

# **Febrile Illness Outbreak Investigation in Sundarharicha-5, Foklan Tapu, Morang District**

**Nepal Health Research Council (NHRC)**

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Prof. Dr. Anjani Kumar Jha  
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## LIST OF ABBREVIATION

DPHO	District Public Health Officer
WHO	World Health Organization
EDCD	Epidemiology and Disease Control Division
BPKIHS	BP Koirala institute of Health Science
ELISA	Enzyme-Linked Immunosorbent Assay
KZH	Koshi Zonal Hospital
NHRC	Nepal Health Research Council
ILI	Influenza like Illness
VA	Verbal Autopsy
KII	Key Informant Interview
ICU	Intensive Care Unit

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## EXECUTIVE SUMMARY

On the date of 24 July 2017, the major national daily newspapers reported that there were two death cases from unknown disease were found in the Morang district, Sundarharicha Municipality 5, Foklan Tapu. According to news, the death cases were the 14-year-old male child and 5-year-old female child. The common symptom of the disease was fever, coryza, headache, drowsiness.

Addressing the outbreak, a descriptive study was conducted with the team of experts in affected area to investigate and identify the etiological and epidemiological causes, how this happens in the outbreak area for the period of July 2017 to December 2017. Verbal Autopsy (VA) and Key in-depth guidelines were used to identify the major causes of febrile illness. Verbal autopsy interview and Key in-depth interview was done and once the diagnosis was confirmed the patient (134 patients, 37 household) was sent for the further full treatment also. The leptospirosis to a human being is contracted through the contaminated urine of infected domestic and wild animals. The disease is common in summer and rainy season. Socioeconomic factors, environmental conditions, and geographical areas are the associated with the risk factors of Leptospirosis. Leptospirosis is a widespread water-borne spirochetal zoonotic disease caused by leptospire.

The analysis was done in SPSS Statistics 20 version. Descriptive and inferential statistics was done to determine the association of major risk factors to the disease occurrence. Qualitative data (VA and KII) was analyzed manually. Ethical approval was obtained from ethical review board at the NHRC (Reg. no. 382/2017) on expedited process according to the outbreak investigation provision of national ethical guidelines.

Findings suggested that out of 83 blood samples 5 were antibody G and antibody M positive for leptospira, 20% were exposed to respiratory illness and three participants below 9 years have fA (H1N1) Pdmo9. Study suggested that large portion i.e. 49% were Igm positive, 375 were Igm negative where as 13% stayed equivocal. Total of 87% of participants were found exposed to domestic animals including pets, 20% exposed to insects bites, 15% with closed exposure with swine and 2.4% exposed to animal necropsy. Participants were found 8.4% with receiving regular immunization vaccine, 1.2% received influenza vaccine and 1.2% were on medications.

Research hence concluded that not just exposure to ill contributed to the risk of developing illness but also increases the risk of infection. Also, Leptospira was responsible agent for the manifestation of an outbreak. Raising awareness of disease patterns and epidemiology of incidence may enrich practices of prevention of leptospirosis. This research finding could be used by concerned stakeholders in emergency and outbreak response to manage risk factors at outbreak sites. This finding may be used for further to assessing the capacity of outbreak response teams, to provide an evidence-based for planning and allocating outbreak management and public health resources and the overall disease burden of leptospirosis.





# BACKGROUND

## About the Febrile illness

On the date of 24 July 2017, the major national daily newspaper reported that there were two death cases from unknown disease were found in the Morang district, Sundarharicha Municipality 5, Foklan Tapu. According to news, the death cases were the 14-year-old male child and 5-year-old female child. The common symptom of the disease was fever, coryza, headache, drowsiness.

In the phone call, District Public Health Officer (DPHO) Morang district, said besides administering treatment to the sick people, the medical team was also conducting sample collection and awareness programme from 25 July 2017. The information provided by the Health Administrator of the Epidemiology and Disease Control Division (EDCD) said with the collaboration of WHO and BPKIHS, screening program is going on. A team consisting of a medical officer, public health researcher, and laboratory technologist was mobilized to Foklan Tapu for the outbreak study.

The purpose of the febrile illness outbreak investigation is to identify the aetiological and epidemiological causes, how this happens in the outbreak area. It also involves implementing control measures to prevent additional illness and evaluating the impact of those control measures to make sure that the problem has been adequately addressed.

## Justification of febrile illness outbreak investigation process

Epidemiology and disease control division (EDCD) was collected the 7 serum sample from Sundarharicha, ward no. 5, Foklan Tapu, Morang at NPHL for analysis of on 2074/04/12. Three different tests like Leptospirosis (rapid), Scrub Typhus (ELISA) and Dengue (rapid) test was done. In Scrub typhus and Dengue laboratory test, the result was negative. Among the 7 serum sample for Leptospirosis, 2 samples was found to be IgM Reactive. (For detail annex 4)

## About febrile illness and Leptospirosis

The infectious sources of febrile illness remain poorly consider in many parts of the world in this century. This is due to lack of human resources, medical facilities, and limited diagnostic facilities. In Nepal, febrile illness is one of the main reasons for seeking medical attention in rural areas, but there is limited information on the incidence of specific infections.

Leptospirosis has been reported as an emerging zoonosis and the widespread zoonotic disease in the world(1). Leptospirosis is presumed to be the widespread zoonosis in the Asia (2). The leptospirosis to a human being is contracted through the contaminated urine of infected domestic and wild animals(1). The disease is common in summer and rainy season(3). Socioeconomic factors, environmental conditions, and geographical areas are the associated with the risk factors of Leptospirosis(4).Leptospirosis is a widespread water-borne spirochetal zoonosis disease caused by leptospire.

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In the Asian region, leptospirosis is largely a water-borne disease. Till date, the frequency, incidence, and prevalence of Leptospirosis have not been well demonstrated in Nepal. Leptospirosis is known to be endemic and sometimes epidemic in India as well as demonstrated in Bangladesh also, which occurred commonly in the urban and rural poor population (6,7). Nepal being in similar tropical and geographical region, the country has also verified Leptospirosis infection occasionally. Only, few studies have been carried out in this regard and there was only few evidence of Leptospirosis in the country (6,8,9). This study has been demonstrated evidence of Leptospirosis infection as one of the etiology of unknown febrile illnesses of Foklan Tapu of Morang district Nepal.

# OBJECTIVES

- To establish existence and magnitude of the outbreak
- To identify the possible source and mode of transmission
- To detect the possible causes/risk factors/vehicles of transmission and
- To make recommendations to implement preventive and control measures

## Case definition

### Clinical case definition of unknown disease

Those people who were suffering from Fever, Corzya, Headache and Drowsiness from last 30 days.

### Laboratory diagnosis

Blood, urine and sputum sample (Was follow the standard guidelines)

### Case classifications:

Clinically confirmed: A case that meets the clinical case definition

**Laboratory-confirmed:** A case that meets the clinical case definition by laboratory procedures

# METHODOLOGY

## Study Design

A descriptive study was conducted Sundar Haraicha Municipality ward no 5, Foklan Tapu of Morang District. In this study all the people were included from the particular pocket area and were screened for the febrile illness and after screening. Once the diagnosis was confirmed the patient was sent for the further full treatment also.

## Study Area

The study population was people residing in the Sundar Haraicha Municipality ward no 5, Foklan Tapu of Morang District. Those people who have met the above case definition were selected. The investigation period was from July 2017 to December 2018.

## Investigation Site

In the 37 households, only 160 populations were residing in the Sundar Haraicha Municipality ward no 5, Foklan Tapu of Morang District. In the screening and data collection time, we got only 134 people.

## Sample Size and Sample Population

A total number of 134 people who were suffering from Fever, Corzya, Headache and Drowsiness from last 30 days were checked by medical doctor during the field visit. Among them, 86 blood sample and 37 sputum samples were taken from the patients for the laboratory analysis. The blood and sputum sample were collected and brought to the National Public Health Laboratory, Kathmandu, for further microbiological analysis.

Two verbal autopsies were done among the household heads of decrease family. Five Key in-depth interviews were taken from local community leader, local health service providers, Medical officer of Koshi Zonal Hospital, District public health officer and Medical director of WHO.

## Data Collection Tools and Techniques

For quantitative data, semi- structured questionnaire was used to collect information from the screened population. Similarly, laboratory transportation form taken from NPHL was used for sample collection and appropriate transportation as well as to remove the duplication. Interview was done before collecting the sample.

Verbal Autopsy (VA) and Key in-depth guidelines were also used to identify the major causes of febrile illness. Verbal autopsy interview and Key in-depth interview was done.

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## Process of data collection and screening camp

The team includes Medical officer (Koshi Zonal Hospital (KZH)), Medical Lab Technologist (KZH and NPHL), Research team (NHRC), upon arrival to Sundar Haricha, reviewed the VDC and health facility data. The secondary information like total number of household, affected population and their distribution, geographical variations, ongoing services, etc. Together with local health workers, Female Community Health Volunteers (FCHVs), newly elected local leaders developed a field work plan.

The team organized health camps covering most affected areas of Foklan Tapu for 5 days. Assessment of febrile illness (Leptospirosis illness, Influenza like Illness (ILI)) cases was done using the standard case definition along with line listing of other patients. An index case was discovered.

### Collection of Blood Specimen:

Whole blood was collected for leptospirosis examination. Serum was separated from blood by using the serological techniques. Blood sample was collected for immediate provisional and detail laboratory diagnosis. In order to separate serum from blood, drawn blood was stored at room temperature for least 30 minutes for complete clot formation. Serum was separated from the clotted blood by centrifugation to prevent hemolysis. After centrifugation was complete, serum (supernatant) was decanted using sterile technique into plastic freezing vials.



Serum was stored at 4 °C for short term and at -20 °C for long time periods. Serum was transported at 0 °C to 4 °C to the NPHL. Based on this findings of NPHL (annex 4), we made our concept for doing the laboratory analysis for Leptospirosis by rapid methods and ELISA. All the process was maintained by Medical lab technician of NPHL. Rapid testing for provisional diagnosis was done on the spot to identify the positive cases.

### Specimen Collection and Transportation of Throat Swab

For influenza suspected people sputum sample was collected. Specimen was collected from the throat Infections people. A plain cotton swab was collected as much exudates as possible from tonsils, posterior pharyngeal wall and other area that is inflamed or bears exudates. After collecting the throat swab, it was immediately placed into a sterile tube for transport to NPHL.

### Data management and analysis

Quantitative data was collected and cleaned as well as checked for consistency and entered in the MS excel. The analysis was done in SPSS Statistics 20 version. Descriptive and inferential statistics was done to determine the association of major risk factors to the disease occurrence. Qualitative data (VA and KII) was analyzed manually.

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## **Process for doing the Laboratory Analysis**

Epidemiology and disease control division (EDCD) was collected the 7 serum sample from Sundarharicha, ward no. 5, Foklan Tapu, Morang at NPHL for analysis of on 2074/04/12. Three different tests like Leptospirosis (rapid), Scrub Typhus (ELISA) and Dengue (rapid) test were done. In Scrub typhus and Dengue laboratory test, the result was negative. Among the 7 serum sample for Leptospirosis, 2 samples were found to be IgM Reactive.

## **Ethical considerations**

Ethical approval was obtained from ethical review board at the NHRC (Reg. no. 382/2017) on expedited process according to the outbreak investigation guidelines. Written consent was taken from all the participants after informing about the study and clarifying their role in the study.

## FINDINGS

### Leptospirosis (rapid) test done by NPHL

Epidemiology and disease control division (EDCD) collected the 7-serum sample from Sundarharicha, ward no. 5, Foklan Tapu, Morang NPHL on 2074/04/12. Three different tests like Leptospirosis (rapid), Scrub Typhus (ELISA) and Dengue (rapid) test were done. In Scrub typhus and Dengue laboratory test, the test result was negative. Among the 7 serum sample, for Leptospirosis 2 sample was found to be IgM Reactive.

### Influenza test from throat swab

37 throat swabs was collected from the febrile disease outbreak area of Foklan Tapu. Influenza test was done at the NPHL by applying the influenza PCR test. The result of Influenza test shows that 3 participants have fA (H1N1) pdm09 positive case. These all participants were below 9 years.

### Rapid testing of Leptospirosis

83 blood samples were collected and sent to NPHL for necessary laboratory diagnosis. Rapid testing was done in the field to identify the positive cases. Findings show that there were 5 (Five) antibody G and antibody M positive cases for leptospira.

### Socio-demographic characteristics of participants

The participants were suffering from fever, coryza, headache, and drowsiness like symptoms onset dates ranged from July 20-July 30, 2017. Among the total 83 cases, the median respondent's age was 18 years (range = 1 year–85 years). The median age among the cases was 17 years (range = 1 year 52 years). The majority (65%) of the participants were female. According to caste/group grouping of Nepal, the majority (84%) of the participants were disadvantage janajati followed by (16%) upper caste groups. Similarly, on the religion, Hindu participants were slightly higher (92%) than the census report 2011, which accounts 81.3% of country's population. (Table 1)

**Table 1: Socio-demographic characteristics**

Characteristics(n=83)	Frequency	Percent
<b>Sex</b>		
Male	29	34.9
Female	54	65.1
<b>Ethnicity</b>		
Disadvantaged janajatis	70	84.3
Upper caste groups	13	15.7
<b>Religion</b>		
Hindu	76	91.6

Buddhist	6	7.2
Christian	1	1.2

## Exposure to Respiratory Illness

Two out of ten (20%) of the participants were exposed with respiratory illness people. Among the exposed participants, majority (69%) of them were contacted with household/intimate people. The data available suggest that exposure to ill either through exposure to partner or during induced family member, increases the risk of developing illness. In addition, the longer the duration of exposure the higher the risk of infection for febrile illness.

**Table 2: Exposures to respiratory illness**

Exposures to respiratory illness	Frequency	Percent
Contact respiratory illness (n=83)		
Yes	16	19.3
No	67	80.7
Types of contact (n=16)		
Household/ Intimate	11	68.8
Institutional setting	5	31.2

“An isolated island, Inadequate sanitation, open defecation, open riversides in all directions, the very low distance of pet shelter and people bedroom with low awareness is prevalent in Foklan Tapu. We keep in touch with the febrile disease outbreak time”. – KII, DPHO Morang

## Animal Exposure

For the Leptospirosis, there is always a risk of contagion for people who have contact with infected soil/water or animals where the Leptospira bacteria are present. Generally, people who work animals or outdoors may be at increased risk for contagion and infection.

**Table 3: Animal Exposures (within past 8 weeks)**

Animal exposure (n=83)	Frequency	Percent
Animals living in his/her home (including pets)	72	86.7
Animal bite (including wild and domestic animals)	4	4.8
Insect bite (e.g. mosquito, tick, and spider)	16	19.3
Close contact with rodents (e.g. rats, mice, squirrels, prairie dogs)	16	19.3
Close contact with rodent droppings or rodent nests	8	9.6
Close contact with birds (includes turkeys and chickens)	16	19.3
Close contact with bird droppings	11	13.3



Close contact with swine	12	14.5
Hunting or fishing	2	2.4
Skin, dress, or eating wild game	3	3.6
Spending time on a farm, rural area or petting zoo	4	4.8
Performing or assisting with an animal necropsy	2	2.4

Out of total participant, a majority (87%) of the participant was exposed to an animal living in his/her home (including pets). One out of five (20%) of the participants were exposed with insect bite (e.g. mosquito, tick, and spider) likewise, close contact with rodents (e.g. rats, mice, squirrels, prairie dogs), and close contact with birds (includes turkeys and chickens). Around (15%) of the participants were close contact with swine and so on. Very few (2.4%) of the participants were exposed to performing or assisting with an animal necropsy and hunting or fishing.

“Most of the people are poor and living unhygienically. In every household, there are a lot of pet animals like a pig, buffalo, goat, duck, and chicken. Their pets entered in their kitchen and rooms easily. Their pet house and their bedroom are nearby”. - KII, Local Health Workers

““Like the darkness under the candle” Foklan Tapu is nearby with the well facilitated Itahari and Biratnagar cities. Many people in the Foklan Tapu have not managed the waste properly. They eat unhealthy food, and meat. Their home has no proper sanitation. They use open space mostly river for defecation”. – KII, FCHV

**Table 4: Medications/Biological exposure of participants**

Characteristics (n=83)	Yes		No	
	Frequency	Percent	Frequency	Percent
Receiving any immunizations	7	8.4	76	91.6
Receiving an influenza vaccine in last one month	1	1.2	82	98.8
Taking any medications including prescription, over the counter, or herbal remedies	1	1.2	82	98.8

After the febrile disease outbreak, only (8.4%) of the participants were receiving the immunization. Only 1.2% of the participants were receiving an influenza vaccine in the last one month. Likewise, only 1.2% of the participants were taking any medications including prescription, over the counter, or herbal remedies.

“Poor people were neglects the minor symptoms. In this febrile disease outbreak cases, most of the villagers were affected with the same symptoms. The team of health post moves to the place of incident. We started the screening camp among the ill people and given medicine for symptomatic treatment”. –KII, Local health

**Table 5: Exposure in public gathering**

Exposure (n=83)	Yes		No	
	Frequency	Percent	Frequency	Percent
Working with any chemicals or toxins	2	2.6	81	97.4
Attending any gathering, Mela (community program) in last 1 month	7	8.4	76	91.6
Attending any party, function in last 1 month	10	10.8	74	89.2

On the exposure of participants, around 11% of the participants were attended the party, function within 1 month. 8.4% of the participants were attended any gathering, Mela (community program) within the last 1 month.

“Local political leader who won in the local election was gathered the local community for winning party (BHOJ). Every one of the village was invited and ate chicken and pulau”.-A Local people, Foklan Tapu

## Leptospirosis case

**Table 6: Laboratory Diagnosis of Leptospirosis**

Laboratory Diagnosis (n=83)	Leptospira immunoglobulin M (IgM)		Leptospira immunoglobulin G (IgG)	
	Frequency	Percent	Frequency	Percent
<b>Positive</b>	41	49.4	21	25.3
<b>Negative</b>	31	37.3	51	61.4
<b>Equivocal</b>	11	13.3	11	13.3

IgM positive antibodies to Leptospira species detected suggesting recent infection. Almost half (49%) of the participants were IgM positive antibodies to Leptospira species followed by 37% negative IgM and Only 13% equivocal. IgG positive antibodies to Leptospira species detected suggesting the prolonged time of time. One out of 4 (25%) participants was IgM positive antibodies to Leptospira species followed by (61%) negative and 13% equivocal.

**Table 7: Association of Symptoms with IgM**

Symptoms	Positive		Negative		Equivocal	
	Frequency	Percent	Frequency	Percent	Frequency	Percent
Fever	11	13.3	6	7.2	3	3.6
Sweats	5	6.0	0	0.0	1	1.2
Chills rigors	1	1.2	0	0.0	0	0.0
Cough	12	14.5	7	8.4	3	3.6
Shortness of breath	4	4.8	6	7.2	1	1.2

Chest pain		7.2	5	6.0	1	1.2
Runny nose	3	3.6	4	4.8	1	1.2
Sore throat	4	4.8	7	8.4	1	1.2
Difficulty swallowing	2	2.4	6	7.2	1	1.2
Ear pain	0	0.0	1	1.2	0	0.0
Redeyes	2	2.4	2	2.4	0	0.0
Muscles ache	0	0.0	5	6	2	2.4
Joint pain	3	3.7	2	2.4	0	0.0
Enlarged gland	2	2.4	0	0.0	1	1.2
Rash	2	2.4	0	0.0	0	0.0
Vomiting	2	2.4	0	0.0	1	1.2
Diarrhea	0	0.0	2	2.4	1	1.2
Dark bloody urine	1	1.2	0	0.0	0	0.0

Among the *Leptospira* (IgM) positive participants on the different symptoms shown by the participants. Among cough symptoms shown participants, (15%) of them were IgM positive followed by (8%) IgM negative and nearly (4%) equivocal. Similarly, around (13%) of the IgM positive participants were shown the fever followed by 6% sweats, (5%) a sore throat and shortness of breath and so on.

Table 8: Lab findings of Leptospirosis according to different variables

Characteristic	Response	No leptospirosis		Leptospirosis		Total		p-value
		Frequency	Percent	Frequency	Percent	Frequency	Percent	
Gender	Male	12	36.4	17	34	29	34.9	0.825
	Female	21	63.6	33	66	54	65.1	
Ethnicity	Disadvantaged Janjati	25	75.8	45	90	70	84.3	0.081
	Upper caste groups	8	24.2	5	10	13	15.7	
Religion	Hindu	31	93.9	45	90	76	91.6	0.671
	Buddhist	2	6.1	4	8	6	7.2	
Animals pets	Christian	0	0	1	2	1	1.2	0.805
	No	4	12.1	7	14	11	13.3	
Animal bite	Yes	29	87.9	43	86	72	86.7	0.096
	No	33	100	46	92	79	95.2	
Insect bite	Yes	0	0	4	8	4	4.8	0.179
	No	29	87.9	38	76	67	80.7	
Contact with rodents	Yes	4	12.1	12	24	16	19.3	0.084
	No	29	87.9	37	74	66	79.5	
Contact with rodent droppings	Yes	3	9.1	13	26	16	19.3	0.131
	No	30	93.8	43	86	73	89	
Contact with birds	Yes	1	3.1	7	14	8	9.8	0.084
	No	29	87.9	37	74	66	79.5	
Contact with bird droppings	Yes	3	9.1	13	26	16	19.3	0.043
	No	31	93.9	40	80	71	85.5	
Contact with swine	Yes	1	3	10	20	11	13.3	0.077
	No	31	93.9	40	80	71	85.5	
Hunting and fishing	Yes	2	6.1	10	20	12	14.5	0.765
	No	32	97	49	98	81	97.6	
Use skin dress or eat wild	Yes	1	3	1	2	2	2.4	0.817
	No	32	97	48	96	80	96.4	
	Yes	1	3	2	4	3	3.6	

## Qualitative

### Verbal Autopsy (14 years child)

According to the participant, her son died of unknown febrile illness. The deceased was 14 years old male. The deceased was exposed to a contaminated water source while swimming. He ate the flesh meat taken from the hilly region of Shankhusawa district. The flesh meat of buffalo was rotten. They have never taken the precaution for freezing the meat. He had a cough with sputum from last 3 days. It was associated with muscle pain, muscle cramps, particularly affecting the muscles in the lower back and calves. Deceased was taken to the BPKIHS, Dharan hospital and admitted there for a week. After the medical testing, the doctor told to the deceased family for taking the cerebrospinal fluid (CSF). Hospital doctor gives him usual treatment in the intensive care unit (ICU). They never allowed visiting the patient during the ICU time. Suddenly, on the 8th day of ICU, when I was inside the ICU of the hospital. He sat down and asked for water meanwhile he dead on the spot.

### Verbal Autopsy (4 years child)

According to the participant, her daughter died of unknown febrile illness. The deceased was 4-years-old female. She had the fever for five days. The deceased had been attended the party organized by a newly elected local leader. The party was for celebrating the winning happiness of local election. Where she ate chicken and pulao (Fried rice). When the fever was very high, her mother gave her medicine, which she had bought from a nearby pharmacy. After taking medicine, she felt better and got back to normal life. At the same night, a buffalo died, after bitten by a dog (sick dog as told by villager) and they just cooked the dead buffalo which everybody ate. In the third day of meat eating date, she suddenly had a high fever in the morning with muscle cramps. She was barely able to open her eyelid. The family members took her to a nearby hospital in Itahari, but in the middle of the day, she died.

## CONCLUSION

Nepal remains a place for the frequent outbreak of febrile illness and the sometimes leptospirosis outbreaks go without identifying the causative agents. Based on the evidence from clinical and laboratory findings, the leptospirosis outbreak was recorded in Foklan Tapu, Sundarharaicha Municipality of Morang district. *Leptospira* was responsible agent for the manifestation of an outbreak.

Awareness rising of disease patterns and epidemiology of incidence may enrich practices of prevention of leptospirosis. This research finding could be used by concerned stakeholders in emergency and outbreak response to manage risk factors at outbreak sites. This finding may be used for further to assessing the capacity of outbreak response teams, to provide an evidence-based for planning and allocating outbreak management and public health resources and the overall disease burden of leptospirosis.

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# ANNEXES

## Annex 1: Work Schedule

SN	Tasks to be performed	Personnel assigned for task	No. of person days required	Justification
1	Proposed blood, urine and sputum sample collection, providing standard guidelines for test and providing test report	Lab Technician	1*7=10	Per day 30 sample was collected including cold chain maintain and transportation
2	Preparation of data collection guidelines, clinical examination, screening, interpretation of laboratory test result	Medical doctor (Clinician)	1*7=10	Per day 30 people was checked
3	Concept paper preparation, Preparation of data collection guidelines, Coordination and data collection	Researcher	2*7=10	Coordination, management and data collection



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**Annex 2: Some pictures from outbreak investigation site**



Preparation for data collection



Clinical investigation



Doctor check up



Blood sample collection



Data Collection



Verbal Autopsy



Developing Screening camp and data collection area



Developing Blood and throat swab sample collection area



Data collection area and people gathering



Deceased children

## Annex 3: Press Release

प्रेस विज्ञप्ती

२०७४ साउन, २३

मोरङ्ग जिल्लाको सुन्दर हरैचा ५, फोकलान टापूमा फैलिएको अज्ञात रोगको अध्ययन र अनुसन्धान ।

विभिन्न सञ्चार माध्यममा प्रकाशित समाचारमा मोरङ्ग जिल्लाको सुन्दर हरैचा ५, फोकलान टापूमा फैलिएको अज्ञात रोगको कारण २ जना बालबालिकाको मृत्यु भएको खबर आए सँगै नेपाल स्वास्थ्य अनुसन्धान परिषद् (स्वास्थ्य मन्त्रालय) को गम्भीर ध्यानाकर्षण भएको छ । उक्त महामारीको कारण पत्ता लगाउनको लागि नेपाल स्वास्थ्य अनुसन्धान परिषद्ले सम्बन्धित निकायहरू (EDCD, NPHL, DPHO Morang, Koshi Regional Hospital) सँग समन्वय गरि यहाँबाट अनुसन्धान टोली पठाएको थियो ।

उक्त अनुसन्धान टोलीले जिल्ला जन-स्वास्थ्य कार्यालय मोरङ्ग, कोशी क्षेत्रीय अस्पताल, नोबेल मेडिकल कलेज, विश्व स्वास्थ्य संगठन विराटनगर कार्यालय, सुन्दर हरैचा स्वास्थ्य चौकी र सम्बन्धित वडा अन्तर्गतका वडा कार्यालय र राजनितिक कर्मीहरूको समन्वय र सहयोगमा महामारी स्थलमा गई स्वास्थ्य परिक्षण, रगतको नमूना संकलन, महामारीको अध्ययन, स्थलगत अध्ययन र निरिक्षण तथा मृत्यु भएका बालबालिकाहरूको घरमा गई मौखिक जानकारी संकलन गरेको थियो ।

प्रारम्भिक अध्ययन अनुसार महामारी स्थलका मानिसहरूमा ३८ डिग्री भन्दा माथी ज्वरो आउने, रिंगटा लाग्ने, टाउको दुख्ने कारणहरूलाई आधारमानि स्वास्थ्य परिक्षण र Rapid Test बाट रगतको नमूना जाँच गर्दा *Leptospira* नामक विषाणुले लाग्ने *Leptospirosis* नामक रोग देखिएको साथै महामारी स्थल फोकलान टापुबाट लिईएको ७ जना बिरामीहरूको रगतको नमूना मध्ये रक्त परिक्षण पश्चात् २ जनामा *Leptospirosis Positive* देखिएको जानकारी समेत NPHL मार्फत प्राप्त भएको थियो । उक्त अनुसन्धान टोलीले फोकलान टापूका सम्पूर्ण बासिन्दाको रक्त नमूना संकलन गरि अन्य विशेष जाँचको लागि NPHL पठाईएको र उक्त परिक्षणको नतिजा आए पश्चात् यस महामारीका बारेमा थप जानकारी प्रतिवेदन मार्फत सार्वजनिक गराउनेछ ।


उक्त रोग सर-सफाईको कमी, घर पालुवा जनावरहरू सँगको अति निकट सम्बन्ध, मरेका वा सडेगलेका मासु खादाँ फैलिएको हुन सक्छ । भौगोलिक बिकटता, जंगलको अत्यन्त नजिक बस्ती, स्वास्थ्य परिक्षणको लागि खोला तरेर आउन पर्ने बाध्यता, गरीबी, निरक्षरता र जन चेतनाको कमीका कारणले महामारी फैलिएको र २ जना बालबालिका समेत गुमाउन परेको देखिन्छ । उक्त महामारीबाट पाठ सिक्दै भविष्यमा यस्ता महामारी जन्म घटनाहरू दोहोरिन नदिन अपनाउनु पर्ने जन चेतना, सर-सफाई तथा महामारी रोगहरूको बारेमा विस्तृत जानकारी सम्पूर्ण नागरिकहरूमा पुऱ्याउनु पर्ने टड्कारो आवश्यकता देखिन्छ ।

.....

प्रा. डा. अन्जनी कुमार भा

कार्यकारी अध्यक्ष

## Annex 4: National Public Health Laboratory Report



Government of Nepal  
Ministry of Health & Population  
Department of Health Services  
**National Public Health Laboratory**  
Toku, Kathmandu

Tel: 4741115  
Fax: 4741175  
E-mail: npht@nepal.gov.np

Date: 2074/04/17

Ref no: 36/74/75

To:  
Epidemiology and Disease control Division  
Toku

**Subject: Regarding report of serum samples**

Dear Sir,

This is to inform you that the report of 7 serum samples received from *Sandhe Tamang, Ward no. 5, Fokland tappu, Morang* at NPHL for analysis on 2074-04-17 is as follows:

S.No	Name of the patient	Leptospira (Rapid)	Serife Typhus (ELISA)	Dengue (Rapid)
1.	Nitu Rai	IgG/IgM- Non reactive	Negative	NSI antigen- Negative
2.	Prabhat Rai	IgG/IgM- Non reactive	Negative	NSI antigen- Negative
3.	Pasang Tamang	IgG- Non reactive IgM- Reactive	Negative	NSI antigen- Negative
4.	Bibek Tamang	IgG/IgM- Non Reactive	Negative	NSI antigen- Negative
5.	Achik Tamang	IgG/IgM- Non reactive	Negative	NSI antigen- Negative
6.	Ajay Rai	IgG- Non reactive IgM- Reactive	Negative	NSI antigen- Negative
7.	Everest Rai	IgG/IgM- Non Reactive	Negative	NSI antigen- Negative

Approved by

*for [Signature]*

Dr. Raj Kumar Mahito  
Director  
NPHL

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## Annex 5: ELISA for Leptospira IgM

Annex 6: ELISA for IgG



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NovaLisa®

# Leptospira IgM

ELISA

CE

Only for in-vitro diagnostic use

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Product Number:      LEPM0660 (96 Determinations)

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## ENGLISH

### 1. INTRODUCTION

Leptospirosis (also known as Weil's syndrome) is probably the most widespread zoonosis in the world. It is caused by infection with spirochete bacteria of the genus *Leptospira* and affects humans as well as a broad spectrum of animal hosts. The incidence is significantly higher in warm climate countries than in temperate regions. The disease is seasonal, with peak incidence occurring in summer or fall in temperate regions, where temperature is the limiting factor in survival of leptospires, and during rainy seasons in warm climate regions, where rapid desiccation would otherwise prevent survival.

Natural reservoirs for the pathogenic *Leptospira interrogans* include rodents as well as a large variety of domesticated mammals (e. g. pigs, cattle and dogs). Leptospires occupy the lumen of nephritic tubules in their natural host and are shed into the urine.

Transmission can occur when humans are directly or indirectly exposed to the urine of infected animals or a urine-polluted environment. Leptospires gain entry into the human blood stream via cuts, skin abrasions or mucous membranes through contact with moist soil, vegetation, and contaminated waters; handling infected animal tissues, and ingestion of food and water. Leptospires are rarely transmitted from human to human.

The incubation period is usually 5-14 days, with a range of 2-30 days.

The spectrum of clinical symptoms is extremely wide. The vast majority of leptospiral infections are either subclinical or result in very mild illness and recover without any complications. Clinical manifestations of leptospirosis range from mild influenza-like symptoms to severe life-threatening disease forms, characterized by jaundice, renal failure, bleeding and severe pulmonary hemorrhage.

The clinical presentation of leptospirosis is biphasic, with the acute or septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine. Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase and thus occur during the second week of the illness. The classical syndrome of Weil's disease represents only the most severe presentation. It is characterized by jaundice, renal failure, hemorrhage and myocarditis with arrhythmias.

Species	Disease	Symptoms	Mechanism of Infection
Leptospira spp.	Leptospirosis	Wide spectrum of clinical symptoms: mild influenza-like symptoms to severe life-threatening disease forms	direct or indirect contact with the urine of an infected animal (via cuts, skin abrasions or mucous membranes)
	Weil's disease	jaundice, renal failure, haemorrhage and myocarditis with arrhythmias	

The presence of pathogen resp. infection may be identified by

- Pathogen detection: dark-field microscopy  
culture from blood, urine, cerebrospinal fluid or tissues  
PCR
- Serology: microscopic agglutination test (MAT), ELISA

### 2. INTENDED USE

The *Leptospira* IgM-ELISA is intended for the qualitative determination of IgM class antibodies against *Leptospira* spp. in human serum or plasma (heparin).

### 3. PRINCIPLE OF THE ASSAY

The qualitative immunoenzymatic determination of IgM-class antibodies against *Leptospira* spp. is based on the ELISA (Enzyme-linked Immunosorbent Assay) technique.

Microtiter strip wells are coated with *Leptospira* antigens to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labelled anti-human IgM conjugate is added. This conjugate binds to the captured *Leptospira*-specific antibodies. The immune complex formed by the bound conjugate is visualized by adding Tetramethylbenzidine (TMB) substrate which gives a blue reaction product.

The intensity of this product is proportional to the amount of *Leptospira*-specific IgM antibodies in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450 nm is read using an ELISA microwell plate reader.

### 4. MATERIALS

#### 4.1. Reagents supplied

- **Leptospira Coated Wells (IgM):** 12 break-apart 8-well snap-off strips coated with *Leptospira* antigens; in resealable aluminium foil.
- **IgM Sample Diluent:** 1 bottle containing 100 ml of phosphate buffer (10 mM) for sample dilution; pH 7.2 ± 0.2; anti-human IgG; coloured green; ready to use; white cap; < 0.1 % MIT; < 0.1 % CMT; < 0.1 % NaN<sub>3</sub>.
- **Stop Solution:** 1 bottle containing 15 ml sulphuric acid, 0.2 mol/l; ready to use; red cap.
- **Washing Solution (20x conc.):** 1 bottle containing 50 ml of a 20-fold concentrated phosphate buffer (0.2 M), pH 7.2 ± 0.2, for washing the wells; white cap; < 1 % Ethanol; < 0.5 % Bromonitrodioxane.
- **Leptospira anti-IgM Conjugate:** 1 bottle containing 20 ml of peroxidase labelled antibody to human IgM in phosphate buffer (10 mM); coloured red; ready to use; black cap; < 1 % Ethanol; < 0.5 % Bromonitrodioxane.

- **TMB Substrate Solution:** 1 bottle containing 15 ml 3,3',5,5'-tetramethylbenzidine (TMB), < 0.04 %; ready to use; yellow cap; < 0.0001 % CMIT; < 0.0001 % MIT; < 0.01 % H<sub>2</sub>O<sub>2</sub>.
- **Leptospira IgM Positive Control:** 1 vial containing 2 ml control (human serum or plasma); coloured yellow; ready to use; red cap; < 0.1 % Bromonitrodioxan; < 0.1 % MIT.
- **Leptospira IgM Cut-off Control:** 1 vial containing 3 ml control (human serum or plasma); coloured yellow; ready to use; green cap; < 0.1 % Bromonitrodioxan; < 0.1 % MIT.
- **Leptospira IgM Negative Control:** 1 vial containing 2 ml control (human serum or plasma); coloured yellow; ready to use; blue cap; < 0.1 % MIT; < 0.1 % CMIT; < 0.1 % NaN<sub>3</sub>.

#### 4.2. Materials supplied

- 1 Strip holder
- 1 Cover foil
- 1 Test protocol
- 1 Distribution and identification plan

#### 4.3. Materials and Equipment needed

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450/620 nm
- Incubator 37 °C
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex tube mixer
- Deionised or (freshly) distilled water
- Disposable tubes

### 5. STABILITY AND STORAGE

Store the kit at 2...8 °C. The opened reagents are stable up to the expiry date stated on the label when stored at 2...8 °C.

### 6. REAGENT PREPARATION

It is very important to bring all reagents, samples and standards/controls to room temperature (20...25 °C) before starting the test run!

#### 6.1. Coated snap-off strips

The ready to use break-apart snap-off strips are coated with Leptospira antigen. Immediately after removal of the strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2...8 °C.

#### 6.2. Washing Solution (20x conc.)

Dilute Washing Solution 1 + 19; e. g. 10 ml Washing Solution + 190 ml fresh and germ free redistilled water. The diluted buffer is stable for 5 days at room temperature. Crystals in the concentrate disappear by warming up to 37 °C in a water bath.

#### 6.3. TMB Substrate Solution

The reagent is ready to use and has to be stored at 2...8 °C, away from the light. The solution should be colourless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away.

#### 6.4. IgM Sample Diluent

The solution contains anti-human IgG class antibodies to eliminate competitive inhibition from specific IgG class antibodies and to remove rheumatoid factors.

### 7. SPECIMEN COLLECTION AND PREPARATION

Use human serum or plasma (heparin) samples with this assay. If the assay is performed within 5 days after sample collection, the specimens should be kept at 2...8 °C; otherwise they should be aliquoted and stored deep-frozen (-70...-20 °C). If samples are stored frozen, mix thawed samples well before testing. Avoid repeated freezing and thawing.

Heat inactivation of samples is not recommended.

#### 7.1. Sample Dilution

Before assaying, all samples should be diluted 1+100 with IgM Sample Diluent. Dispense 10 µl sample and 1 ml IgM Sample Diluent into tubes to obtain a 1+100 dilution and thoroughly mix with a Vortex.

### 8. ASSAY PROCEDURE

#### 8.1. Test Preparation

Please read the test protocol carefully **before** performing the assay. Result reliability depends on strict adherence to the test protocol as described. If performing the test on ELISA automatic systems we recommend increasing the washing steps from three to five and the volume of Washing Solution from 300 µl to 350 µl to avoid washing effects. Pay attention to chapter 12. Prior to commencing the assay, the distribution and identification plan for all specimens and standards/controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder.

Please allocate at least:

1 well	(e. g. A1)	for the Substrate Blank,
1 well	(e. g. B1)	for the Negative Control,
2 wells	(e. g. C1+D1)	for the Cut-off Control and
1 well	(e. g. E1)	for the Positive Control

It is recommended to determine standards/controls and patient samples in duplicate.

Perform all assay steps in the order given and without any appreciable delays between the steps.

A clean, disposable tip should be used for dispensing each standard/control and sample.

Adjust the incubator to  $37 \pm 1$  °C.

1. Dispense 100 µl standards/controls and diluted samples into their respective wells. Leave well A1 for the Substrate Blank.
2. Cover wells with the foil supplied in the kit.
3. **Incubate for 1 hour  $\pm$  5 min at  $37 \pm 1$  °C.**
4. When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300 µl of Washing Solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be  $> 5$  sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step.  
Note: Washing is critical! Insufficient washing results in poor precision and falsely elevated absorbance values.
5. Dispense 100 µl *Leptospira* anti-IgM Conjugate into all wells except for the Substrate Blank well (e. g. A1). Cover with foil.
6. **Incubate for 30 min at room temperature.** Do not expose to direct sunlight.
7. Repeat step 4.
8. Dispense 100 µl TMB Substrate Solution into all wells.
9. **Incubate for exactly 15 min at room temperature ( $20...25$  °C) in the dark.**
10. Dispense 100 µl Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate.  
Any blue colour developed during the incubation turns into yellow.
11. Measure the absorbance of the specimen at 450/620 nm within 30 min after addition of the Stop Solution.

## 8.2. Measurement

Adjust the ELISA Microwell Plate Reader to zero using the **Substrate Blank in well A1**.

If - due to technical reasons - the ELISA reader cannot be adjusted to zero using the Substrate Blank in well A1, subtract the absorbance value of well A1 from all other absorbance values measured in order to obtain reliable results!

**Measure the absorbance** of all wells at **450 nm** and record the absorbance values for each standard/control and patient sample in the distribution and identification plan.

Dual wavelength reading using 620 nm as reference wavelength is recommended.

Where applicable calculate the **mean absorbance values** of all duplicates.

## 9. RESULTS

### 9.1. Run Validation Criteria

In order for an assay to be considered valid, the following criteria must be met:

- **Substrate Blank** in A1: Absorbance value **< 0.100**
- **Negative Control** in B1: Absorbance value **< 0.200 and < Cut-off**
- **Cut-off Control** in C1 and D1: Absorbance value **0.150 – 1.300**
- **Positive Control** in E1: Absorbance value **> Cut-off**

If these criteria are not met, the test is not valid and must be repeated.

### 9.2. Calculation of Results

The Cut-off is the mean absorbance value of the Cut-off Control determinations.

Example: Absorbance value Cut-off Control 0.44 + absorbance value Cut-off control 0.42 =  $0.86 / 2 = 0.43$   
Cut-off = 0.43

#### 9.2.1. Results in Units [NTU]

$\frac{\text{Patient (mean) absorbance value} \times 10}{\text{Cut-off}} = [\text{NovaTec Units} = \text{NTU}]$

Example:  $\frac{1.591 \times 10}{0.43} = 37 \text{ NTU (Units)}$



### 9.3. Interpretation of Results

Cut-off	10 NTU	-
Positive	> 11 NTU	Antibodies against the pathogen are present. There has been a contact with the antigen (pathogen resp. vaccine).
Equivocal	9 – 11 NTU	Antibodies against the pathogen could not be detected clearly. It is recommended to repeat the test with a fresh sample in 2 to 4 weeks. If the result is equivocal again the sample is judged as <b>negative</b> .
Negative	< 9 NTU	The sample contains no antibodies against the pathogen. A previous contact with the antigen (pathogen resp. vaccine) is unlikely.
Diagnosis of an infectious disease should not be established on the basis of a single test result. A precise diagnosis should take into consideration clinical history, symptomatology as well as serological data. In immunocompromised patients and newborns serological data only have restricted value.		

## 10. SPECIFIC PERFORMANCE CHARACTERISTICS

### 10.1. Precision

Intraassay	n	Mean (OD)	CV (%)
Sample #1	23	0.478	1.9
Sample #2	24	0.893	1.8
Sample #3	24	0.448	1.6
Interassay	n	Mean (NTU)	CV (%)
Sample #1	12	19.15	3.2
Sample #2	12	10.10	4.2

### 10.2. Diagnostic Specificity

The diagnostic specificity is defined as the probability of the assay of scoring negative in the absence of the specific analyte. It is 96.0 %.

### 10.3. Diagnostic Sensitivity

The diagnostic sensitivity is defined as the probability of the assay of scoring positive in the presence of the specific analyte. It is > 98 %.

### 10.4. Interferences

Interferences with hemolytic, lipemic or icteric specimen are not observed up to a concentration of 10 mg/ml hemoglobin, 5 mg/ml triglycerides and 0.5 mg/ml bilirubin.

### 10.5. Cross Reactivity

It cannot be excluded that Cytomegalovirus, Treponema pallidum and Coxiella specimens may result in false-positive IgM antibody results. In addition, it should be noted that IgM class antibodies directed against Leptospira generally remain detectable for months or even years but at low titer.

Note: The results refer to the groups of samples investigated; these are not guaranteed specifications.

## 11. LIMITATIONS OF THE PROCEDURE

Bacterial contamination or repeated freeze-thaw cycles of the specimen may affect the absorbance values.

## 12. PRECAUTIONS AND WARNINGS

- In compliance with article 1 paragraph 2b European directive 98/79/EC the use of the in vitro diagnostic medical devices is intended by the manufacturer to secure suitability, performances and safety of the product. Therefore the test procedure, the information, the precautions and warnings in the instructions for use have to be strictly followed. The use of the testkits with analyzers and similar equipment has to be validated. Any change in design, composition and test procedure as well as for any use in combination with other products not approved by the manufacturer is not authorized; the user himself is responsible for such changes. The manufacturer is not liable for false results and incidents for these reasons. The manufacturer is not liable for any results by visual analysis of the patient samples.
- Only for in-vitro diagnostic use.
- All materials should be regarded and handled as potentially infectious.
- All components of human origin used for the production of these reagents have been tested for anti-HIV antibodies, anti-HCV antibodies and HBsAg and have been found to be non-reactive.
- Do not interchange reagents or strips of different production lots.
- No reagents of other manufacturers should be used along with reagents of this test kit.
- Do not use reagents after expiry date stated on the label.
- Use only clean pipette tips, dispensers, and lab ware.
- Do not interchange screw caps of reagent vials to avoid cross-contamination.

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## Annex 6: ELISA for IgG



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NovaLisa®

# Leptospira IgG

ELISA

CE

Only for in-vitro diagnostic use

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Product Number:      LEPG0660 (96 Determinations)

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## ENGLISH

### 1. INTRODUCTION

Leptospirosis (also known as Weil's syndrome) is probably the most widespread zoonosis in the world. It is caused by infection with spirochete bacteria of the genus *Leptospira* and affects humans as well as a broad spectrum of animal hosts. The incidence is significantly higher in warm climate countries than in temperate regions. The disease is seasonal, with peak incidence occurring in summer or fall in temperate regions, where temperature is the limiting factor in survival of leptospires, and during rainy seasons in warm climate regions, where rapid desiccation would otherwise prevent survival.

Natural reservoirs for the pathogenic *Leptospira interrogans* include rodents as well as a large variety of domesticated mammals (e. g. pigs, cattle and dogs). Leptospires occupy the lumen of nephritic tubules in their natural host and are shed into the urine.

Transmission can occur when humans are directly or indirectly exposed to the urine of infected animals or a urine-polluted environment. Leptospires gain entry into the human blood stream via cuts, skin abrasions or mucous membranes through contact with moist soil, vegetation, and contaminated waters; handling infected animal tissues; and ingestion of food and water. Leptospires are rarely transmitted from human to human.

The incubation period is usually 5-14 days, with a range of 2-30 days.

The spectrum of clinical symptoms is extremely wide. The vast majority of leptospiral infections are either subclinical or result in very mild illness and recover without any complications. Clinical manifestations of leptospirosis range from mild influenza-like symptoms to severe life-threatening disease forms, characterized by jaundice, renal failure, bleeding and severe pulmonary hemorrhage.

The clinical presentation of leptospirosis is biphasic, with the acute or septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine. Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase and thus occur during the second week of the illness. The classical syndrome of Weil's disease represents only the most severe presentation. It is characterized by jaundice, renal failure, hemorrhage and myocarditis with arrhythmias.

Species	Disease	Symptoms	Mechanism of Infection
<i>Leptospira</i> spp.	Leptospirosis	Wide spectrum of clinical symptoms: mild influenza-like symptoms to severe life-threatening disease forms	direct or indirect contact with the urine of an infected animal (via cuts, skin abrasions or mucous membranes)
	Weil's disease	jaundice, renal failure, haemorrhage and myocarditis with arrhythmias	

The presence of pathogen resp. infection may be identified by

- Pathogen detection: dark-field microscopy  
culture from blood, urine, cerebrospinal fluid or tissues  
PCR
- Serology: microscopic agglutination test (MAT), ELISA

### 2. INTENDED USE

The *Leptospira* IgG-ELISA is intended for the qualitative determination of IgG class antibodies against *Leptospira* spp. in human serum or plasma (heparin).

### 3. PRINCIPLE OF THE ASSAY

The qualitative immunoenzymatic determination of IgG-class antibodies against *Leptospira* spp. is based on the ELISA (Enzyme-linked Immunosorbent Assay) technique.

Microtiter strip wells are coated with *Leptospira* antigens to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labelled anti-human IgG conjugate is added. This conjugate binds to the captured *Leptospira*-specific antibodies. The immune complex formed by the bound conjugate is visualized by adding Tetramethylbenzidine (TMB) substrate which gives a blue reaction product.

The intensity of this product is proportional to the amount of *Leptospira*-specific IgG antibodies in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450 nm is read using an ELISA microwell plate reader.

### 4. MATERIALS

#### 4.1. Reagents supplied

- **Leptospira Coated Wells (IgG):** 12 break-apart 8-well snap-off strips coated with *Leptospira* antigens; in resealable aluminium foil.
- **IgG Sample Diluent:** 1 bottle containing 100 ml of phosphate buffer (10 mM) for sample dilution; pH 7.2 ± 0.2; coloured yellow; ready to use; white cap; < 0.1 % MIT; < 0.1 % CMIT; < 0.1 % NaN<sub>3</sub>.
- **Stop Solution:** 1 bottle containing 15 ml sulphuric acid, 0.2 mol/l; ready to use; red cap.
- **Washing Solution (20x conc.):** 1 bottle containing 50 ml of a 20-fold concentrated phosphate buffer (0.2 M), pH 7.2 ± 0.2, for washing the wells; white cap; < 1 % Ethanol; < 0.5 % Bromonitrodioxane.
- **Leptospira anti-IgG Conjugate:** 1 bottle containing 20 ml of peroxidase labelled antibody to human IgG in phosphate buffer (10 mM); coloured blue; ready to use; black cap; < 1 % Ethanol; < 0.5 % Bromonitrodioxane.

- **TMB Substrate Solution:** 1 bottle containing 15 ml 3,3',5,5'-tetramethylbenzidine (TMB), < 0.04 %, ready to use; yellow cap; < 0.0001 % CMIT; < 0.0001 % MIT; < 0.01 % H<sub>2</sub>O<sub>2</sub>.
- **Leptospira IgG Positive Control:** 1 vial containing 2 ml control (human serum or plasma), coloured yellow; ready to use; red cap; < 0.1 % Bromonitrodioxan; < 0.1 % MIT.
- **Leptospira IgG Cut-off Control:** 1 vial containing 3 ml control (human serum or plasma), coloured yellow; ready to use; green cap; < 0.1 % Bromonitrodioxan; < 0.1 % MIT.
- **Leptospira IgG Negative Control:** 1 vial containing 2 ml control (human serum or plasma), coloured yellow; ready to use; blue cap; < 0.1 % MIT; < 0.1 % CMIT; < 0.1 % NaN<sub>3</sub>.

#### 4.2. Materials supplied

- 1 Strip holder
- 1 Cover foil
- 1 Test protocol
- 1 Distribution and identification plan

#### 4.3. Materials and Equipment needed

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450/620 nm
- Incubator 37 °C
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex tube mixer
- Deionised or (freshly) distilled water
- Disposable tubes

### 5. STABILITY AND STORAGE

Store the kit at 2...8 °C. The opened reagents are stable up to the expiry date stated on the label when stored at 2...8 °C.

### 6. REAGENT PREPARATION

It is very important to bring all reagents, samples and standards/controls to room temperature (20...25 °C) before starting the test run!

#### 6.1. Coated snap-off strips

The ready to use break-apart snap-off strips are coated with Leptospira antigen. Immediately after removal of the strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2...8 °C.

#### 6.2. Washing Solution (20x conc.)

Dilute Washing Solution 1 + 19; e. g. 10 ml Washing Solution + 190 ml fresh and germ free redistilled water. The diluted buffer is stable for 5 days at room temperature. Crystals in the concentrate disappear by warming up to 37 °C in a water bath.

#### 6.3. TMB Substrate Solution

The reagent is ready to use and has to be stored at 2...8 °C, away from the light. The solution should be colourless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away.

### 7. SPECIMEN COLLECTION AND PREPARATION

Use human serum or plasma (heparin) samples with this assay. If the assay is performed within 5 days after sample collection, the specimens should be kept at 2...8 °C; otherwise they should be aliquoted and stored deep-frozen (-70...-20 °C). If samples are stored frozen, mix thawed samples well before testing. Avoid repeated freezing and thawing. Heat inactivation of samples is not recommended.

#### 7.1. Sample Dilution

Before assaying, all samples should be diluted 1+100 with IgG Sample Diluent. Dispense 10 µl sample and 1 ml IgG Sample Diluent into tubes to obtain a 1+100 dilution and thoroughly mix with a Vortex.

### 8. ASSAY PROCEDURE

#### 8.1. Test Preparation

Please read the test protocol carefully **before** performing the assay. Result reliability depends on strict adherence to the test protocol as described. If performing the test on ELISA automatic systems we recommend increasing the washing steps from three to five and the volume of Washing Solution from 300 µl to 350 µl to avoid washing effects. Pay attention to chapter 12. Prior to commencing the assay, the distribution and identification plan for all specimens and standards/controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder.

Please allocate at least:

1 well	(e. g. A1)	for the Substrate Blank,
1 well	(e. g. B1)	for the Negative Control,
2 wells	(e. g. C1+D1)	for the Cut-off Control and
1 well	(e. g. E1)	for the Positive Control

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It is recommended to determine standards/controls and patient samples in duplicate.

Perform all assay steps in the order given and without any appreciable delays between the steps.

A clean, disposable tip should be used for dispensing each standard/control and sample.

Adjust the incubator to  $37 \pm 1^\circ\text{C}$ .

1. Dispense 100  $\mu\text{l}$  standards/controls and diluted samples into their respective wells. Leave well A1 for the Substrate Blank.
2. Cover wells with the foil supplied in the kit.
3. **Incubate for 1 hour  $\pm$  5 min at  $37 \pm 1^\circ\text{C}$ .**
4. When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300  $\mu\text{l}$  of Washing Solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be  $> 5$  sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step.  
Note: Washing is critical! Insufficient washing results in poor precision and falsely elevated absorbance values.
5. Dispense 100  $\mu\text{l}$  Leptospira anti-IgG Conjugate into all wells except for the Substrate Blank well (e. g. A1). Cover with foil.
6. **Incubate for 30 min at room temperature.** Do not expose to direct sunlight.
7. Repeat step 4.
8. Dispense 100  $\mu\text{l}$  TMB Substrate Solution into all wells.
9. **Incubate for exactly 15 min at room temperature ( $20\text{--}25^\circ\text{C}$ ) in the dark.**
10. Dispense 100  $\mu\text{l}$  Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate.  
Any blue colour developed during the incubation turns into yellow.
11. Measure the absorbance of the specimen at 450/620 nm within 30 min after addition of the Stop Solution.

## 8.2. Measurement

Adjust the ELISA Microwell Plate Reader to zero using the **Substrate Blank in well A1**.

If - due to technical reasons - the ELISA reader cannot be adjusted to zero using the Substrate Blank in well A1, subtract the absorbance value of well A1 from all other absorbance values measured in order to obtain reliable results!

**Measure the absorbance** of all wells at **450 nm** and record the absorbance values for each standard/control and patient sample in the distribution and identification plan.

Dual wavelength reading using 620 nm as reference wavelength is recommended.

Where applicable calculate the **mean absorbance values** of all duplicates.

## 9. RESULTS

### 9.1. Run Validation Criteria

In order for an assay to be considered valid, the following criteria must be met:

- **Substrate Blank** in A1: Absorbance value  $< 0.100$
- **Negative Control** in B1: Absorbance value  $< 0.200$  and  $< \text{Cut-off}$
- **Cut-off Control** in C1 and D1: Absorbance value  $0.150 - 1.300$
- **Positive Control** in E1: Absorbance value  $> \text{Cut-off}$

If these criteria are not met, the test is not valid and must be repeated.

### 9.2. Calculation of Results

The Cut-off is the mean absorbance value of the Cut-off Control determinations.

Example: Absorbance value Cut-off Control 0.44 + absorbance value Cut-off control 0.42 =  $0.86 / 2 = 0.43$   
Cut-off = 0.43

#### 9.2.1. Results in Units [NTU]

$\frac{\text{Patient (mean) absorbance value} \times 10}{\text{Cut-off}} = [\text{NovoTec Units} = \text{NTU}]$

Example:  $\frac{1.501 \times 10}{0.43} = 37 \text{ NTU (Units)}$

### 9.3. Interpretation of Results

Cut-off	10 NTU	-
Positive	> 11 NTU	Antibodies against the pathogen are present. There has been a contact with the antigen (pathogen resp. vaccine).
Equivocal	9 – 11 NTU	Antibodies against the pathogen could not be detected clearly. It is recommended to repeat the test with a fresh sample in 2 to 4 weeks. If the result is equivocal again the sample is judged as <b>negative</b> .
Negative	< 9 NTU	The sample contains no antibodies against the pathogen. A previous contact with the antigen (pathogen resp. vaccine) is unlikely.
Diagnosis of an infectious disease should not be established on the basis of a single test result. A precise diagnosis should take into consideration clinical history, symptomatology as well as serological data. In immunocompromised patients and newborns serological data only have restricted value.		

## 10. SPECIFIC PERFORMANCE CHARACTERISTICS

### 10.1. Precision

Intraassay	n	Mean (OD)	CV (%)
Sample #1	23	0.437	3.2
Sample #2	24	0.994	2.4
Sample #3	24	0.544	2.4
Interassay	n	Mean (NTU)	CV (%)
Sample #1	12	21.97	8.2
Sample #2	12	13.10	3.5

### 10.2. Diagnostic Specificity

The diagnostic specificity is defined as the probability of the assay of scoring negative in the absence of the specific analyte. It is 97.4 %.

### 10.3. Diagnostic Sensitivity

The diagnostic sensitivity is defined as the probability of the assay of scoring positive in the presence of the specific analyte. It is > 98 %.

### 10.4. Interferences

Interferences with hemolytic, lipemic or icteric specimen are not observed up to a concentration of 10 mg/ml hemoglobin, 5 mg/ml triglycerides and 0.5 mg/ml bilirubin.

### 10.5. Cross Reactivity

It cannot be excluded that polyclonal B-cell activation induced by Epstein-Barr virus (EBV) or the presence of Rheumatoid Factors may result in false-positive *Leptospira* IgG antibody results.

Note: The results refer to the groups of samples investigated; these are not guaranteed specifications.

## 11. LIMITATIONS OF THE PROCEDURE

Bacterial contamination or repeated freeze-thaw cycles of the specimen may affect the absorbance values.

## 12. PRECAUTIONS AND WARNINGS

- In compliance with article 1 paragraph 2b European directive 98/79/EC the use of the in vitro diagnostic medical devices is intended by the manufacturer to secure suitability, performances and safety of the product. Therefore the test procedure, the information, the precautions and warnings in the instructions for use have to be strictly followed. The use of the testkits with analyzers and similar equipment has to be validated. Any change in design, composition and test procedure as well as for any use in combination with other products not approved by the manufacturer is not authorized; the user himself is responsible for such changes. The manufacturer is not liable for false results and incidents for these reasons. The manufacturer is not liable for any results by visual analysis of the patient samples.
- Only for in-vitro diagnostic use.
- All materials should be regarded and handled as potentially infectious.
- All components of human origin used for the production of these reagents have been tested for anti-HIV antibodies, anti-HCV antibodies and HBsAg and have been found to be non-reactive.
- Do not interchange reagents or strips of different production lots.
- No reagents of other manufacturers should be used along with reagents of this test kit.
- Do not use reagents after expiry date stated on the label.
- Use only clean pipette tips, dispensers, and lab ware.
- Do not interchange screw caps of reagent vials to avoid cross-contamination.
- Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.

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- After first opening and subsequent storage check conjugate and standard/control vials for microbial contamination prior to further use.
  - To avoid cross-contamination and falsely elevated results pipette patient samples and dispense reagents without splashing accurately to the bottom of wells.
  - The ELISA is only designed for qualified personnel who are familiar with good laboratory practice.
  - The concentrations of the hazardous materials mentioned in point 4.1. are very low. Therefore there is hardly any toxicological risk. Nevertheless rinse with plenty of water upon contact with eyes, skin or mucous membranes and consult a doctor in case of irritations. All solutions should be handled with adequate care.

### **12.1. Disposal Considerations**

Residues of chemicals and preparations are generally considered as hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations. Contact your local authorities or waste management companies which will give advice on how to dispose hazardous waste.

### **13. ORDERING INFORMATION**

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Prod. No.:      LEPG0660      Leptospira IgG-ELISA (96 Determinations)