. Its specificity should also be reasonable, so that the number of false positives subjected to the confirmatory test remains economically justifiable. Negative reactions to the screening test are considered definitive negatives, and are not submitted to any further tests. Any positive screening test result is subjected to a confirmatory test. This test can



require more technical expertise and more sophisticated equipment, and may be more expensive, because it is only applied to a reduced number of samples. But it has to be highly specific, and any positive reaction to the confirmatory test is considered a definitive positive.

Accuracy and Precision

The accuracy of a test relates to its ability to give a true measure of the substance being measured. To be accurate, a test need not always be close to the true value, but if repeat tests are run, the average of the results should be close to the true value. The precision of a test relates to how consistent the results of the test are. If a test always gives the same value for a sample (regardless of whether or not it is the correct value), it is said to be precise.

Reliability

The value of a diagnostic test is also judged by its reliability, that is, the extent to which its results are stable. This can be explored by running the test two or more times on the same samples in the same laboratory under the same conditions, and assessing the repeatability of the results. Tests that are used in several laboratories (e.g., those that are recognized as international standards) also require their reproducibility to be determined.

Also, the characteristics of each disease should be known before either a testing strategy is developed or the results can be meaningfully interpreted.

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Point of care diagnostics for leptospirosis

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The definite diagnosis of leptospirosis is made by isolation of the organism from blood samples or other clinical specimens. However, the sensitivity of this technique strongly varies with the stage of illness. Furthermore, culture does not provide a rapid result and many laboratories in endemic areas do not have culture facilities. Therefore, the diagnosis often relies on serologic testing. The gold standard serological test is Micro agglutination Test (MAT) and other serological tests include enzyme-linked immune sorbent assay (ELISA). However these tests require a laboratory set up and difficult to carry out at on field situations. Diagnostics available at field level will aid in rapid diagnosis. Such diagnostics are called Point of care diagnostics.

Point-of-care testing (POCT) is defined as medical testing at or near the site of animal or patient care. It is also called bedside or bedside test. The driving notion behind POCT is to bring the test conveniently and immediately to the patient. This increases the likelihood that the patient, physician, and care team will receive the results quicker, which allows for immediate clinical management decisions to be made. POCT is often accomplished through the use of transportable, portable, and handheld instruments and also through technologies such as Lateral flow assays.

Point of care testing for leptospirosis is much useful to the veterinarians when handling aborted animals for taking extra precautions. Not all physicians in areas that are endemic for leptospirosis have access to laboratory facilities, and in these problematic situations a rapid field test with a high sensitivity and specificity are required.

Because of the complexity of the MAT, rapid screening tests for leptospiral antibodies in acute infection have been developed. Screening tests use broad reacting genus-specific antigens to detect the patient's immune response to the infecting leptospire. Since the prevalence of leptospiral serogroups varies geographically, antigenic characteristics of the pathogen causing infection may vary from one location to another. The sensitivity of the screening test in any given setting therefore, depends on the ability of test antigens to detect antibodies produced against the site-specific leptospiral serovars.

Microcapsule agglutination test (MCAT), LEPTO Dipstick, Macroscopic slide agglutination test (Macroscopic SAT), LEPTO Lateral flow, Indirect haemagglutination assay (IHA) and LEPTO Dri Dot are some of the rapid diagnostic tests used for screening. LEPTO Dipstick and LEPTO Lateral flow are IgM immunoassays whereas LEPTO Dri Dot is a latex agglutination test. The principle of MCAT is similar to that of latex agglutination assay.

Generally, the sensitivity of these tests is usually low during the first week of illness, then increases to a peak by 10-12 days or during the second week of the disease. IgM antibodies become detectable during the first week of illness allowing the diagnosis to be confirmed and treatment initiated while it is likely to be most effective. Antibody levels are generally low or absent during the first 3 days of illness. IgM detection has been shown to be more sensitive than MAT when the first specimen is taken early in the acute phase of the illness.



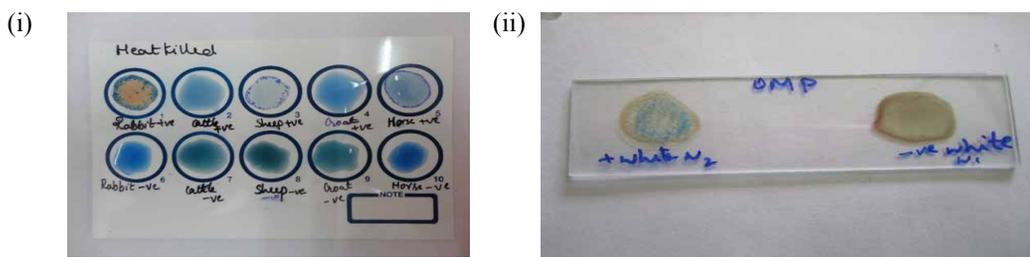
It is easy to perform and read and results can be obtained within 30 seconds to 15 minutes. It does not require any special expertise or equipment. The reagents have long shelf lives even at room temperature and it can be performed where electric supply is erratic or absent. But all these rapid tests are costly and not a confirmatory test.

Latex agglutination test

Latex Agglutination Test is an agglutination immuno assay for the detection of leptospira specific antibodies. The co`loured latex beads are sensitized with the broadly reactive and specific antigen (recombinant antigen/OMP antigen/ heat killed antigen) from leptospire and suspended in storage buffer. When the specimen is mixed with suspended latex, antibodies present in the specimen interact with the antigen that is coated on the surface of the latex particles leading to the formation of fine and clearly visible granular agglutination.

Protocol

The vial containing colored latex beads along with antigen was shaken well and 20 ul of latex beads were transferred on a slide. 20 ul of serum was added near the latex. Serum was mixed thoroughly with the latex beads and spread uniformly in circular fashion. The slide was rotated slowly and gently. The results were recorded in 3 minutes. (Fig.)



LAT (i) using heat killed leptospiral antigen in rabbit, cattle, sheep, goat and horse positive and negative serum and (ii) using OMP leptospiral antigen in rabbit hyper immune and negative serum

Fine clearly visible agglutination occurs within 3 minutes. The intensity of the agglutination depends on concentration of the antibodies in a serum sample. Clearly visible granular agglutination indicates the presence of specific antibodies to leptospire. In stronger reactions fine granular clumps tend to settle at the edge of the circle. Agglutination that occurs beyond 3 minutes may be due to evaporation and should not be considered.

LEPTO-LAT

Lepto–LAT is a latex agglutination test for detection of antibodies to leptospire in body fluids.

Materials provided

- Sensitized latex beads in suspension
- A positive control
- A negative control
- Plastic sticks for mixing

Materials required

- Clean glass slides (Please do not use already used ones)
- Micro pipette (5 µl – 10 µl)
- Micropipette tips

Principle

Lepto LAT is a agglutination immuno assay for the detection of leptospira specific antibodies. The blue coloured latex beads are sensitized with the broadly reactive and specific antigen prepared from pathogenic leptospire and suspended in storage buffer. When the specimen is mixed with suspended latex, antibodies present in the specimen interact with the antigen that is coated on the surface of the latex particles leading to the formation of fine and clearly visible granular agglutination within 60 seconds.

Procedure

- Shake well the vial containing coloured latex beads.
- Transfer 10 ul of latex beads on a slide.
- Add 5 µl of serum near to the latex.
- Mix the serum thoroughly with the latex beads and spread uniformly in circular fashion. (The diameter of the circle should not be more than 1 cm).
- Rotate the slide slowly and gently.
- Record the results within 60 seconds

Interpretation of the test results

Fine clearly visible agglutination occurs within 30-60 seconds. The intensity of the agglutination depends on concentration of the antibodies in a serum sample. Clearly visible granular agglutination indicates the presence of specific antibodies to leptospire. In stronger reactions, fine granular clumps tend to settle at the edge of the circle. Agglutination that occurs beyond 60 seconds may be due to evaporation and should not be considered.

Storage

The coated latex particles are stable up to one year at 4°C– 8°C but can be stored for up to six months at room temperature (32–37°C).

Advantages

- Simple to perform and easy to read and doesn't require any special equipment or expertise
- The Lepto-LAT have long shelf life even at room temperature

Cost effective.

Disadvantages

- Results need to be confirmed.

Leptorapidae- (Linnodee, Ireland kit)

A Simple, Rapid, one step for detection of the presence of Leptospira-specific antibody in Human sera by LAT

- Simple to perform



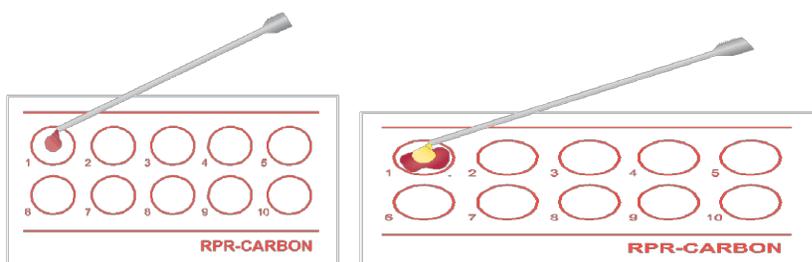


- No requirement for laboratory equipment
- Rapid results in less than 3mins
- Highly sensitive and specific
- Cost-effective

Kit Contents: Leptorapidae Leptospira bead suspension; Dispensing pipettes; Agglutination cards

Procedure:

- Using a dispensing pipette apply 5µl of Leptorapidae to the agglutination card
- Using a new dispensing pipette add 5µl of test sera to the 5µl of Leptorapidae and mix
- Rock agglutination card gently for 2-3minutes and interpret the score card

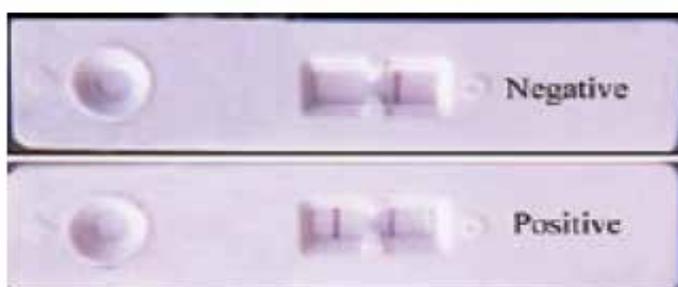


Interpretation:

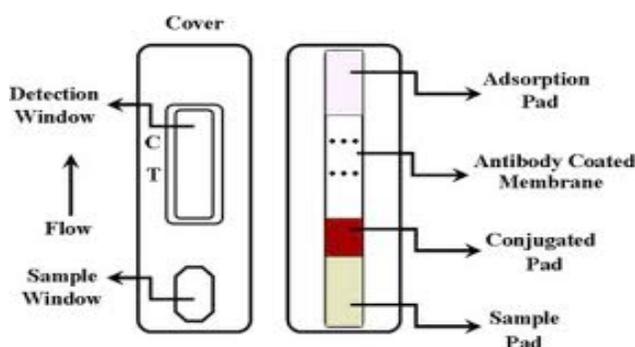
- A positive / negative result will appear within 3minutes of mixing.
- Score extent to agglutination according to the scale

Lateral Flow Assay

Lateral flow assays employ interplay of antibodies and antigens, to diagnose a host of physiological conditions. The LFA device for the serodiagnosis of leptospirosis consists of a porous nitrocellulose detection strip flanked at one end by a reagent pad and at the other end by an absorption pad. A sample application pad flanks the reagent pad in turn. The composite strip is contained in a plastic assay device with a round sample well positioned above the sample application pad and a test result window positioned above the detection zone of the strip. The detection zone contains two distinct lines, a test line and a control line. The test line is done by spraying *Leptospira* antigen/OMP onto the nitrocellulose strip and the control line by spraying immunoglobulin G (IgG) antibodies. A detection reagent is prepared by conjugating affinity purified antibodies against species (in which to be tested) Ig (H+L) antibodies to 40 nm colloidal gold particles and this conjugate are sprayed onto the conjugate pad of the composite strip. Tests are performed by the addition of 5 µl serum to the sample pad of the assay device followed by the



addition of 130 μ l sterile running fluid consisting of phosphate-buffered saline, pH 7.6, containing 1.67% bovine serum albumin and 3% Tween 20. Test results read after 10 min by visual inspection for staining of the antigen and control lines. Tests are scored negative when no staining is observed at the test line and scored positive when the test line stains. The control line should stain in all cases. The test line may stain at different intensities, and positive results may be subjectively rated 1+ when staining is weak, 2+ when staining is moderately strong, 3+ when staining is strong, and 4+ when staining is very strong. Devices sealed in a humidity resistant foil and containing silica may be stored at 4–27°C without loss of activity. The stain of exposed tests is stable after drying.



Lepto lateral flow is based on the binding of specific IgM antibodies to the broadly reactive heat extracted antigen prepared from non-pathogenic Patoc 1 strain. Ig M antibodies bound to the broadly reactive antigen are detected with an anti-human Ig M gold conjugate contained within the test device.

Materials Required: Lepto lateral flow device; Sample fluid (diluent), Micropipettes (10 μ l); Disposable pipette tips

Procedure

- Add five ml of undiluted serum or 10 μ l of whole blood added to the sample application.
- Add 130 μ l of sample fluid (diluent).
- Wait for up to 15 min.

Reading results

- If only the control band became stained, the test is negative.
- If both test and control bands became stained, the test is considered as positive.

Advantages: Very quick. Both serum as well as blood can be used **Disadvantages:** Expensive.

Leptocheck-Rapid test for IgM antibodies to Leptospira (Zephyr Biomedicals)

Leptocheck is a rapid, qualitative, sandwich immunoassay for the detection of Leptospira specific IgM antibodies in human serum/plasma or whole blood specimen. It is useful for the serodiagnosis of current or recent leptospirosis. The broadly reactive genus specific antigen employed in the test allows the detection of Leptospira infections caused by a wide range of strains of different



serovars. It qualitatively detects the presence of IgM class of *Leptospira* specific antibodies in human serum/plasma or whole blood specimen.

Principle: utilizes the principle of immunochromatography, a unique two-site immunoassay on a membrane. As the test sample flows through the membrane assembly of the test device, the anti-human IgM -colloidal gold conjugate forms a complex with IgM antibodies in the sample. This complex moves further on the membrane to the test window 'T' where it is immobilized by the broadly reactive *Leptospira* genus specific antigens coated on the membrane, leading to the formation of a red to deep purple coloured band at the test region 'T' which confirms a positive test result. Absence of this coloured band in test region 'T' indicates a negative test result. The unreacted conjugate and the unbound complex if any, along with rabbit globulin colloidal gold conjugate move further on the membrane and are subsequently immobilized by the anti-rabbit antiserum coated at the control region 'C' of the membrane assembly, forming a red to deep purple coloured band. The control band serves to validate the test results.

Kit Contents:

- Device: Membrane test assembly pre-dispensed with Anti Human IgM -colloidal gold conjugate, Rabbit globulin Colloidal gold conjugate, *Leptospira* genus specific antigens at test window 'T' and anti-rabbit antiserum pre-dispensed at the control window 'C'.
- Desiccant pouch.
- Pipette: Disposable 5µl Sample loop.
- Buffer: 0.1 M Tris buffer with 0.1% sodium azide.

Specimen Collection and Preparation:

- Fresh serum / plasma is preferable. Anticoagulated whole blood can also be used as specimen. Serum / plasma may be stored at 2-8°C up to 24 hours in case of delay in testing.
- For long-term storage, freeze the specimen at -20°C for 3 months or -70°C for longer periods. Whole blood should be used immediately and should not be frozen.
- Do not use haemolysed, clotted, contaminated, viscous/turbid specimen.
- Specimen containing precipitates or particulate matter must be centrifuged and the clear supernatant only should be used for testing.

Testing Procedure

- Bring kit components to room temperature before testing.
- Add 10µl of serum/ plasma or whole blood with a micropipette into the sample port 'A', OR using the 5µl sample loop.
- Provided with the kit, dip the loop into the sample and then blot into the sample port 'A'.
- Repeat this step twice for each sample. Ensure that the loop does not retrieve clots or debris from the sample.
- Immediately dispense 5 drops of sample running buffer in the buffer port 'B', by holding the

plastic dropper bottle vertically.

- At the end of **15 minutes**, read the results as follows:

Interpretation



Negative: Only one coloured band appears in the control window ‘C’.

Positive: In addition to the control band, another red to deep purple coloured band appears in the test window ‘T’

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Molecular Diagnosis of Leptospirosis

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Polymerase chain reaction (PCR)-based assays are now used in some diagnostic and many reference laboratories for the detection of leptospires in clinical samples of animals and humans. A variety of primer sets for the conduct of PCR assays have been described. However, only two PCR assays have been extensively evaluated and gained widespread use for diagnosis. One is the genus-specific assay which amplifies DNA from both pathogenic and non-pathogenic serovars and the another is the approach described by Gravekamp et al. (1993) and evaluated by Brown et al. (1995), requires two sets of primers in order to detect all leptospira species containing pathogens. Improved sensitivity has been achieved by quantitative- PCR either using TaqMan probes or SYBR green fluorescence. Quality control of PCR assays used for diagnosis of leptospirosis requires careful attention to laboratory design and workflow to prevent contamination of reagents, and use of appropriate control samples. This assay, gives relatively quick results in the early stage of the disease when antibodies are not yet developed at detectable level, but it has some demerits like need of sophisticated equipment and skilled/ technical expertise, complicated and expensive, etc.

Identification of isolates to serovar level is an essential step to understand the epidemiology of the disease in both humans and animals in any geographic region. However, serovar identification remains a relatively blunt tool with which to investigate fine details of epidemiology. Because of the difficulties associated with serological identification of leptospiral isolates, there has been great interest in molecular methods for identification and subtyping. Molecular tools employed for the classification of *Leptospira* spp. include pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), arbitrarily primed PCR, fluorescent amplified fragment length polymorphism (FAFLP) and variable number tandem repeat (VNTR). However, these techniques lack reproducibility or have low sensitivity or specificity.

Detection of Leptospiral DNA from clinical material by various methods has been developed. Dot-Blotting, In-situ Hybridization were used before the age of PCR and the sensitivity of radio labelled probes was lower approx. 10^3 leptospires /ml. Polymerase chain reaction (PCR) based assays are increasingly used for the detection of leptospires in tissues and body fluids of humans and animals because of their perceived sensitivity and capacity to give an early diagnosis. Real-time PCR is faster than regular PCR and less sensitive to contamination (Picardeau, 2013).

Nucleic Acid based detection assays are generally categorised into two:

- i) detection of universal genes like *gyrB*, *rrs* (16S r RNA gene), *sec Y* and
- ii) detection of genes restricted to pathogenic *Leptospira* like *lipL21*, *lipL32*, *lipL41*, *ligA* and *ligB*

Most of the primer pairs designed were evaluated for human diagnosis and very few are used for animal leptospira diagnosis and the most commonly used one is *lipL32* gene.

Advantages:

- PCR can rapidly confirm the diagnosis in the early phase of the disease, when bacteria may be present and before antibody titres are at detectable levels.
- Leptospiral genomo species can be differentiated by combining PCR and native PAGE staining
- Rapid detection of pathogenic leptospires.
- Distinguishes between pathogenic and non-pathogenic serovars.

Limitations:

- PCR assays cannot identify infecting serovar which has limited role in diagnosis but plays significant role in epidemiological and public health point of view.
- PCR requires special equipment and dedicated laboratory space, and also highly skilled personnel for operating the equipment.
- False-positive results in the presence of minute amounts of extraneous DNA that may contaminate working areas.
- False-negative results because inhibitors are present in the clinical materials that are being examined.

The validity of PCR data depends essentially on the quality controls included in the test. Rapid diagnosis of leptospirosis has not been evaluated worldwide as it is not yet widely used, particularly in tropical and subtropical countries.



Nucleic acid extraction from various clinical /cultured samples for diagnosis of Leptospirosis

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Nucleic acids are large biological molecules essential for all known forms of life. They include DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). Together with proteins, nucleic acids are the most important biological macromolecules; each is found in abundance in all living things, where they function in encoding, transmitting and expressing genetic information. DNA isolation is a routine procedure to collect DNA for subsequent molecular or forensic analysis. The test samples from which DNA can be extracted most commonly for leptospirosis diagnosis include tissues from neonates or aborted fetuses, milk, whole blood, serum, semen, body fluids, etc.,. Some samples are easily obtained from animals for DNA extraction, including milk and blood. Blood samples are often used in PCR-based diagnosis of human leptospirosis. However, inhibitors frequently affect PCR results. Washing the blood a few times with water or lysis buffer until all the hemoglobin disappears before extracting the DNA increases the PCR sensitivity substantially.

Principle

There are three basic and two optional steps in a DNA extraction:

1. Breaking the cells commonly referred to as cell disruption or cell lysis, to expose the DNA from the cells and achieved by grinding or sonicating the sample.
2. Removing membrane lipids by adding a detergent preferably SDS, anionic type.
3. Removing proteins by adding a protease (optional but almost always done).
4. Removing RNA by adding an RNase (often done).

5. Precipitating the DNA with an alcohol usually ice-cold ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a pellet upon centrifugation. This step also removes alcohol-soluble salt. Optionally, a chelating agent commonly EDTA to sequester divalent cations such as Mg^{2+} and Ca^{2+} , which prevents enzymes like DNase from degrading the DNA. Cellular and histone proteins bound to the DNA can be removed either by adding a protease or by having precipitated the proteins with sodium or ammonium acetate, or extracted them with a phenol-chloroform mixture prior to the DNA-precipitation.

DNA isolation from tissues

Materials required: Centrifuge, microcentrifuge tubes, Pestle and mortar, ice-water bath, $-20^{\circ}C$ deep freezer; Liquid nitrogen; Lysis buffer (10mM Tris HCl pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% SDS; 10mg/ml Proteinase K; 20 μ g/ml DNase free from RNase); 0.5M Tris-HCl saturated phenol and Ethanol Phenol:chloroform (1:1)Ethanol/acetate solution (95% ethanol/0.12 M sodium acetate); TE buffer (10 mM Tris-HCl, pH 7.6-8.0, 0.1 mM EDTA).

Procedure:

For fresh tissues (aborted/fetal contents) weighing ~1g should be initially reduced to powder form using liquid nitrogen in mortar and pestle before lysing the sample and homogenizing with Phenol. To get good amount of genomic DNA, they should be first lysed with lysis buffer properly

before homogenization with 0.5M Tris-HCl saturated phenol and Ethanol precipitation of DNA for elution of genomic DNA. Then an equal volume of buffer-saturated phenol:chloroform (1:1) to the DNA sample lysate is added and mixed well. Most DNA solutions can be vortexed for 10 sec except for high molecular weight DNA which should be gently rocked. It should be spinned in a microcentrifuge for 3 min and the aqueous layer should be carefully removed to a new tube, being careful to avoid the interface (these steps can be repeated until an interface is no longer visible). To remove traces of phenol, an equal volume of chloroform can be added to the aqueous layer. Then, 2.5 to 3 volumes of an ethanol/acetate solution is added to the DNA sample in a microcentrifuge tube, which is placed in an ice-water bath for at least 10 min. Frequently, this precipitation is performed by incubation at -20°C overnight. To recover the precipitated DNA, the tube is centrifuged, the supernatant is discarded, and the DNA pellet is rinsed with a more dilute ethanol solution. After a second centrifugation, the supernatant again is discarded, and the DNA pellet is dried. Dried DNA pellet is then dissolved in TE.

It is advisable to aliquot the DNA purified in large scale into several small (0.5 ml) microcentrifuge tubes for frozen storage to avoid repeated freezing and thawing. Isopropanol can also be used instead of ethanol. However, the precipitation efficiency of the isopropanol is higher making one volume enough for precipitation. However, isopropanol is less volatile than ethanol and needs more time to air-dry in the final step. The pellet might also adhere less tightly to the tube when using isopropanol.

DNA isolation from blood

Materials required: Centrifuge, microcentrifuge tubes, shaker incubator; Erythrocyte lysis solution (5 mM MgCl_2 , 320 mM sucrose, 12mM Tris HCL, 1% TritonX-100[pH 7.5]), Deionized water; Nucleic lysis buffer (60 mM NH_4Cl , 24mM Na_2EDTA [pH 8]); Sodium dodecyl sulfate (1%); Proteinase K 1mg/ml

NaCl (6M); Cold ethanol; Nuclease free water (NFW).

Procedure: A minimum of 2ml of blood collected in a citrated tube should be resuspended in 5ml erythrocyte lysis solution, mixed and centrifuged at 15,000Xg for 2min. Treatment with erythrocytes lysis solution should be repeated, followed by treatment with deionized water until the leukocyte pellets loss all reddish coloring. Template DNA is obtained from the leukocytes by adding 400 μl of nucleic lysis buffer, sodium dodecyl sulfate (1%)and proteinase K 1mg/ml, followed by mixing and incubation for 2h at 55°C or overnight at 37°C with slow shaking. A quantity of 100 μl of NaCl (6M) to be added, followed by centrifugation at 15,000Xg for 10 min. The supernatant containing total DNA is to be transferred to a fresh tube and then two volumes of cold ethanol is to be added and shaken until the DNA is precipitated, then pelleted and dissolved in 30 μl NFW.

In addition to above mentioned conventional method of DNA isolation, there are ample number of commercially available DNA extraction kits from many multinational companies to make the process simple, accurate and to get high quality pure DNA from diverse sources of human and animals. They are

- Delivers high-purity nucleic acids for use in most downstream applications
- Fast, inexpensive
- No silica-slurry carry over, no alcohol precipitation



Examples:

- Qiagen DNeasy Blood and tissue kit, Spin column based
- AuPrep Miniprep DNA extraction kit (Life Technologies),

Reference

Molecular Cloning: A Laboratory Manual (Third Edition) by Joseph Sambrook, Peter MacCallum Cancer Institute, Melbourne, Australia; David Russell, University of Texas Southwestern Medical Center, Dallas.

Isolation of genomic DNA from bacterial cultures by Qiagen QIAmp DNA Mini Kit

Materials required: Centrifuge, vortexer, water bath, microcentrifuge tubes, ethanol (96-100 %)

Qiagen QIAmp DNA Mini Kit includes QIAamp spin column, collection tubes; Buffer ATL Buffer AL; Proteinase K; Buffer AW1; Buffer AW2; Buffer AE

- Transfer one ml of bacterial culture into a 1.5 ml microcentrifuge tube and centrifuge for 5 min at 13,000 rpm.
- Discard the supernatant without disturbing the pellet or concentrate.
- To the pellet add 180 μ l of Buffer ATL and 20 μ l of Proteinase K, mix by vortexing and incubate at 56°C for 20 minutes (until the pellet is completely lysed).
- Briefly centrifuge the tube to remove the drops from the inside of the lid.
- Add 200 μ l of Buffer AL to the sample, mix by pulse-vortexing for 15 seconds and incubate further at 70°C for 10 min.
- Add 200 μ l ethanol (96-100 %) to the sample and mix by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the tube to remove the drops from inside the lid.
- Carefully apply the mixture (including the precipitate if any) to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 13,000 rpm for 1-2 min. Discard the filtrate from the collection tube.
- Add 500 μ l of Buffer AW1 without wetting the rim and centrifuge at 13,000 rpm for 1-2 min. Discard the filtrate from the collection tube.
- Add 500 μ l of Buffer AW2 without wetting the rim and centrifuge at 13,000 rpm for 2 min. Discard the collection tube containing filtrate.
- Place the QIAamp spin column in a new collection tube (provided) and centrifuge at 14,000 rpm for 2 min.
- Discard the collection tube containing the filtrate and Place the QIAamp spin column in a clean 1.5 ml microcentrifuge tube.
- Add 50 μ l of Buffer AE and incubate at room temperature for 3-5 min and centrifuge at 13,000 rpm for 2 min.
- Repeat elution with 50 μ l of Buffer AE in same tube and incubate at room temperature for 3-5 min and centrifuge at 13,000 rpm for 2 min and store the DNA at -20°C for further use.

DNA thus extracted can be used as a template for diagnosis of Leptospirosis by molecular methods such as PCR, Real time PCR, Multiplex PCR, PCR-RFLP etc.,

Diagnosis of leptospirosis by Polymerase chain reaction

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The gold standard for the diagnosis of leptospirosis is isolation of *Leptospira* bacteria. However, to isolate bacteria is time and resource-intensive; it requires level biocontainment facilities and highly skilled technical personnel to handle samples and live bacteria for eventual identification and typing. Handling all live leptospira involves risk of laboratory infection and very strict bio safety rules must be observed. In order to avoid these disadvantages, methods based on the polymerase chain reaction (PCR) are becoming very useful and considerable progress has been made recently to improve their sensitivity, specificity, and technical ease and to lower costs.

Polymerase chain reaction: PCR is a molecular technique to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. The steps involved are denaturation, annealing and extension followed by final extension to complete the products length. PCR was invented in 1984 by Dr. Kary Banks Mullis & he received the Nobel Prize in chemistry in 1993, for his invention.

The following are the different phases in PCR assay. Exponential phase: If 100% efficiency—exact doubling of products have been taken place that is very specific and precise. Linear phase: High variability. Reaction components are being consumed and PCR products are starting to degrade. Plateau phase: End-point analysis. The reaction has stopped and if left for long, degradation of PCR products will begin.

Primers: Each primer should be 20-30 nucleotides in length and contain approximately equal number of four bases (A, T, G and C) with a balanced distribution of G and C residues and a low propensity to form stable secondary structures. Standard reactions contain non limiting amount of primers, typically 0.1-0.5 μM of each primer.

Template DNA: The DNA can be extracted directly from blood or serum from livestock and human. It is also possible to extract DNA from milk, CSF and tissues. DNA extracted from confirmed cultures can be used as a positive control.

Deoxynucleoside triphosphates (dNTPs): Standard PCRs contain equimolar amounts of dATP, dTTP, dCTP and dGTP. Concentrations of 200-250 μM of each dNTP are recommended for reaction containing 1.5mM MgCl_2 .

DNA Polymerase: A thermostable DNA polymerase, routinely *Taq* polymerase is used to catalyse template-dependent synthesis of DNA. Usually 0.5 to 2.5 units per standard 25-50 μl reaction is used.



Protocol

Equipment / plastic ware

- a. Thermal cycler
- b. Micropipettes
- c. PCR tubes

Reagents

- a. Oligonucleotide primers: The oligonucleotide primers synthesized and supplied in lyophilized form from the company needs to be reconstituted to 100 pmol/ μ l stocks in sterile TE buffer. Then working primers should be made to 10 pmol/ μ l in sterile nuclease free water (NFW).
- b. dNTP (10 mM)
- c. *Taq* DNA polymerase (1 U/ μ l)
- d. PCR buffer (10X)
- e. MgCl₂ (25mM) (If PCR buffer contains MgCl₂, no need of adding separately)

PCR reaction

PCR is to be carried in a 50 μ l reaction mixture in PCR tube.

The PCR reaction mixture includes.

PCR buffer (10X)	:	5.00 μ l
dNTP (10 mM)	:	1.00 μ l
Forward primer (10 pmole/ μ l)	:	1.00 μ l
Reverse primer (10 pmole/ μ l)	:	1.00 μ l
<i>Taq</i> DNA polymerase (1U/ μ l)	:	1.00 μ l
Template DNA	:	2.00 μ l
Nuclease free water	:	<u>39.00 μl</u>
		50.00 μ l

The DNA amplification should be carried out by keeping PCR tube along with reaction mixture in a thermal cycler using following conditions.

Steps	Temperature	Duration	No. of cycles
Initial denaturation	94°C	3-5 min	1
Denaturation	94°C	30 sec	
Annealing	55°C	30 sec	30-40*
Extension	72°C	30 sec	
Final extension	72°C	5-7 min	1

*Varies according to PCR to be performed.

The end reaction is checked for the amplification in 1 or 1.5 % agarose gel and the gel image is capture in the gel documentation under the UV – Trans illuminator.

Agarose gel electrophoresis

Equipments

- a. Weighing balance
- b. Horizontal electrophoresis apparatus with power pack
- c. Microwave oven
- d. UV transilluminator

Reagents

- a. Agarose
- b. TBE buffer (Tris - Borate EDTA buffer) (10X, pH 8.2)

Tris base	:	108.0 g
Boric acid	:	55.0 g
EDTA disodium salt	:	8.3 g
Double distilled water up to	:	1000.0 ml

The stock solution was sterilized by autoclaving and made to 0.5X before use.

- c. Gel loading dye (6X)

Bromophenol blue	:	0.25% (w/v)
Xylene cyanol	:	0.25% (w/v)
Sucrose	:	40% (w/v) in distilled water

Stored at 4°C.

- d. Ethidium bromide (10 mg/ ml)

Ethidium bromide (Biogene, USA):	100 mg
Double distilled water	: 10 ml

The suspension was stirred to ensure that the dye was completely dissolved. The container was then wrapped in aluminum foil and stored at 4°C until use.

Procedure:

- The edges of a clean, dry, gel casting tray to be sealed at both the ends using adhesive tape. An appropriate comb should be placed to form a sample slot in the gel.
- Agarose solution is prepared by dissolving required quantity of agarose in a proportionate volume of 0.5X TBE buffer and melted in a microwave oven for one min.
- (For 0.8% gel, 0.32g agarose in 40ml of 0.5X TBE buffer)



- Once the molten gel cooled, 0.5 μg of ethidium bromide to be added and mixed thoroughly by gentle swirling.
- Warm agarose solution is then to be poured into the gel casting tray avoiding formation of air bubbles and allowed to solidify.
- Once agar gets solidified, a small amount of electrophoresis buffer is to be poured on the top of the gel to remove the comb. Then the buffer to be poured off and the tape removed.
- The gel casting tray is to be mounted in the electrophoresis tank and the electrophoresis buffer is to be added just enough to cover the gel to a depth of 1 mm.
- Five μl of sample DNA to be mixed with 1 μl volume of 6X gel loading dye and slowly loaded into the slots of submerged gel using a micropipette.
- The gel tank to be closed with the lid and electrical leads attached so that the DNA will migrate towards the anode.
- The electrophoresis to be carried out at 5V/cm at room temperature until the bromophenol blue dye migrated to an appropriate distance through the gel.
- Following electrophoresis, the gel / bands needs to be visualized at 300 nm wavelengths using a UV trans-illuminator and recorded in a gel documentation unit.

Reference:

1. Molecular Cloning: A Laboratory Manual (Third Edition) by Joseph Sambrook, Peter MacCallum Cancer Institute, Melbourne, Australia; David Russell, University of Texas Southwestern Medical Center, Dallas.

Multiplex PCR for detection of pathogenic *Leptospira*

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Confirmed diagnosis of leptospirosis depends upon the isolation of leptospire from clinical specimens or the demonstration of sero diagnosis in paired acute and convalescent serum samples. Both of these approaches require expertise and are time consuming. Also not routinely available in clinical laboratories and usually result in delayed diagnosis. Conventional PCR assays have been developed, but all have limitations which have restricted their widespread use. In order to overcome these limitations, multiplex/Duplex PCR using two primers targeted at 331bp, 242bp and 434bp, which are conserved in pathogenic *Leptospira*. Using around 18 pathogenic *Leptospira* and one non - pathogenic *Leptospira* DNA was used to standardize the PCR along with the non-template negative control.

Principle: Any serum/blood/plasma/urine (DNA extracted) samples showing the symptoms of the *Leptospira* such as fever, pyrexia and jaundice etc., can be tested for the presence of pathogenic *Leptospira*. The primers set used are Lept 1 & 2 (targeted at 331bp), LipL32 (targeted at 242bp) and Lig B (targeted at 434bp), Loa 22 (targeted 205 bp).

Materials Required:

Thermal Cycle, Agarose Electrophoretic Unit along with the gel casting tray and gel comb, Gel documentation, PCR vials (0.5 ml and 1.5ml), Pipette (2µl, 10µl and 100µl), Micro tips (2µl, 10µl and 100µl). DNA extraction kit (OIA amp DNA mini kit Cat # 51306), Master mix (Amplicon Cat # - 180303), Agarose (Amresco Cat # N605), 100bp pulse DNA ladder (Fermentas Cat # SM0321), Ethidium Bromide, Nuclease free water and TAE buffer (1X and 0.5X - Fermentas Cat# - LT - 02241).

Primer:

(Lep1) 5'-GGCGGCGCGTCTTAAACATG-3'

(Lep2) 5'-TTCCCCATTGAGCAAGATT-3'

(FLigBL3) and (RLigBL4); (Loa22 F and Loa22R); (LipL32 F and LipL32 R)

Standard reference Serovars: Australis, Bankinang, Canicola, Hardjo, Hebdomadis, Pyrogenes, Tarassovi, Icterohaemorrhagiae, Pomona, Patoc, Shermani, Kaup, Grippotyphosa, Hurstbridge, Javanica, Panama, Djasiman, Copenhageni and Bataviae.

Procedure

- Extract DNA of the Standard Serovars and the test sample using Quigen DNA extraction kit as described earlier.



- Add 2-3µl of the standard reference serovar DNA into PCR tube 0.2ml vial each and label.
- Add around 8 -10µl of the test sample DNA to PCR tube 0.2ml vial each and label.
- Take a 1.5 ml vial and add the following mixture:
 - Total Reaction = 25µl * total no. of reaction (no. of standard reference serovar reactions+ test samples + negative control)
 - Master Mix = 12.5µl * total No. of reaction
 - Primer Lept 1 = 1µl * total no. of reaction
 - Primer Lept2 = 1µl * total no. of reaction
 - Primer Lig BL3 = 1µl * total no. of reaction
 - Primer Lig BL4= 1µl * total no. of reaction
 - NFW = total reaction mixture – (total primer quantity + total master mix)

Note: for the Negative control NFW is used instead of template DNA. Positive controls are the standard reference serovar DNA.

- Vortex the mixture and spin.
- Aliquot the equal amount into the 0.5ml labeled with the different serovar.
- The PCR tubes are vortexed and spinned briefly.
- The tubes are placed in the thermal cycle and the PCR reaction is set at the following conditions (Step up PCR).

Steps	Temperature	Duration	No. of cycles
Initial denaturation	94°C	3-5 min	1
Denaturation	94°C	30 sec	5
Annealing	52°C step up 1 °C /cycle	45 sec	
Extension	72°C	60 sec	
Denaturation	94°C	30 sec	25
Annealing	57°C	45 sec	
Extension	72°C	60 sec	
Final extension	72°C	10 min	1

The end reaction is checked for the 331bp and 434bp amplification in 2.5 or 3% agarose gel and documented in gel doc.

Agarose Gel verification procedure:

- 3% agarose is weighed and added to the 1X TAE buffer in a conical flask.
- The conical flask is then placed into the microwave and a time period of 30 s is set for boiling the

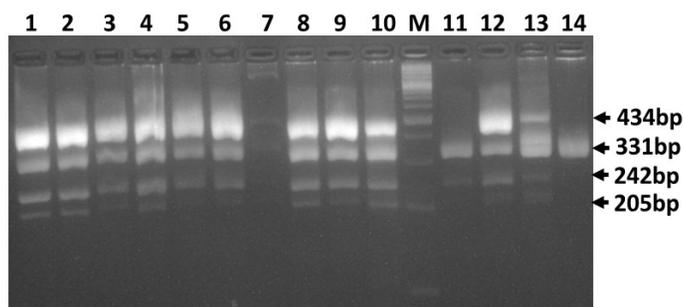
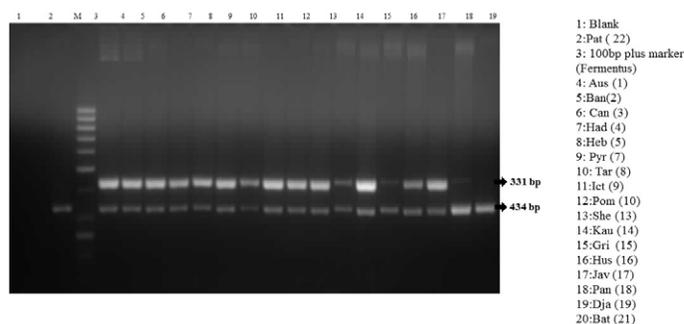
agar. Then the microwave is stopped and the mixture is mixed well. This step is repeated until all the agarose is dissolved.

- As the clear boiled mixture cool to about 50°C Ethidium Bromide solution of 0.1% of the Gel mixture is added.
- The mixture is immediately poured into the clean gel casting tray and the comb is placed.
- The gel is allowed to solidify. The comb is slowly removed and the gel is placed in 0.5X TAE buffer filled gel tank after the gels is completely solidified.

Note:

- 3% gel solidifies very quickly hence needs to be alert and the mixture needs to be poured from all the four corner of the gel tray). Place the comb before pouring.
- The Comb needs to be wiped by the spirit/alcohol just before adding the agarose gel mixture. This will help in the easy removal of the comb after the gel solidified. Otherwise which is quite difficult and hence can avoid the damage to the wells.
- Load the Biflexia (serovar Patoc) standard in the 1st well, Blank is added in the 2nd well, rest of the standard reference are added from 3rd well on wards, then the 100bp plus ladder is added and finally the test samples. (Any one serovars can be used as positive control)
- The gel is runs at the 100 volts current until the dye front reached the $\frac{3}{4}$ th of the gel.
- Then the gel image is capture in the gel documentation under the UV – Trans illuminator.

Multiplex PCR for the standard reference serovar with Lept 1 & 2 and Lig B L 3 & 4 primers



Multiplex PCR products from Lane 1-10: Aus; Ban; Can; Had; Heb; Pyr; Tar; Ict; Pom; She; M: 100bp DNA ladder plus; lane 11-13 ; Kau; Gri; Hur; Pat serovars, respectively.



SYBR Green Real time PCR: Principle and its application for diagnosis of Leptospirosis

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What is Real-Time PCR?

The most powerful tool for quantitative nucleic acid analysis is the real-time PCR, which is a refinement of the original PCR developed in the mid-1980 and found useful for medical and scientific arena in diagnostic applications (Kubista *et al.*, 2006). In real-time PCR, the amount of product formed is monitored during the course of the reaction by monitoring the fluorescence of dyes or probes incorporated into the reaction that is proportional to the amount of product formed, and the number of amplification cycles required to obtain a particular amount of DNA molecules is registered. In conventional PCR, the amplified product, or amplicon, is detected by an end-point analysis, by running DNA on an agarose gel after the reaction has finished. In contrast, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in “real time”. Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequence-specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle. Real time PCR has the ability to directly measure the PCR reaction as amplification is taking place with the use of fluorescent molecules.

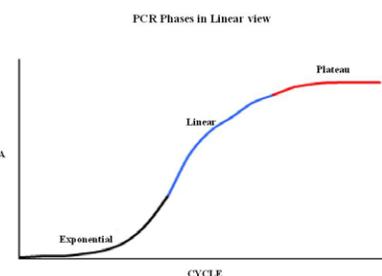
Principle of real-time is based on the detection and quantitation of a fluorescent reporter. Three general methods for the quantitative detection by using 1. DNA-binding dyes/ agents (SYBR Green), 2. Hydrolysis probes (TaqMan, Beacons, Scorpions), 3. Hybridisation probes (Light Cycler). Others Includes, Dye-labeled, sequence-specific oligonucleotide primers or probes (molecular beacons and TaqMan, hybridization, and Eclipse probes, and Amplifluor, Scorpions, LUX, and BD QZyme primers, etc).

The following are the different phases in PCR assay (Fig. 1).

- **Exponential phase**: If 100% efficiency – exact doubling of products have been taken place that is very specific and precise.
- **Linear Phase**: High variability. Reaction components are being consumed and PCR products are starting to degrade.

Plateau phase: End-point analysis. The reaction has stopped and if left for long, degradation of PCR

products will begin.



RT_PCR Chemistry

The most commonly used chemistries for real-time PCR are the DNA-binding dye SYBR Green I and TaqMan hydrolysis probes. In real time PCR, DNA binding dyes are used as fluorescent reporters to monitor the real time PCR reaction. If a graph is drawn between the log of the starting amount of template and the corresponding increase the fluorescence of the reporter dye fluorescence during real time PCR, a linear relationship is observed. Based on the molecule used for the detection, the real time PCR techniques can be categorically placed under two heads namely non-specific detection using DNA binding dyes and specific detection target specific probes.

1. SYBR Green I technique

Non-specific detection using DNA binding dyes: SYBR Green I exhibits little fluorescence when it is free in solution, but its fluorescence increases up to 1,000-fold when it binds dsDNA. Therefore, the overall fluorescent signal from a reaction is proportional to the amount of dsDNA present, and will increase as the target is amplified. SYBR Green remains stable under PCR conditions and the optical filter of the thermocycler can be affixed to harmonize the excitation and emission wavelengths. Ethidium bromide can also be used for detection but its carcinogenic nature renders its use restrictive.

At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis. After annealing of the primers, a few dye molecules can bind to the double strand. SYBR Green I binds all double stranded molecules, emitting a fluorescent signal of a defined wavelength on binding (Fig. 2)

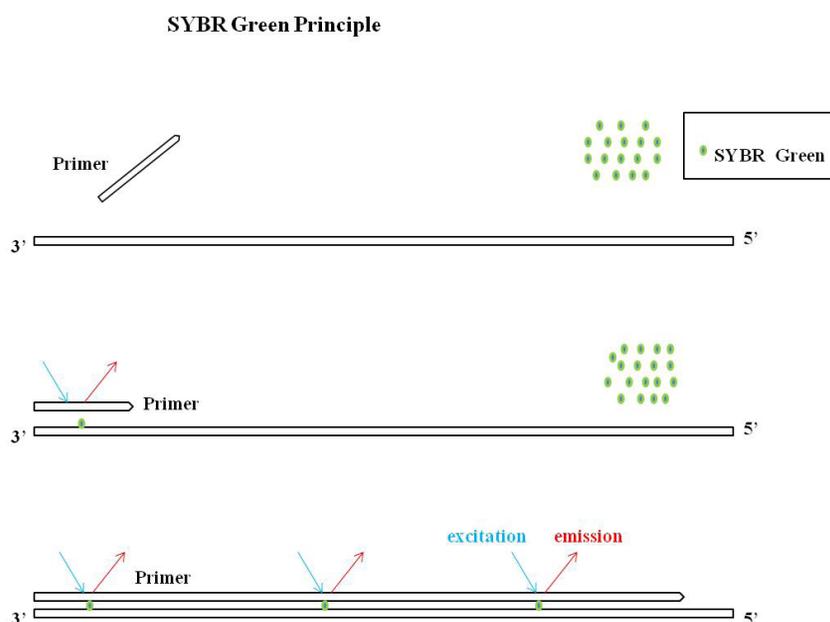


Fig. 2 Schematic diagram to show the Principle of SYBR Green I based detection of PCR products in real-time PCR

It is compatible with PCR up to a point, at very high concentrations it starts to inhibit the PCR reaction. Detection takes place in the extension step of real-time PCR. Use of fluorescent



dyes enables analysis of many different targets without having to synthesize target-specific labeled probes. However, nonspecific PCR products and primer-dimers will also contribute to the fluorescent signal. Therefore, high PCR specificity is required when using SYBR Green I. The biggest advantage of SYBR is that it binds to any dsDNA; there is no designing and optimizing of probes required. The advantages of using dsDNA-binding dyes include simple assay design (only two primers are needed; probe design is not necessary), ability to test multiple genes quickly without designing multiple probes (e.g., for validation of gene expression data from many genes in a microarray experiment), lower initial cost (probes cost more), and the ability to perform a melt-curve analysis to check the specificity of the amplification reaction.

Melt-curve analysis can be used to identify different reaction products, including non-specific products. After completion of the amplification reaction, a melt curve is generated by increasing the temperature in small increments and monitoring the fluorescent signal at each step. As the dsDNA in the reaction denatures (i.e., as the DNA “melts”), the fluorescence decreases. The negative first derivative of the change in fluorescence is plotted as a function of temperature. A characteristic peak at the amplicon’s melting temperature (T_m , the temperature at which 50% of the base pairs of DNA duplex are separated) distinguishes it from other products such as primer-dimers, which melt at different temperatures.

Although these double-stranded DNA-binding dyes provide the simplest and cheapest option for real time PCR, the principal drawback to intercalation based detection of PCR product accumulation is that both specific and nonspecific products generate signal. The major drawback of DNA-binding dyes is their lack of specificity, that is, DNA-binding dyes bind to any dsDNA. As a result, the presence of non-specific products in a real-time PCR reaction may contribute to the overall fluorescence and affect the accuracy of quantification. Another consequence is that DNA-binding dyes cannot be used for multiplex reactions because fluorescent signals from different amplicons cannot be distinguished. Instead, you can set up parallel reactions to examine multiple genes, such as a gene of interest and reference gene, in a real-time PCR assay with SYBR Green I.

Careful optimization of the PCR reaction can usually reduce primer dimers to a level that is only important for very low copy detection. Melting curve analysis of the reaction can help to determine the fraction of the signal coming from the desired product and the fraction coming from primer dimer. Once the melting point of the product has been determined the Light Cycler Instrument’s flexible programming allows the user to acquire fluorescence above the melting temperature of the primer dimers, but below the melting temperature of the product.

When to choose SYBR Green: Assays that do not require specificity of probe based assays. Detection of 1000s of molecules of target. General screening of transcripts prior to moving to probe based assays. When the PCR system is fully optimized, where no primer dimers or non-specific amplicons, e.g. from genomic DNA.

II. Specific detection target specific probes:

In the simplest and most economical format, that reporter is the double-strand DNA-specific dye. Specific detection of real time PCR is done with some oligonucleotide probes labeled with both a reporter fluorescent dye and a quencher dye. Probes based on different chemistries are available for real time detection: these include Molecular Beacons, *TaqMan* probes, FRET Hybridization Probes and Scorpion Primers.

Reporter, Quencher and Internal Reference Dyes:

The classical reporter dye is 6-FAM (6 carboxy fluorescein), HEX (5-hexa chloro fluorescein) and Cy5 (carbocyanin-cyanine dyes). Other reporters used for multiplexing are JOE (4-5 Dichloro carboxy fluorescein) and VIC (4, 7, 2 trichloro carboxy fluorescein). Some other real-time machines, such as the Stratagene Mx4000, can use red dyes as reporters. The classic quencher dye has been TAMRA (6-carboxy tetramethyl rhodamine). Newer quenchers are the dark dyes, DABYCL (Fluorophore 4-dimethylamino phenyl azobenzoic acid) and the black hole quenchers-BHQ I and II (Biosearch Technologies). TAMRA-quenched probes do not require a reference dye; they can use the TAMRA itself. Single probe reactions quenched by dark dyes should use an internal reference dye, classically ROX (5 or 6-carboxy-x-rhodamine-dark red). Multiplex reactions usually use dark quenchers and ROX.

Threshold Cycle:

The Amplification Plot contains valuable information for the quantitative measurement of DNA or RNA. The Threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the Cycle Threshold, C_T . These values are very important for data analysis using the 5' nuclease assay. The $C_{T \text{ value}}$ is the cycle at which a significant increase in DR_n is first detected. It is the parameter used for quantitation. $C_{T \text{ value}}$ of 40 or more means no amplification and cannot be included in the calculations.

DRn :

R_n^+ is the R_n value of a reaction containing all components (the sample of interest); R_n^- is the R_n value detected in NTC (baseline value). DR_n is the difference between R_n^+ and R_n^- . It is an indicator of the magnitude of the signal generated by the PCR. DR_n is plotted against cycle numbers to produce the amplification curves and gives the C_T value (Fig.7)

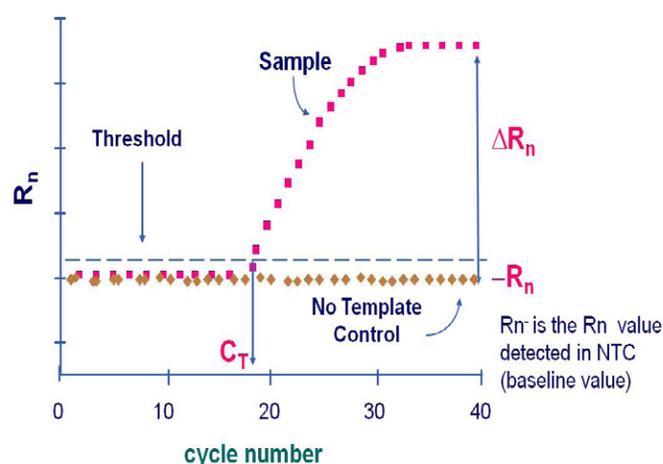


Fig. 3. DR_n is plotted against cycle numbers to produce the amplification curves and gives the C_T value



Efficiency

- The slope of the log-linear phase is a reflection of the amplification efficiency.
- The efficiency of the reaction can be calculated by the following equation.

Efficiency = $10^{(-1/\text{slope})} - 1$. The efficiency of the PCR should be 90-100% (ideal slope = 3.3).

$$X_n = X_0(1+E)^n$$

X_n = PCR product after cycle n
 X_0 = initial copy number
E = amplification efficiency
n = cycle number

If the C_T values for each of the dilutions are plotted against concentrations, the result should be a linear graph with a high correlation coefficient (> 0.99). The slope of this graph is also a measure of efficiency, and can be readily used to calculate efficiency - this is done by most software.

Assay Performance and Evaluation:

Real-time quantification is based on the relationship between initial template amount and the C_T value obtained during amplification. A powerful way to determine the quantitative PCR (qPCR) assay optimization is to run serial dilutions of a template and use the results to generate a standard curve. The template used for this purpose can be with known concentration of nanograms of genomic DNA or copies of plasmid DNA or a sample of unknown quantity (e.g., cDNA). The standard curve is constructed by plotting the log of the starting quantity of template (or the dilution factor, for unknown quantities) against the C_T value obtained during amplification of each dilution. The equation of the linear regression line, along with Pearson's correlation coefficient (r) or the coefficient of determination (R^2), can then be used to evaluate whether the qPCR assay is optimized. To determine and assess the repeatability and reproducibility of real time mPCR assay, the coefficient of variation (CV) to be calculated by testing in three to five consecutive runs (Inter- assay) and three to five times in the same run (intra-assay) using serial dilutions of standard / plasmid DNA copies/ genomic DNA. After standardizing all the parameters using standard plasmid and purified gDNA, the real time mPCR has to be applied over known cell culture/field isolates and also known clinical specimens to assess the diagnostic efficacy in terms of specificity and sensitivity.

Quantitation:

There are three type of quantitation methods for quantitation of DNA or copy numbers. **Absolute Quantitation:** Standard curve to be prepared, standards must be accurately quantified, Best used for viral load determination. **Relative Quantitation:** standard curve to be prepared, standards are serial dilutions of a calibrator template, best used for gene expression studies. They are two methods in this, namely Relative standard method (relative fold change) and Comparative threshold method. **Comparative Quantitation:** mathematical determination, Calibrator sample used as standard. Best used when particular ratios are expected or to verify trends.

Advantages

- Not influenced by non-specific amplification
- Amplification can be monitored real-time
- No post-PCR processing of products (high throughput, low contamination risk)
- Ultra-rapid cycling (30 minutes to 2 hours)
- Wider dynamic range of up to 10^{10} fold
- Detection is capable down to a 2-fold change
- Confirmation of specific amplification by melting point analysis
- Not much more expensive than conventional PCR

Disadvantages

- Not ideal for multiplexing in general
- Setting up requires high technical skill and support
- High equipment cost
- Intra- and inter-assay variation

Application of Real Time PCR in diagnosis of Leptospirosis:

(LipL32 based SYBR Green based real time PCR)

A DNA-binding dye binds to all double-stranded (ds) DNA in a PCR reaction, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified. However, dsDNA dyes such as SYBR Green® will bind to all dsDNA PCR products, including nonspecific PCR products (such as “primer dimers”).

FastStart Universal SYBR Green Master includes a novel reference dye that enables its use on all real-time PCR instruments requiring normalization with ROX, without modifications or adjustments to the specific instrument or protocol. This ready-to-use, 2x concentrated master mix contains all reagents (except primers and template) needed for running quantitative, real-time DNA detection assays.

Components: FastStart Universal SYBR Green Master, 2x concentrated master mix that contains FastStart Taq DNA Polymerase, Reaction Buffer, Nucleotides (dATP, dCTP, dGTP, dUTP), SYBR Green I, and a reference dye, ROX. FastStart Universal SYBR Green Master can be used for the amplification and detection of any DNA or cDNA target, including those that are GC- or AT-. Real time PCR machine (AriaMx Realtime PCR System, Agilent technologies- Agilent Genomics / LightCycler® 480 Instrument II - Roche).

Materials and Methods

- DNA (as made earlier)



- SYBR Green master Mix
- LipL 32 specific primers
- Nuclease free water
- Reaction is conducted in 20µl volume using

10µl SYBR Green PCR Master mix,
2µl DNA,
1µl (5pmol/µl) forward primer
1µl (5pmol/µl) reverse primer

- Volume is adjusted up to 20µl with nuclease free water. Details of RT-PCR condition with thermal profile set up are given below:

Segment 1

Hot start PCR	95°C	10 min	1 cycle
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(To activate *Taq* DNA polymerase)

Segment 2

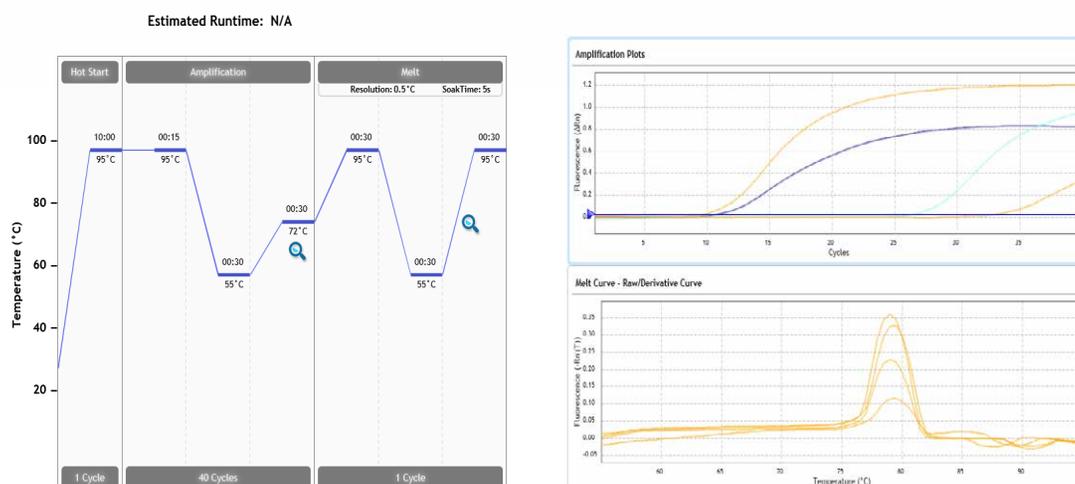
Denaturation	94°C	15 Sec	} 40 cycles *
Annealing	55°C	30 Sec	
Extension	72°C	30 Sec	

*(to detect and report fluorescence during the annealing/extension step of each cycle)

Segment 3

With a final dissociation curve analysis program of 94°C 30 sec -1 cycle with ramping down (95-55°C) at a rate of 0.2°C per sec and collecting fluorescence data continuously on the 95-55°C ramp.

Specificity of the amplified product is assessed by dissociation curve, generated at temperature 55°C through 95°C. The result is expressed as threshold cycle values (Ct). Ct value is the cycle number when the fluorescence of the reporter dye is appreciably higher than the background fluorescence. The threshold automatically adjusted by the instrument is used for the generation of Ct values. During a melting curve run, the reaction mixture is slowly heated up to 95°C, which causes melting of dsDNA and a corresponding sharp decrease of SYBR Green I fluorescence when the temperature reaches the T_m of a PCR product present in the reaction. The Instrument continuously monitors fluorescence over temperature transitions. In Melting Curve analysis using Software these data are then displayed as a melting curve chart (fluorescence [F] vs temperature [T]). The T_m of a PCR product present in the reaction can be estimated from the inflection point of the melting curve. But the T_m is more easily discerned using the Software T_m Calling analysis module by plotting a derivative melting curve (-dF/dT) where the center of a melting peak corresponds to the point of inflection. If the PCR generated only one amplicon, Melting Curve analysis will show only one melting peak. If primer-dimers or other non-specific products are present, they will be shown as additional melting peaks. Checking the T_m of a PCR product can thus be compared with analyzing a PCR product by length in gel electrophoresis.



Analysis of *Leptospira*-specific LipL32 gene products by melting dissociation curve showed $T_m =$

General precautions: SYBR Green/ Real time PCR master mix should never be exposed to light. Before real time PCR estimations allow a minimum warm up time of 15 minutes.

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Application of GIS in understanding the Spatial Epidemiology of Leptospirosis in India (GLPs)

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A geographic information system (GIS) is a system designed to capture, store, manipulate, analyse, manage, and present all types of spatial or geographical data in a computer. GIS is a system of computer software, hardware and data, and the personnel to enter, manipulate, analyse the data. The hardware component of computer is on which a GIS operates. The software components of GIS rely on the underlying Database Management System (DBMS). The data is the most important component of GIS comprising of geographic features and attribute data. There are two types of data stored in the database of GIS. The attribute data gives information about the data like for example the livestock population. The spatial feature gives the information about where the feature is located in spatial domain, for example Leptospirosis cases/outbreaks in different districts of India. Spatial data can be either stored in raster or a vector format. A raster data is a continuous surface and the attribute data has rows and columns of number with a Digital Value/Number (DN) for each cell. Units are usually represented as square grid cells that are uniform in size. The satellite images, aerial photography or scanned images can all be stored in raster format. The vector data are discrete features of spatial data and they can be of three different forms- points, lines and polygons. Vector data are stored as x and y coordinates or a series of x & y coordinates.

Application of GIS in understanding the Spatial Epidemiology of Leptospirosis

The GIS can be very helpful in mapping spatial distribution of Leptospirosis in India. The GIS can help us to understand the spatial features of Leptospirosis cases/outbreaks revealing hidden patterns, trends etc., which may not be apparent in spreadsheets. The application of GIS is not only restricted to better visualization but can also be used for many other purposes as discussed below.

Field surveys to collect the epidemiological data using GPS: Leptospira cases are known to occur in water logged areas and the recording of latitude/longitude will be very helpful to map the location of cases. The GPS can be integrated into GIS software and point maps can be developed to know the clustering of cases which will help in identifying source of infection and also to suggest appropriate control measures.

Mapping the point data and interpolation: Point data can be used for interpolation of outbreaks/cases in the areas where the samples have not been collected. There are many interpolation techniques available in GIS software. The interpolated maps can be of great help in planning future surveillance activities and also in strategizing disease control measures.

Choropleth mapping: A map that uses graded differences in shading or colour or the placing of symbols inside defined areas on the map in order to indicate the average values of some property or quantity in those areas. A choropleth map showing human cases of leptospira is shown in Fig.1. The cases of Leptospirosis is more concentrated in coastal parts in Karnataka, Maharashtra and

Kerala. However, cases of leptospires are distributed in northern and central districts of Tamil Nadu, but maximum number of cases is reported from coastal districts (e.g. Nagapattinam). The spatial distribution of Leptospirosis may not be true picture and the real situation may be different.

Similarly, choropleth map percentage seropositivity of Leptospirosis in animals is shown in Fig.2. The percentage positivity of Leptospirosis is more in coastal districts of Karnataka, Maharashtra, Gujarat and Orissa. Surprisingly there is high Seropositivity of Leptospirosis in Orissa, but not much human cases have been reported.

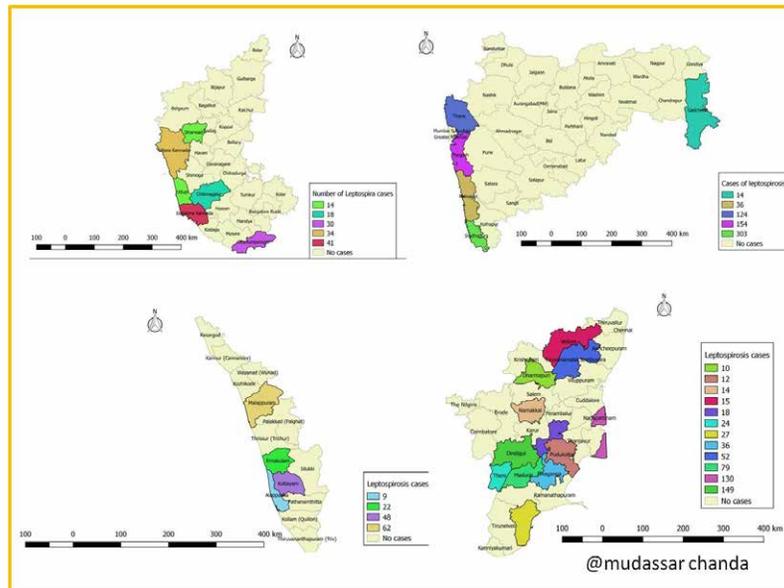


Fig.1: Choropleth maps showing spatial distribution of Human leptospira cases in Karnataka, Maharashtra, Kerala and Tamil Nadu.

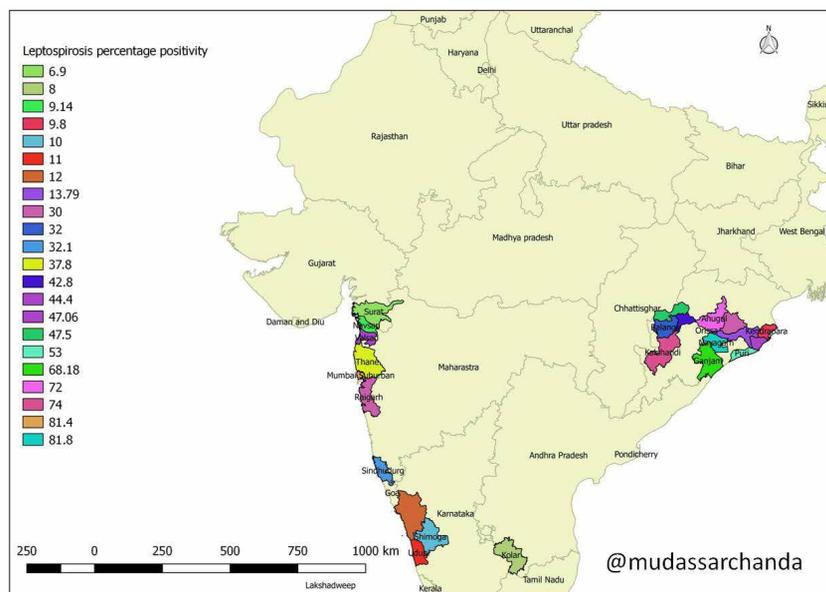


Fig. 2: Seropositivity of Leptospirosis in animals

Overlaying disease data with other layers: The leptospira cases data can be over layered with other probable risk factors. Over layering can be used to understand the epidemiology of Leptospirosis and the probable factors responsible for the outbreaks can be identified. The point map can be overlaid with other layers such as river map, vegetation map, forest map etc. Likewise it can be over layered with other probable risk factors which can be identified by quantitative analysis.

Analysis of disease data: The GIS can also be used to quantify the relationship between Leptospirosis cases/outbreak data and the environmental factors so that it can be used in prevention and control of the disease. Heavy rainfall or flooding is believed to be the important precipitating factor for the occurrence of Leptospirosis. Comparison of mean rainfall and Leptospirosis cases for Karnataka is shown in Fig.3. The Leptospirosis cases are more in areas of high average rainfall compared to the areas with low rainfall. However, one district (Chamarajanagar) with relatively lower average rainfall shows high percentage positivity of Leptospirosis indicating the role of other important environmental factors which needs to be explored.

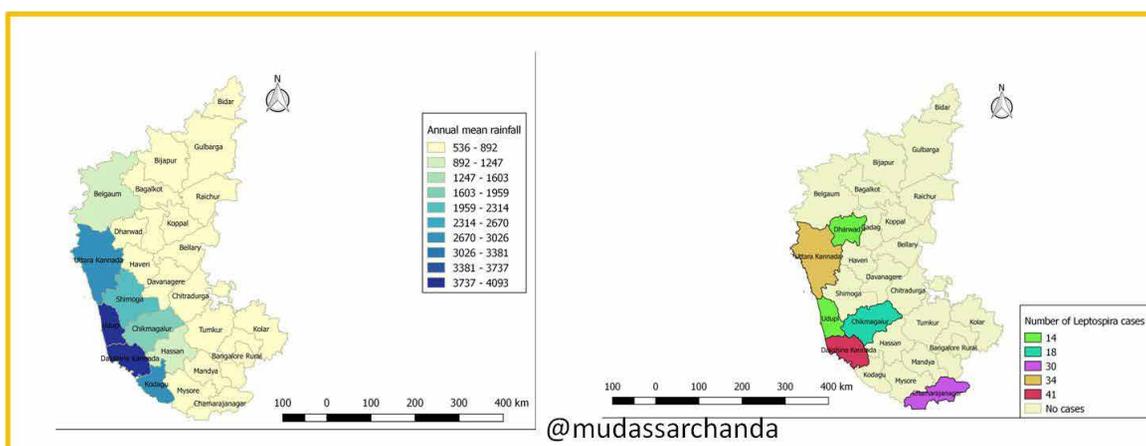


Fig.3: Comparison of rainfall and leptospira cases in Karnataka



Economic impact of leptospirosis in animals and human and KAP studies

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Leptospirosis is the most widespread zoonosis in the world and has emerged as an important public health problem in large urban centres of developing countries and is caused by infection with the spirochete *Leptospira*. Leptospirosis is an important public health concern in most developing countries since it affects livestock and humans significantly and its prevention, control and eradication are very important for any country. A large variety of animal species act as carriers for leptospirosis transmission. Though it prevails throughout the world, high incidence has been recorded in tropical and sub-tropical regions where the climatic conditions highly favour the existence of leptospires. The *Leptospira* affect human and animals. Till date research world over was focused mainly on the study of human leptospirosis because of the explicit disease manifestations in man. In animals, the disease escapes early attention of the veterinary clinician as the symptoms are invariably masked. In animal the production losses are abortion, still berth, milk loss etc. It also acts as an indirect carrier for human transmission. In humans the disease reduces household production and also incurs cost on public health regulation. The household cost includes, medical cost, productivity loss, pain and suffering etc., The public health sector cost includes disease surveillance cost, cost of investigating the outbreak, and investment on control measures. Some of the costs are monetizable and some are non-monetizable in nature. The disease incidence can be mitigated through various actions of the individuals as well as the actions of the state through public funding. Since the public funding alone will not suffice for effective mitigation, the willingness of different stakeholder's participation to reduce *Leptospira* risk is also essential.

Sampling procedure and methodology

- Secondary data collection of outbreaks in different regions to identify the risk regions
- Data collection from sample hospitals to identify the individual infected households
- Primary data collection from different leptospirosis affected households.
- Collection of data from leptospirosis affected livestock farms.
- Fitting deterministic models for quantifying the direct economic loss in humans and livestock

Before implementing any public health programme for prevention or control or eradication, the Knowledge, Attitude and Practice (KAP) evaluation study is essential for knowing the base level of KAP across individual/groups. It will help the policy makers for designing better public health programme. In the past, the assessment of knowledge, attitudes and practices for diseases, such as avian influenza (AI), has been helpful for policy makers to develop control strategies and health education campaigns. The studies on awareness of the disease and the disease routes of transmission among the different risk groups is a pre-requisite for implementing any public health programme. KAP study is also an important tool for ex-post assessment of any intervention programmes. The KAP study can be performed for different risk groups separately, since the degree of risk vary depending on the extent of association of individuals with livestock.

To assess the knowledge, attitudes and practices (KAPs) of different risk groups with regards to leptospirosis a systematic sampling frame need to be developed. Depending on the size of the risk-groups the sample size may be determined. A systematic questionnaire need to be developed for different at-risk groups separately since KAP vary depending on risk groups and their socio-economic status. A systematic cross-section survey may be conducted with the developed questionnaire. The negative statements may be included as a part of the Knowledge/attitude evaluation in order to differentiate the assessment clearly. Based on the statement type (positive and negative) the scores are decided. The two/multi point continuum scores may be considered depending on the response (two/three) types. Besides simple percentage analysis, the Knowledge Index, Attitude Index and Practice/Adoption Index can also be calculated.



Annexure

LABORATORY REQUEST FORM TO BE SUBMITTED

FOR LEPTOSPIROSIS DIAGNOSIS TO ICAR-NIVEDI, BENGALURU

(LEPTOSPIROSIS SURVEILLANCE - HUMAN)- Format made with IDSP team

1. Sl No/Regn No/Bed No:
2. Name of the patient:
3. Sex:
4. Age:
3. Address:
 - Name of guardian:
 - Village/Ward:
 - Sub-centre:
 - Block:
 - Contact No:
4. Occupation:
5. Pregnant: Yes/ No
6. Religion:
7. Category: General/SC/ST/OBC / Others
8. Presence of animals in house: Yes/ No; If yes mention: Cattle/ Sheep/ Goat/ Dog/ Buffaloes
9. Presence of water bodies nearby: Yes/ No
10. Contact with animals: Livestock/ Wildlife/ Rodents/ Dogs/ Other
11. Contact with water:
 - Standing fresh water (eg: lake, pond): Yes/No
 - River/ Stream: Yes/No
 - Wet soil: Yes/No
 - Sewage: Yes/No
 - Flood water: Yes/No
 - Other:
12. Rat infestation at house: Yes/ No
13. House surrounding: Wet/ Dry
14. Drinking water source: Bore well/ Tap water/ Other (Mention)
15. Type of house: Hut/ Brick/ Wood/ Others
16. Type of floor: Mud/ Cement



17. Attached latrines: Yes/ No

18. Personal habits:

- Smoking: yes/ No
- Alcohol: Yes/ No
- Use of foot wear: Yes/ No
- Handles animals: Yes/ No
- Bath in river/ pond: Yes/ No
- Work in field: Yes/ No

19. Medical history: Diabetic: Yes/ No; Hypertension: Yes/ No; Tuberculosis: Yes/ No;
Allergic Disorders: Yes/ No; Asthama: Yes/ No; Other

20. Date of onset of symptoms:

21. History of travel: Yes/ No; If yes mention Date and Place of visit:

22. Duration of Fever/ Illness: <10 days/ >10 days

23. History of similar illness in the family/community: Yes/ No

24. Clinical symptoms and signs : Headache/ Chill/ Fever/ Cough/ Body ache/ Arthritis/
Haemoptysis/ Bleeding gums/ Epistaxis/ Breathlessness/ Vomiting/ Abdomen pain/ Oliguria/
Rigors/ Haemetemesis/ Haematuria/ Diarrhoea/ Conjunctival suffusion/ Meningitis/ Muscle
pain/ Jaundice/ Yellow urine

25. Any other symptoms (Mention):

26. Antibiotic taken: Yes/ No; Mention name of antibiotic and duration:

27. Nature of specimen collected: Blood/ Plasma/ Serum/Urine

28. Date of sample collection:

29. Date of dispatch of specimen:

30. Investigation/s required:

31. Previously positive for leptospirosis: Yes/ No

32. Similar or other symptoms in the past: Yes/ No

33. What treatment received in the past (Details):

Name of the Medical Officer

Address:

Contact No:



PATIENT CONSENT FORM

To be signed by the patient

I hereby give my consent for clinical information related to me to be used for Leptospirosis surveillance. I understand that my name and identity will be concealed. Once signed, I cannot revoke my consent.

Name of patient:

Signature of patient (or signature of the person giving consent on behalf of the patient):

Relationship to the patient in case of other person signing the consent:

Address:

Date:

Facility available at ICAR-NIVEDI, Bengaluru Leptospirosis Research

- ICAR_NIVEDI is a pioneering institute in the *Leptospira* research in animals.
- Institute has all the facilities required for conducting basic, applied and molecular research work on *Leptospira*.
 - Dark field microscopic examination
 - Microscopic Agglutination Test.
 - Isolation and Maintenance of reference leptospira serovars
 - Molecular Diagnostic PCR techniques
 - Typing of leptospiral isolates to species level by molecular based approaches.
 - Imparting training program or “hands-on” training to the research scholars, or research / medical officers /personnel in the *leptospira* research area.
- The laboratory has made a mark for its technical expertise in the field in veterinary fraternities in the country.
- Providing Leptospirosis diagnostic services to the Livestock Farmers, Veterinarians as well as Medical doctors.
- Surveillance /prevalence of leptospirosis study in livestock in endemic states of India using Serum repository facility of institute. Institute is having a National livestock serum repository (NLSR) with sera of all the livestock species from different parts of the country, which have been screened for economically important livestock diseases in the country.
- The research activities in leptospirosis since inception has led to
 - Development or formulation of a new user friendly, sensitive simple *Leptospira* staining technique for diagnosis of leptospirosis (*Leptospria* Staining kit)-commercially available kit and are being used widely in the country
 - Development of transport medium for sending the field materials to laboratory.
 - Recording of the *Leptospira* abortions in bovines and other animal species. Isolation of *Leptospira* spp. from hosts
 - First isolation of *L. inadai* from rodent reservoir hosts and a rabbit and also from two fatal human cases.
- Scientists of the Institute are delivering lectures on Epidemiology and Diagnosis of Leptospirosis” in the Veterinary College, National and International seminars and Institute training courses.
- Institute scientists are guiding M.V.Sc and Ph.D scholars on the topics related to *Leptospira* research with major emphasis on the diagnosis.