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# INVESTIGATION OF HANTAVIRUS AND LEPTOSPIRA AS POSSIBLE CONTRIBUTING FACTORS OF THE UNEXPLAINED KIDNEY DISEASE EPIDEMIC IN NICARAGUA

Nicole J. Delgado  
*UTHealth School of Public Health*

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INVESTIGATION OF HANTAVIRUS AND *LEPTOSPIRA* AS POSSIBLE  
CONTRIBUTING FACTORS OF THE UNEXPLAINED KIDNEY DISEASE EPIDEMIC  
IN NICARAGUA

by  
NICOLE DELGADO, BSPH

APPROVED:

---

CATHERINE TROISI, PHD  
ACADEMIC ADVISOR

---

REBECCA FISCHER, PHD  
THESIS SUPERVISOR

---

RUOSHA LI, PHD  
COMMITTEE MEMBER

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2018

## DEDICATION

To Miguel and Judy Delgado

INVESTIGATION OF HANTAVIRUS AND *LEPTOSPIRA* AS POSSIBLE  
CONTRIBUTING FACTORS OF UNEXPLAINED KIDNEY DISEASE EPIDEMIC IN  
NICARAGUA

by

NICOLE DELGADO  
BSPH, UNIVERSITY OF SOUTH FLORIDA, 2015

Presented to the Faculty of The University of Texas

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for the Degree of

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SCHOOL OF PUBLIC HEALTH  
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INVESTIGATION OF HANTAVIRUS AND *LEPTOSPIRA* AS POSSIBLE  
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NICARAGUA

NICOLE DELGADO, BSPH, MS  
The University of Texas  
School of Public Health, 2018

Thesis Chair: Rebecca Fischer, PhD

Throughout Latin America, an epidemic of a kidney disease of an unknown etiology has been occurring since the late 1990s, and this disease is being called “Mesoamerican nephropathy.” Mesoamerican nephropathy predominantly affects male sugarcane workers. In Chichigalpa, Chinandega, Nicaragua, there is sugarcane plantation that is being heavily impacted by the Mesoamerican nephropathy epidemic, and they invited researchers from Baylor College of Medicine to investigate the epidemic. The prospective epidemic investigation began in 2015, and it is an ongoing investigation. Based on the compilation of data collected during the preliminary investigation, our hypothesis is that a possible zoonotic disease, such as hantavirus and/or *Leptospira*, could be causing Mesoamerican Nephropathy due to the large rodent population in the fields. Our specific aims were to determine the prevalence of hantavirus and *Leptospira* among the study population and to describe and evaluate the differences between potential risk factors for MeN. We tested for

IgM and IgG antibodies using ELISA kits for hantavirus and *Leptospira*. For hantavirus, we tested 149 cases and 50 controls. Due to kit validation issues, we tested 92 controls for *Leptospira* IgM and IgG antibodies, and we tested 104 cases for *Leptospira* IgM antibodies and 45 cases for *Leptospira* IgG antibodies. We also built a multivariate logistic model using the purposeful model selection method to evaluate potential risk factors for the disease. The model was tested for goodness of fit and validated. We found that hantavirus had an overall prevalence of 12.1% and *Leptospira* had 27% prevalence for IgM antibodies with 1.5% for IgG antibodies. Hantavirus and *Leptospira* were not statistically found to be probable causes for the epidemic. The results from the multivariate model found that the use of some types of protective equipment and access to safe drinking water help to reduce the odds of disease. Having an immediate family member also increases the odds. While the results of this study allow us to eliminate hantavirus and *Leptospira*, it does not eliminate a possible zoonotic pathogen. Implementing the use of protective equipment and providing access to safe drinking water may be possible prevention strategies. Continued investigation is needed to determine the etiology of the epidemic.



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## **BACKGROUND**

### **Literature Review**

Throughout Central America, an unexplained kidney disease epidemic has been occurring since the 1990s.<sup>1</sup> The cause(s) of this kidney disease remains unknown. The disease is being called Mesoamerican nephropathy (MeN).<sup>1</sup> MeN is characterized by a rapid progression of kidney disease that is identified when patients present for medical care with chronic kidney disease of unknown etiology (CKDu).<sup>1,2,3</sup> Curiously, the commonly known renal disease risk factors, such as diabetes and hypertension, are not present in the individuals with MeN.<sup>1,4, 5</sup> Previous studies have shown that MeN can also present in cases with acute kidney injury (AKI).<sup>1,6-10</sup> However, most studies investigating MeN focus on patients in the late stages of disease (CKDu) rather than in the earliest, acute phase (AKI).

One of the countries highly impacted by this epidemic is Nicaragua.<sup>1,11,12</sup> Within Nicaragua, MeN is disproportionately impacting sugarcane workers in Chichigalpa, Chinandega.<sup>1,13,14</sup> One leading hypothesis suggests an infectious agent causing the epidemic.<sup>1</sup> Researchers noted the warm, tropical weather patterns for this area alongside occupational and environmental exposures that could be conducive to infectious disease transmission.<sup>1</sup> A large rodent population was discovered, which could harbor pathogens potentially responsible for causing kidney disease that the workers are exposed to in the sugarcane fields.<sup>1</sup> Two possible infectious rodent-borne pathogens are hantavirus and *Leptospira*.<sup>1</sup> However, the hypothesis of one of these, or any other agents, as the cause of MeN has not yet been fully examined.

### **Hantavirus**

Hantavirus is a relatively recently described pathogen infecting humans. Hemorrhagic fever with renal syndrome (HFRS) is one of the major clinical scenarios that results from

hantavirus infection in humans.<sup>13-15</sup> HFRS manifests typically through 4 clinical phases.<sup>14,16</sup> In the first phase, the majority of HFRS cases experience high fevers, flulike symptoms, abdominal pain, and headaches accompanying the renal syndrome.<sup>14</sup> Renal syndrome is characterized by elevated creatine levels and reduced renal filtration. The real danger of HFRS is the damage to the vascular system that occurs throughout the body, which can lead to internal hemorrhaging throughout the capillaries.<sup>14</sup> When symptoms are present, antibody detection with seroconversion and virus isolation are the best diagnostic methods.<sup>15</sup> IgM assays can detect antibodies to hantavirus in serum during the acute infection phase. Using IgM and IgG assays on paired (acute and convalescent) sera samples can be used to demonstrate seroconversion.<sup>15</sup>

Throughout the world, rodent population dynamics are strongly connected to hantavirus epidemics.<sup>17,18</sup> In geographical areas highly populated with rats, there are higher numbers of HFRS cases.<sup>19</sup> Rats and mice are the predominant reservoirs and carriers of hantavirus that can transmit the infection to humans.<sup>17</sup> The most common mode of transmission is when infected rat urine or feces becomes airborne and inhaled by a human.<sup>17</sup> For this route of transmission, these factors must occur: a highly dense carrier population, the carrier's habitat must be available to the human, and the human needs to come in contact with the virus while it is viable outside of the carrier.<sup>17</sup> Thus, breaking the link between the carrier and the human could be beneficial to preventing hantavirus infection. Understanding specific exposures putting people at risk in settings where hantavirus is known to circulate can ultimately lead to targeted prevention measures.

In recent years, the frequency of hantavirus infections is rising.<sup>17</sup> One study conducted by Montoya-Ruiz, et al examined the prevalence of hantavirus in 10 nations located in Central and South America.<sup>20</sup> Within the Americas, the monitoring of hantavirus is a more recent

development since the 1990s.<sup>20</sup> Reports state that there are over 30 different strains of hantavirus within Central and South America.<sup>20</sup> In their research, they found an outbreak of hantavirus infection in humans in Panama that was most likely transmitted from rodents.<sup>20</sup> Overall, they found that there is need for improved surveillance of hantavirus in Latin America.

## **Leptospirosis**

Spirochetes of the genus *Leptospira* cause leptospirosis.<sup>19-22</sup> Of all zoonotic infections, leptospirosis is the most common in the world.<sup>21-22</sup> Globally, there are more than 1 million people infected by leptospirosis every year.<sup>22</sup> This creates a significant global burden of disease. Nations with tropical and subtropical climates have the highest transmission rates of leptospirosis.<sup>19,21-22</sup> As global warming continues to raise temperatures and alter weather patterns worldwide, the risk of *Leptospira* infections will greatly increase.<sup>22</sup> In addition, tropical and subtropical climates are susceptible to leptospirosis epidemics following strong storm systems with large amounts of rain and flooding.<sup>22</sup>

Like Hantavirus, rodents are the main carriers of *Leptospira*, but other mammals can also carry and transmit *Leptospira*.<sup>22</sup> Also similar to hantavirus, transmission of *Leptospira* to humans involves exposure to infected urine.<sup>21-22</sup> This can occur through exposure to water and soil that have been contaminated with infected urine. Within endemic areas, people living in impoverished areas and people with occupational exposures are the most vulnerable groups for contracting leptospirosis.<sup>22</sup> The occupation with the highest prevalence of leptospirosis is sugarcane workers.<sup>22</sup> A study conducted in Chinandega, Nicaragua, found that almost three-fifths of sugarcane workers have leptospirosis.<sup>22-23</sup> Some possibility of *Leptospira* infection transmission to sugarcane workers are exposure to infected rats living in the fields, aerosolization

of infected field soil, and contact with run-off from fields that collect in irrigation and drainage ditches.<sup>22-23</sup>

Leptospirosis manifests itself in humans in different ways. There is typically a range of people experiencing mild to severe symptoms, with only a few individuals will be asymptotic.<sup>22</sup> For minor illness, people typically experience a fever, headache, and myalgia, but there can be more negative consequences from infection.<sup>24</sup> For instance, leptospirosis can negatively impact people through the kidneys by causing renal insufficiency or acute renal failure.<sup>24</sup> This can lead to AKI and CKD in individuals without other risk factors for kidney disease.<sup>22</sup> As with hantavirus, a common practice is to test for IgM and IgG antibodies to *Leptospira* in humans presenting with symptoms of leptospirosis.

Numerous studies have examined leptospirosis cases in endemic nations. One example is a cohort study conducted in Taiwan following a flood which monitored communities to assess a possible relationship between CKD and leptospirosis.<sup>21</sup> Data from that study suggest that CKD may result from *Leptospira* infection. A retrospective cohort study conducted by Daher et al in Brazil looked at clinical data from patients with confirmed leptospirosis.<sup>25</sup> Of these cases, nearly 90% presented with AKI.<sup>25</sup> Many patients had the common symptoms of leptospirosis such as fever, headache, myalgia, etc.<sup>25</sup> Most of those patients were young adults.<sup>25</sup> Within that population, they also found that individuals working as farmers had a greater risk of leptospirosis.<sup>25</sup>

### **Public Health Significance**

The Pan American Health Organization estimates that over 50,000 individuals have died prematurely as a result of MeN.<sup>1,6,26</sup> Despite the vast number of lives this epidemic has claimed, the cause of MeN remains unknown. One of the primary goals of epidemiology is to determine

the cause(s) of disease in order to stop epidemics and to prevent more individuals from becoming diseased. Since most individuals with MeN do not have the expected risk factors to develop kidney disease, something else, possibly an infectious pathogen or pathogens, is causing MeN.<sup>1</sup> For this reason, improved surveillance and testing of leptospirosis and hantavirus may be beneficial in endemic nations experiencing CKDu epidemics. As previously mentioned, prior studies investigating MeN focused primarily on CKDu cases. The studies that have researched AKI have done so to document that AKI is a part of the early progression of MeN.<sup>6-10</sup> Inasmuch, there is a need to investigate whether the AKI in the early disease process is linked to leptospirosis or hantavirus infection and to understand the potential occupational exposures and behavioral exposures associated with AKI in MeN.

### **Specific Aims**

In Chichigalpa, Chinandega, Nicaragua, there are over 500 sugarcane workers with MeN working at ISA.<sup>1</sup> As throughout Latin American, the cause of the MeN remains a mystery.<sup>1</sup> In Chichigalpa, the sugarcane fields have a high-density population of rodents, and sugarcane workers spend extended periods of time within these fields, exposing them to infectious diseases transmitted by rodents. The epidemiology of rodent-borne diseases, such as leptospirosis and hantavirus, has not been described within this specific population of sugarcane workers and has not yet been evaluated as the cause of MeN.<sup>1</sup>

The long-term goal of this study is to discover the cause(s) of MeN and to prevent new cases from occurring. The overall objective of this proposed thesis is to investigate hantavirus and leptospirosis as potential causes of MeN. The central hypothesis of this study is that hantavirus and/or leptospirosis will be more prevalent in individuals with MeN than healthy individuals. The central hypothesis will be objectively tested through 2 specific aims:



**Aim 1:** To determine the prevalence of hantavirus and leptospirosis among cases and controls through laboratory testing.

**Aim 2:** To describe and evaluate the differences potential risk factors for MeN through statistically analyzing interview data from cases of MeN and controls

The expected outcomes of this study are as follows: the cases will have a higher prevalence of antibodies to *Leptospira* than controls and higher prevalence of antibodies to hantavirus than controls. Differences in possible risk factors for MeN between cases and controls are also expected, with the cases having risk factors for rodent-borne pathogen transmission than the controls have. The alternative, null hypothesis is that the prevalence of antibodies to hantavirus and leptospirosis will not be different between cases and controls. Additionally, the alternative, null hypothesis predicts that there will not be differences in exposures between cases and controls.

## **METHODS**

### **Study Design**

This is a cross-sectional study to determine if hantavirus infection and/or leptospirosis may be involved with the MeN epidemic in Nicaragua. This study is being conducted as part of a larger investigation into the MeN epidemic entitled “Investigation into the Etiology of Unexplained Kidney Disease in Nicaragua” by researchers at Baylor College of Medicine in Houston, Texas, and Texas A&M University Health Science Center in College Station, Texas.

### **Study Setting**

The study setting is a commercial sugarcane plantation, Ingenio San Antonio (ISA), in Chichigalpa, Chinandega, Nicaragua. The area surrounding ISA has been identified as one of the MeN “hotspots” in Central America as well as a possible epicenter of the MeN epidemic.<sup>27</sup> Due

to this high burden of MeN, this study site is ideal for the etiologic investigation. In February 2015, researchers initiated active surveillance for early-stage MeN disease at ISA's large, private hospital, which is located on the plantation grounds. The ISA workers and their families use this hospital for their primary healthcare needs. Surveillance is currently ongoing.

### Study Subjects

To be included in the study, an individual must work at ISA. Over 70% of the subjects live in Chichigalpa, as well. Most are under the age of 35. See table 1 for a description of the study population.

**Table 1: Sample Demographics**

		<b>Controls (N=160)</b>	<b>Cases (N=584)</b>	<b>P-value for Pearson Chi2</b>
<b>Age 30+</b>	Yes	86	235	Pr = 0.002
	No	74	349	
<b>Sex</b>	Male	150	527	Pr = 0.489
	Female	13	57	
<b>Smoking Status</b>	No, Never	77	396	Pr = <0.001
	Yes, Former	38	83	
	Yes, Current	41	130	
<b>Number of years worked at ISA</b>	$\leq 2$	24	136	Pr=0.003
	3 to 6 years	54	171	
	7 to 10 years	32	118	
	>10	53	112	

A case is defined as an individual who presented to the emergency department at the estate's private hospital and was diagnosed with AKI suspected as due to MeN by the local

attending physician. For the overarching study, local physicians at the hospital completed case report forms on each case, and the hospital laboratory reported clinical laboratory findings (urine analysis, blood chemistry, and hematology). Common complaints were fever, nausea, vomiting, arthralgia, myalgia, headache, neck and back pain, weakness, and paresthesia. Furthermore, most cases had evidence of systemic inflammation or immune activation.<sup>1</sup> After a case was discharged from the hospital, a trained study nurse would obtain informed consent into the overarching study prior to administering an interview about the case's possible exposures and behaviors at both work and home. Cases of MeN occur throughout the year. For the purpose of this study, cases were selected from individuals presenting within the time frame of January 2015 – February 2017.

A control is defined as an individual who works at the ISA sugarcane plantation and who has never been diagnosed with MeN or other type of kidney disease. Controls were recruited in October 2016, during the routine, annual occupational health screenings that are held before each harvest season. A trained study nurse obtained informed consent and administered an interview similar to the interview administered to infected cases. The information for cases and controls has been previously coded with study and sample IDs.

### **Sample Size Calculation**

To calculate the sample size for Aim 1 to determine the prevalence of leptospirosis and hantavirus infection, the prevalence of *Leptospira* antibodies in a study sample was first calculated in a preliminary study, which is 60%.<sup>27</sup> The formula used to calculate sample size:

$$n = \frac{p(1 - p)(z_{\alpha/2})^2}{d^2}$$

For this formula,  $p$  is 0.60,  $z_{\alpha/2}$  is 1.96 for 95% confidence intervals, and  $d$ , the absolute error, was 6.8%. This gave a sample size of 199.39, which was rounded up to 200 for the total sample

size. The sample size calculation was confirmed through OpenEpi.<sup>28</sup> For the ELISA assays, a sample of 100 controls and 100 cases were drawn for the acute sample testing only. An additional sample of 75 cases were added to test seroconversion using paired (acute and convalescent) samples. For aim 2, all individuals on whom interview data was collected will be used in analysis. The entire universe of data will be analyzed, and no sample will be drawn. For this reason, a sample size was not calculated. Data include case report forms on 610 case patients and a subset of interview data on 244 case patients and 163 controls. These data will be included in the statistical analysis portion.

### **Data Collection**

As part of the larger investigation into the etiology of MeN, the cases and controls are currently being tested for evidence of exposure to hantavirus and *Leptospira* via enzyme-linked immunosorbent assay (ELISA) to detect antibodies (IgM and IgG). For both hantavirus and *Leptospira* infections, use of ELISA is common practice to detect antibodies. The ELISA kits for hantavirus are Focus Diagnostics IgM, and IgG DxSelect™ (Langenhagen-Hannover, Germany). The ELISA assay kits for leptospirosis are Virion-Serion (Wurzburg, Germany) classic *Leptospira* IgG and IgM tests. The ELISA assays are performed following the instructions for each kit. The specimens to be tested are selected using simple random sampling from the sera stored in Houston. The samples are brought to room temperature gradually by either thawing in the refrigerator overnight or thawing on the lab bench the day of the ELISA assay. The recommended number of positive controls, negative controls, cut offs, and blanks are used for each plate run as specified by the kit instructions; all procedures were carried out according to kit specifications (see Appendix I and II). Serum samples are stored in -80°C freezers in Houston.

During the overarching study, any worker presenting with AKI to the ISA hospital were asked for consent and had a serum sample taken and stored. These individuals were monitored and followed up with trained cases professionals to see if they would develop MeN. Convalescent samples were taken after an individual was classified as a case. The sera samples used for this study are from individuals who did develop MeN. All workers undergo occupational health screenings prior to employment each harvesting season.<sup>1</sup> The serum samples from controls in this analysis were drawn during the October 2016 screenings, which is when control workers were enrolled in the study and interviewed about potential exposures of interest, including demographics, medical history, and possible risk factors (environmental, occupational, behavioral). For example, some of the information gathered includes drinking water sources, handwashing practices, exposure to rodents, type of occupation at ISA, etc.

For the cases, the serum collected during hospital admission at the onset of acute kidney disease were analyzed. In addition, approximately  $\geq 3$  months following discharge from the hospital, convalescent samples were also drawn from cases. For 75 of the cases, paired acute and convalescent samples were analyzed to evaluate seroconversion from IgM (acute) to IgG (convalescent) antibodies for hantavirus and *Leptospira*.

## **Data Analysis**

The statistical analysis portion of the proposed thesis will be broken into two main parts.

Aim 1:

### **a) Prevalence of Antibodies**

The results from the ELISA assays for both hantavirus and leptospirosis will be analyzed separately, for prevalence ratios between cases and controls. The dependent variable is acute kidney disease (case status). For each infection, the main independent variable is positive

serology. A serum sample exhibiting a seropositive test result is defined as ELISA test which is positive for either IgM or IgG, according to manufacturer protocol, indicating a current, recent, or past antibody response to pathogen. For a subset of cases, the acute seroconversion rate between IgM and IgG for hantavirus and *Leptospira* infections will be calculated for the paired acute and convalescent samples. Seroconversion is defined as:

1. having IgM negative acute specimen with IgM or IgG positive convalescent specimen  
or
2. having IgM positive acute specimen with IgG positive convalescent specimen.

#### b) Descriptive Statistics

Using data from the case reports and interviews, the prevalence of hantavirus and leptospirosis will be calculated for each variable individually: sex, age, residence, drug use, the number of years working at the sugarcane field, type of position at the sugarcane farm, working in contact with soil, and working in contact with water. Each variable will first be tested against Pearson's Chi Test or Fischer's Exact test if there are too few observations. Then, for the significant variables, the prevalence ratios with confidence intervals will be calculated for all of these, using Poisson regression to estimate. In addition, statistical assessment for potential confounders will be performed. This descriptive analysis will be conducted separately for *Leptospira* and hantavirus infection, each using both seropositive and seroconverted outcomes.

#### Aim 2: Logistic Regression:

In order to evaluate the impact of hantavirus or *Leptospira* infection positivity and other potential exposures in MeN, data from the interviews will also be analyzed alongside the laboratory testing results to build a logistical model to evaluate each characteristic as a potential

risk factor, comparing patients with MeN to control workers. The odds ratios with confidence intervals for the variables selected in the multivariate model will be reported. See Table 2 for a list of variables to be tested for the model. The response variable is MeN case status. A subset of the dataset with complete interview information will be used for model building. The purposeful model selection steps as defined by Hosmer, Lemeshow, and Sturdivant will be used for model selection.<sup>29</sup>

**Table 2: Variables to be included in model selection**

Sex	Age	Residence location	Drug use
Number of years working at ISA	Type of position at ISA	Contact with soil while working	Contact with water while working
Have seen rodents in the fields at work	Have seen rodents at home	Hand washing practices at work and home	Drinking water sources at work and home
Protective gear use	Own pets	Gardening as a hobby	Medical history
Family history of Mesoamerican Nephropathy			

First, a univariable analysis will be conducted individually for each potential variable using the likelihood ratio (LR) test. Categorical variables with multiple levels will be tested using the LR test followed by a contingency table analysis to confirm the decision to keep or drop the variable. All variables that have a p-value less than 0.25 will be used to run one large multivariate logistical model. The LR test will be used to determine which variables to exclude and to include using a p-value of 0.05 or less. Each excluded variable will be individually added to the model and tested again using the LR test to see if it is a significant variable. If it is significant, a LR test between the model with the added variable compared to the null model will determine if the variable will be included in the next step of model building. Retained variables will form the preliminary main effects model. Prior to moving to the next step of the purposeful

model selection, the variables that were excluded during the univariate analysis will be added to the model individually and then tested using the LR test. If they are significant, they will be added to the model.

For all the continuous variables in the model, linearity will be tested using a Lowess smooth plot. Variables that demonstrate linearity will be included in the model. The variables that break linearity will be transformed through fractional polynomials and cubic spline functions to see if the linearity is fixed. The models generated will then be tested against the original preliminary effects model. If the LR test is significant, the transformed variable will be included in the model.

Before accepting the final model, any biologically plausible interactions will be tested. Each biologically plausible interaction term will be tested individually in the model. The LR test will be kept in the model if the results are significant. The final preliminary model will be complete, and the AIC will be tested.

Prior to accepting the model, two methods will be used to assess the goodness of fit: The Pearson  $\chi^2$  Goodness of Fit test and Hosmer-Lemeshow Test using 10 groups. Comparison of the AICs, log likelihoods, LR test,  $R^2$ , Pearson  $\chi^2$  Goodness of fit, and the Hosmer-Lemeshow test will be used to assess the model. Finally, the model will be also be validated in STATA. The dataset will be run through STATA's stepwise model building program, and the generated model will be compared to the model built through the purposeful selection.

After the final model is built, the data will then be interpreted through the model. The odd ratios with confidence intervals of each variable included in the final model will be assessed. This information will be used to guide future steps in the investigation of MeN.



## **Human Subjects**

The IRB approval for this study is from the Baylor College of Medicine. The Principal Investigator Dr. Kristy Murray granted permission to analyze data and specimens. The study was also reviewed and approved by the ethics boards at the Nicaragua Ministry of Health and by the Medical Directors of the Hospital Alfredo Chamorro Pellas and Gerencia de Salud Ocupacional at ISA. Participants who were interviewed gave consent for the use of the interview data and samples. Data from individuals who were not interviewed were exempted from consent by all ethics boards. All data from participants used in the study has been deidentified to protect participants. The thesis proposal was submitted to iRIS of the UThealth system on August 13, 2018, for approval, and it was approved on September 6, 2018.

## **RESULTS**

### **Specific Aim 1: ELISA Lab Results for Hantavirus and *Leptospira***

A total of 199 patients were tested for IgG and IgM antibodies to hantavirus (149 cases and 50 controls). A total of 90 paired acute and convalescent sera samples from cases were tested for seroconversion in antibodies to hantavirus. A total of 196 patients were tested for IgM antibodies to *Leptospira* (104 cases and 92 controls), and a total of 137 patients were tested for IgG antibodies to *Leptospira* (92 controls and 45 cases). The results of the ELISA Hantavirus IgM and IgG are displayed below in Table 3.

The prevalence of hantavirus IgG antibodies is 9.6% among all tested samples with 6.7% of cases testing positive and 18% of controls testing positive. The odds of testing negative for IgG among cases were more than 3 times as likely than among controls. The test of homogeneity between cases and controls was significant for hantavirus IgG results, but not for hantavirus IgM results. For IgM hantavirus antibodies, only 3.4% of the cases tested positive with none of the

controls testing positive. Of the 90 paired acute and convalescent samples, there was 6.67% (n=6) seroconversion rate.

**Table 3: ELISA Results**

<b>ELISA Results</b>			
	<b>Negative</b>	<b>Positive</b>	<b>Total</b>
<b>Hantavirus IgG</b>			
<b>Control</b>	41 (82%)	9 (18%)	50
<b>Case</b>	139 (93%)	10 (7%)	149
<b>Total</b>	180 (90%)	19 (10%)	199
		Test of Homogeneity P-value =0.191	
<b>Hantavirus IgM</b>			
<b>Control</b>	50 (100%)	0 (0%)	50
<b>Case</b>	144 (97%)	5 (3%)	149
<b>Total</b>	194 (97.5%)	5 (2.5%)	199
		Test of Homogeneity P-value=0.019	
<b>Leptospira IgG</b>			
<b>Control</b>	90 (98%)	2 (2%)	92
<b>Case</b>	45 (100%)	0 (0%)	45
<b>Total</b>	135 (98.5%)	2 (1.5%)	137
		Test of Homogeneity P-value =0.3208	
<b>Leptospira IgM</b>	<b>Negative</b>	<b>Positive</b>	<b>Total</b>
<b>Control</b>	63 (68%)	29 (32%)	92
<b>Cases</b>	80 (77%)	24 (23%)	104
<b>Total</b>	143 (73%)	53 (27%)	196
		Test of homogeneity p-value= 0.1852	

The possible exposure factors were each tested against the positive hantavirus ELISA results for IgM and IgG. None of them were significant, except for three variables. The first one was the difference between cases and controls testing positive for hantavirus IgG antibodies.

More controls than cases tested positive for IgG antibodies (PR 0.373 [CI 0.160-0.867];

p=0.032). The second is the IgM result for individuals who have contact with rain water while working. Individuals testing positive for hantavirus were more than 10 times as likely to have contact with rain water than those who did not (PR 10.6 [1.64-68.8]; p=0.018). The third and last significant result was for an IgG hantavirus positive antibody result and for those who currently drink alcohol and/or used to drink alcohol. For the individuals who have ever drank alcohol, they were significantly less likely to test positive for IgG hantavirus antibodies than those who have never drank alcohol (PR 0.108 [0.014-0.860]; p=0.035).

### **Specific Aim 2: MeN Multivariate Model**

The results from the univariate analysis are displayed in Table 4. See Appendix VI for the results of all the variables examined. A total of 107 variables were examined prior to being analyzed to build a statistical model. The final model is  $\log[\text{casecontrol}] = -1.958[\text{work involves contact with the field}] - 1.343[\text{handwash using clean municipal water}] + 2.567[\text{drank from a particular well in the field}] - 2.597[\text{drinks from wells in general}] - 0.949[\text{uses protective gloves}] + 1.38[\text{wears protective long sleeves}] + 1.402[\text{has an immediate family member with MeN}] - 0.793[\text{has smoked}] - 1.132[\text{has had moonshine}] + 1.474[\text{sees rats in the field while working}] - 1.94[\text{works in the field in general}] - 1.472[\text{wears protective eye glasses}] + 1.01$ . Table 5 displays the results of the final statistical model predicting MeN. The model passed the goodness of fit testing as well as the validation step.

**Table 4: Univariate Analysis with Whole Dataset**

Variable	Obs	Control (n/N)	Cases (n/N)	OR	Std Error	Z	P-Value	Confidence Intervals	
<b>Sex (Males)</b>	747	150/163 (92%)	527/584 (90%)	1.248	0.401	0.690	0.490	0.665	2.341
<b>AGE CATEGORIES</b>									
<b>Above the age of 30</b>	744	86/160 (23%)	235/584 (40%)	0.579	0.104	- 3.040	0.002	0.407	0.824
<b>Above the age of 25</b>	744	119/160 (74%)	389/584 (66.7%)	0.687	0.138	- 1.860	0.062	0.463	1.020
<b>Above the age of 35</b>	744	45/160 (28%)	132/584 (23%)	0.746	0.151	- 1.450	0.147	0.503	1.108
<b>Resides in Chichigalpa</b>	739	100/159 (63%)	377/580 (65%)	1.096	0.204	0.490	0.623	0.761	1.577
<b>DRUG USE</b>	721								
<b>Yes, former</b>		12/161 (7.5%)	28/560 (5%)	0.661	0.237	-1.16	0.248	0.328	1.333
<b>Yes, current</b>		28/560 (5%)	17/560 (3%)	1.606	1.017	0.75	0.454	0.464	5.557
<b>Have Ever Used Drugs</b>		15/161 (9%)	45/560 (8%)	0.850	0.266	- 0.520	0.604	0.461	1.569
<b>TOTAL YEARS WORKED AT ISA</b>									
	700								
<b>&lt;2 years</b>		24/163 (15%)	136/537 (25%)						
<b>3-10 years</b>		86/163 (53%)	289/537 (54%)	0.593	0.150	-2.06	0.039	0.361	0.974
<b>&gt;10 years</b>		53/163 (33%)	112/537 (21%)	0.373	0.103	-3.56	<0.001	0.217	0.642
<b>OCCUPATION</b>									
<b>Workings in carrying/hauling</b>	407	5/163 (3%)	18/244 (7%)	2.517	1.299	1.790	0.074	0.915	6.920
<b>Works as General Harvester</b>	407	27/163 (17%)	15/244 (6%)	0.330	0.112	- 3.260	0.001	0.170	0.642

Variable	Obs	Control (n/N)	Cases (n/N)	OR	Std Error	P-Value	Confidence Intervals	
OCCUPATION CONTINUED								
Works in planting	407	39/163 (24%)	43/244 (18%)	0.680	0.169	0.121	0.418	1.108
Pest Control	407	5/163 (3%)	15/244 (6%)	3.389	3.729	0.267	0.392	29.278
Weed Control	407	2/163 (1%)	18/244 (7%)	6.411	4.824	0.014	1.467	28.019
Works in Irrigation/Drainage	407	29/163 (18%)	42/244 (17%)	0.961	0.255	0.880	0.571	1.618
Auto mechanic	407	15/163 (9%)	8/244 (3%)	0.334	0.151	0.015	0.138	0.808
occup_fabrica	407	2/163 (1%)	6/244 (2.5%)	2.029	1.670	0.390	0.405	10.181
Work involves contact with the field	370	115/163 (71%)	89/207 (43%)	0.315	0.07	<0.001	0.204	0.486
Works with soil	175	103/163 (63%)	10/12 (83%)	2.913	2.305	0.177	0.617	13.739
Works with dry soil	239	68/163 (42%)	5/76 (7%)	0.098	0.048	< 0.001	0.038	0.257
Works with wet soil	239	71/163 (44%)	4/76 (5%)	0.072	0.039	< 0.001	0.025	0.206
Works with soil in the field	239	69/163 (42%)	7/76 (9%)	0.138	0.059	< 0.001	0.060	0.319
RODENT EXPOSURE IN HOUSE								
Sees rats in house	202	116/148 (78%)	43/54 (80%)	1.078	0.423	0.848	0.500	2.327
Sees rat feces in house	184	60/130 (46%)	18/54 (33%)	0.583	0.197	0.111	0.301	1.132
Sees rats in house every day	202	19/148 (13%)	15/54 (28%)	2.611	1.020	0.014	1.214	5.617
Sees rats in house frequently	196	66/147 (45%)	18/49 (37%)	0.713	0.242	0.318	0.366	1.386
HANDWASH PRACTICES								
Handwashes hands with water from house	407	120/163 (74%)	161/244 (66%)	0.695	0.155	0.103	0.449	1.077
Handwashes with clean municipal water	407	27/163 (17%)	8/244 (3%)	0.171	0.071	0.000	0.075	0.386

Variable	Obs	Control (n/N)	Cases (n/N)	OR	P-Value	Confidence Intervals	
<b>HANDWASH PRACTICES</b>							
Handwash with from municipal truck water	407	7/163 (4%)	19/244 (8%)	1.882	0.164	0.773	4.584
Handwashes with ditch water	407	4/163 (2.5%)	12/244 (5%)	2.056	0.219	0.651	6.490
Handwashes with water from drainage tube	407	1/163 (0.6%)	16/244 (7%)	11.368	0.019	1.493	86.586
Handwashes with well water	407	5/163 (3%)	13/244 (5%)	1.778	0.283	0.622	5.087
Always washes hands before eating	407	138/163 (85%)	182/244 (75%)	0.532	0.016	0.318	0.889
Usually washes hands before eating	407	17/163 (10%)	30/244 (12%)	1.204	0.564	0.641	2.263
Rarely washes hands before eating	407	4/163 (2.5%)	12/244 (5%)	2.056	0.219	0.651	6.490
Never washes hands before eating	407	4/163 (2.5%)	12/244 (5%)	2.056	0.219	0.651	6.490
<b>DRINKING WATER SOURCE</b>							
Drink from clean municipal water	407	35/163 (21%)	11/244 (5%)	0.173	0.000	0.085	0.352
Drink from potable water truck	407	9/163 (6%)	11/244 (5%)	0.808	0.644	0.327	1.995
Drink from a particular well in ISA's field	407	7/163 (4%)	22/244 (9%)	2.208	0.076	0.921	5.297
Drink from water in a ditch	407	1/163 (0.6%)	6/244 (2%)	4.084	0.195	0.487	34.243
Drink from drainage pipe	407	1/163 (0.6%)	9/244 (4%)	6.204	0.085	0.778	49.446
Drink from drainage tube	407	2/163 (1%)	15/244 (6%)	5.273	0.029	1.189	23.376
<b>USE OF PROTECTIVE EQUIPMENT</b>							
Wears gloves	376	126/142 (89%)	157/234 (67%)	0.259	< 0.001	0.144	0.466
Wears protective eye glasses	378	98/124 (79%)	84/236 (36%)	0.248	< 0.001	0.159	0.387
Wears mask over mouth	373	64/140 (46%)	49/233 (21%)	0.316	< 0.001	0.200	0.500
Wears mask over mouth and nose	372	40/140 (29%)	35/232 (15%)	0.444	0.002	0.266	0.742
Wears long sleeves	379	113/141 (80%)	228/238 (96%)	5.650	< 0.001	2.652	12.037

Variable	Obs	Control (n/N)	Cases (n/N)	OR	P-Value	Confidence Intervals
<b>Wears boots</b>	380	139/141 (99%)	236/239 (99%)	1.132	0.893	0.187 6.857
<b>FAMILY HISTORY OF MeN</b>						
<b>Family member with MeN</b>	371	48/141 (34%)	140/230 (61%)	3.014	< 0.001	1.946 4.668
<b>Immediate family member with MeN (parent or sibling)</b>	371	32/141 (23%)	119/141 (84%)	3.652	< 0.001	2.279 5.851

**Table 5: Prediction Model for MeN**

<b>Case Control</b>	<b>Odds Ratio</b>	<b>Std. Err.</b>	<b>P&gt;z</b>	<b>[95% Conf.</b>	<b>Interval]</b>
<b>OCCUPATION BASED</b>					
Work involves contact with field	0.141	0.053	<.001	0.067	0.296
Sees rats in the field while working	4.368	2.038	0.002	1.751	10.899
Works as a general harvester	0.303	0.168	0.032	0.102	0.900
<b>WATER SOURCES</b>					
Handwashing with clean municipal water sources	0.261	0.157	0.026	0.080	0.850
Drinking from a particular well in the field	13.027	9.829	0.001	2.969	57.158
Drinking water from well water in general	0.074	0.037	<0.001	0.028	0.199
<b>USE OF PROTECTIVE EQUIPMENT/GEAR</b>					
Wear gloves	0.387	0.164	0.025	0.169	0.889
Wear protective eye glasses	0.229	0.092	<0.001	0.105	0.503
Wear long sleeves	3.961	2.103	0.010	1.399	11.211
Family history of CKD (sibling or parent)	4.063	1.472	<0.001	1.998	8.264
History of smoking	0.452	0.157	0.023	0.229	0.895
History of drinking moonshine	0.323	0.165	0.027	0.119	0.878
_cons	2.745	2.124	0.192	0.602	12.508



## DISCUSSION

The results from the ELISAs do not support the hypothesis that hantavirus is causing MeN. The prevalence of testing positive to hantavirus IgG antibodies signaling a possible previous infection is low with an 18% prevalence among controls and 6.7% among cases. The significant result of the homogeneity test suggests that cases may be less likely to have had a hantavirus infection in the past than the controls. The POR of testing positive for IgG antibodies to hantavirus for cases was 0.373 against controls which also supports that cases are less likely to have had hantavirus in the past. However, only 3.4% of cases have IgM antibodies to the hantavirus with none of the controls testing positive for IgM antibodies to the hantavirus. While there was a significant POR for those who work with rain water and testing positive for hantavirus IgM antibodies, the confidence interval was very broad. Overall, the IgM and IgG ELISA results do not support further investigation into hantavirus as a potential causative agent for MeN in Nicaragua.

The univariate analysis results suggest numerous risk factors for MeN as well as some of confounding factors. For instance, age is found to be highly significant within the dataset. Individuals younger than 30 appear to have nearly 50% greater odds of suffering MeN than those 30 or older than 30. However, most of the workers are 30 years of age or younger, which potentially skews the result. Another example of this is the total number of years worked at ISA, which suggests the more years worked at ISA as being protective against MeN. However, the majority of the workers are young, seasonal workers with less totaled years accrued at ISA, and this could skew the results as well.

The data also suggest that the type of position that the study population work at ISA impact their risk of MeN. For the individuals working with weed control, their risk of MeN is

greater than 6 times greater than other positions. Another protective factor against MeN was for individuals that worked as automotive mechanics as opposed to workers who worked in other locations. Working as an automotive mechanic most likely has very different occupational exposures as compared to those working in the fields, and it is very possible that they are not exposed to the risk factor(s) that increase the likelihood of a person developing MeN. In our analysis, the individuals with positions for the harvest or dealing with soil had significantly less risk for developing MeN. However, this relationship is unclear, since MeN has long been identified as a disease of agricultural field workers. While the variables dealing specifically for working with soil had too few observations to be examined later in the model building portion, there were enough data points to include a joint variable for working with soil or water in the model building step.

Another significant risk factor was exposure to rodents at home or during work. There were not enough data on individuals seeing rats at home to be examined in the model building step. However, the univariate analysis found that those seeing rats within the home daily had significantly higher odds of developing MeN than those who did not. Furthermore, individuals who saw rats in the field were 3.615 times more likely to have MeN than those who did not. There was enough data on seeing rats in the field to be included in the model building process, and it was included in the final model as displayed in Table #. The significance of being exposed to rats does support the hypothesis that a rodent-borne agent could be a causative agent of the MeN epidemic. since workers who reported encountering rodents in the field were more likely to be MeN case patients.

Water exposures were also found to be significantly associated with disease. For instance, handwashing using fountain water was highly protective, and handwashing with drainage tube

water drastically increased the odds of the MeN. Similarly, drinking water from the fountain significantly protected against MeN, and drinking water from the drainage pipe in the field severely increased the odds of MeN. The type of water exposures for individuals also played a significant role in the final predicting model as well. Unclean water is a known risk factor for many diseases due to containing infectious pathogens, including *Leptospira*, and harmful chemicals. The unsafe water exposures support an environment exposure contributing to and/or causing the MeN epidemic. When examining our findings about a potential role of drinking water source in conjunction with the added risk for MeN associated with exposure to rats, these data support to the hypothesis of *Leptospira* or a different zoonotic disease being a possible causative agent of MeN.

In both the univariate analysis and predictive model, the use of protective personal equipment was mostly found to be protective against MeN. In particular, the use of gloves, protective eye glasses, face mask, and nose protective equipment all significantly reduce the odds of MeN. It is possible that these types of personal protective equipment may help to reduce or prevent exposure(s) that lead to the development of MeN, such as by interrupting the transmission route of a pathogen or other agent. Interestingly, wearing long sleeves was found to increase the odds of MeN in both the univariate analysis and the final model. This does warrant further investigation as it could potentially suggest that heat exhaustion may be a contributing factor or possibly exacerbate the condition of MeN.

Lastly, having a family member with MeN was found to increase the odds of an individual having MeN in both the univariate analysis and the predictive model. It is important to note that the cases do not have any of the risk factors to develop kidney at a young age, but it is possible that there is a genetic component that make individuals more susceptible to developing

MeN. It is also possible that the family members work together or within the same area at ISA, which would mean that they potentially have the same occupational exposures.

The predictive logistic model results mostly agreed with the univariate results, but there were a few differences in the predictive model. Curiously, drinking well water in the field was found to significantly increase the odds of MeN, but drinking well water at home or work was found to be protective. This does merit further investigation as it could suggest that there is a specific exposure that the well in the field contains that the other wells do not contain. The confidence interval was bit broad for the variable which a larger data sample may be able to address. Within the model, the factors of being a fieldworker, using fountain water to wash one's hands, drinking well water at home and work (but not in the field), history of smoking, and drinking local moonshine/lija were all found to reduce the odds of MeN. The factors of seeing rats in the field, drinking from a well in the field, wearing long sleeves, and having a family member with MeN increased the odds of MeN among cases and controls.

When examining the logistic predictive model in Table 5, the model fits well given the constraints from the dataset. Most of the confidence intervals were narrow, except for the variables representing the well in the field, wearing sleeves, having a family member with MeN, and seeing rats in the field. These all had increased odds ratios with significant p-values, but the confidence intervals were not narrow. Due to working with a smaller dataset, these variables do necessitate further investigation with more data.

Despite the valuable information gleaned from this analysis, there are a few weaknesses. Since we analyzed data and biologic specimens that had already been collected, we were only able to analyze what was available. Not all individuals with MeN completed interviews, and some workers in this study declined to answer some questions during the interview, which means

we did not have complete information for all individuals for all variables of interest. For the multivariate analysis, in particular, we were limited to analyzing a subset of individuals with more complete dataset. The data subset had a total of 407 observations with some missing values for variables. Most of the variables were able to be included in the model building process, but there were a few variables that were excluded due to having too many missing observations. However, the univariate analysis and the statistical model still provide valuable information for future studies. In addition, statistical power was met with the sample sizes for the ELISAs, and the results help to guide future studies to investigate other possible infectious agents. A prospective case control study in the future would be highly beneficial to further investigate the MeN epidemic in Nicaragua, as well as define specific opportunities to interrupt transmission of hantavirus and *Leptospira*.

## CONCLUSION

We found a very low level of exposure to hantavirus in this agriculture worker population in Nicaragua. We also found a low prevalence of *Leptospira* among cases and controls. While these results do not support hantavirus or *Leptospira* as sources of the MeN epidemic, the data do not eliminate a different infectious pathogen as a possible source of MeN. The association between seeing rats in the field and MeN do suggest a possible zoonotic disease causing MeN, and the results of this study support eliminating *Leptospira*. From the ELISA results, it may be useful for the local health departments to have routine testing and treatment available for *Leptospira*. Other useful applications of the results of this study would be explore any factors that put the agricultural workers at risk for exposure to hantavirus. Since we found controls in this study had more often been exposed to hantavirus, there may be specific high risk groups for that disease that could warrant investigation for future preventive measures.

We evaluated potential risk factors for MeN. We found an association between several important environmental exposures and MeN, since individuals with MeN working at ISA had more frequent exposures to potential environmental and occupational risk factors than their healthy worker counterparts. We also discovered that several factors seemed to protect against MeN and could lead to possible intervention strategies. Namely, the provision of safe drinking water and use of PPE could reduce the occurrence of MeN in this population. Given that risk factors for MeN identified herein overlap with known risk factors for infectious disease, particularly hantavirus and *Leptospira*, our data do support the hypothesis of an infectious agent or an environmental agent as possible contributing factors to MeN within this study population. Our findings contribute to a deeper understanding of the environmental exposures for the sugarcane workers, and continued investigations into possible infectious agents contributing to the MeN epidemic are needed.

## APPENDICES

### Appendix I: Hantavirus ELISA Kit Instructions

#### Hantavirus IgG DxSelect™ (OUS)

**REF** EL1600G

Rev. P

Enzyme-linked immunosorbent assay (ELISA) for the qualitative detection of human IgG class antibodies to hantavirus

This package insert is for export only and not for distribution in the United States.

Outside of the United States:  
For *in vitro* Diagnostic Use.



#### INTENDED USE

Focus Diagnostics' Hantavirus IgG DxSelect™ test is intended for the qualitative detection of human IgG class antibodies to Hantavirus in human sera.

#### SUMMARY AND EXPLANATION OF TEST

Hantavirus (HTV) is a novel genus in the *Bunyaviridae* family, in which it stands as the only non-arthropod-borne human pathogen. Transmission to man occurs via inhalation of viral particles from aerosolized excreta from HTV-infected rodents. Phylogeny and epidemiology of HTV's are closely linked to those of their respective rodent reservoirs. To date, up to 30 different HTV strains have been isolated or characterized, of which at least 13 have pathogenic significance for man. The clinically most important strains are murine Hantaan (HTN) and Seoul (SEO) in Asia, Puumala (PUU) and Dobrava (DOB) in Europe, and Sin Nombre (SNV) in the America's.<sup>1</sup> Whereas HTN, PUU, SEO, and DOB all have the kidney as the main target organ in man, SNV and SNV-like agents primarily affect the lung. The HTV strains typically have regional distribution; however, geographic overlap between strains does exist.

Over 150,000 cases of hemorrhagic fever with renal syndrome (HFRS) occur world wide each year.<sup>2</sup> HFRS is characterized by fever, renal failure and, at times, hemorrhagic manifestations. The clinical manifestations of HFRS are more severe when caused by HTN and less severe when due to SEO and PUU. In central Europe HFRS due to DOB may also cause a severe form of disease with mortality of up to 20%.<sup>3</sup>

Hantavirus pulmonary syndrome (HPS) is a disease of rapid onset characterized by fever and severe pulmonary dysfunction with mortality approaching 50%. SNV in North America and the Andes virus in South America have been the causative agents of HPS<sup>3</sup> to date.

The HTV are enveloped and have a tripartite, negative-stranded RNA which encodes several proteins including an RNA-dependent RNA polymerase, 2 envelope glycoproteins and a nucleocapsid protein (NP). The small (S) segment of the genome encodes for the NP and the medium segment encodes for 2 envelope proteins, G1 and G2.<sup>4,5</sup> The NP is the predominant target of the antibody response; however, antibodies directed to the NP are highly cross-reactive between the HTV species. The envelope proteins tend to induce a lower antibody response, but the antibody is species specific. Previously Rossi, et al.<sup>2</sup> found that the HTN NP was a potential candidate for the target antigen in an ELISA-based format to detect IgG.

Recently Elgh, et al.<sup>4</sup> studied the kinetics of the antibody response to recombinant (rNP) in nephropathia epidemica patients caused by Puumala virus. They found that IgG was detected within 2 to 8 days of disease onset, remained high for 2 to 5 months and gradually declined over 2 to 3 years. Nearly all patients remained positive for IgG after 2 to 3 years. The IgM was detectable in most cases within 2 to 8 days of disease onset with nearly all patients positive at 5 to 15 days. The IgM titers declined rapidly and most patients are negative at 2 to 5 months after disease onset.

As is true with other viral diseases, it is difficult to diagnose HTV infection based on clinical grounds alone; thus, serologic methods to detect HTV have evolved as the test of choice for aiding the diagnosis of HTV infection. The Focus Diagnostics Hantavirus DxSelect™ kit uses a cocktail of baculovirus-derived recombinant NP of HTV strains. Using a rNP cocktail allows for detecting antibodies to a broad range of pathogenic species of HTV. The Focus Diagnostics Hantavirus DxSelect™ will detect antibodies to the most clinically relevant pathogenic strains of Hantaviruses, i.e., SEO, HTN, PUU, DOB, and SNV.

#### TEST PRINCIPLE

In the Focus Diagnostics Hantavirus IgG DxSelect™ assay, the polystyrene microwells are coated with Hantavirus antigens. Diluted serum samples and controls are incubated in the wells to allow specific antibody present in the samples to react with the antigen. Nonspecific reactants are removed by washing and peroxidase-conjugated anti-human IgG is added and reacts with specific IgG. Excess conjugate is removed by washing. Enzyme substrate and chromogen are added, and the color is allowed to develop. After adding the Stop Reagent, the resultant color change is quantified by a spectrophotometric reading of optical density (OD) which is directly proportional to the amount of antigen-specific IgG present in the sample. Sample OD readings are compared with reference cutoff OD readings to determine results.

#### MATERIALS SUPPLIED

The Focus Diagnostics Hantavirus IgG DxSelect™ Test kit contains sufficient materials to perform 96 determinations. Allow the supplied reagents to warm to room temperature before use. All un-opened materials are stable at 2 to 8°C until the expiration date stated on the reagent label.

##### Antigen Wells, 96 wells

12 eight-well polystyrene microwell strips on a frame. Each well is coated with recombinant proteins. Each strip may be broken down into individual wells for cost effective use. To avoid condensation, allow the antigen strips to warm to room temperature before opening the sealed packets.

##### IgG Conjugate, 16 mL

1 vial of affinity-purified and peroxidase-conjugated goat anti-human IgG (Fc fragment specific). Contains protein, buffer, and preservatives.

##### IgG Detectable Control, 0.30 mL

1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

##### Non-Detectable Control, 0.30 mL

1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

##### IgG Cut Off Calibrator, 0.30 mL

1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

REF	EL1601	Ag	
REF	EL1604	CONJ	IgG
REF	EL1611	CONTROL	>
REF	EL1612	CONTROL	<
REF	EL1606	CONTROL	CAL



**Sample Diluent, 100 mL**

1 vial of protein, surfactant, and preservatives in PBS.

REF	EL1608	DIL	SPE
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**10X Wash Buffer, 100 mL**

1 vial of surfactant in PBS with preservatives. Prepare a 1X wash buffer solution before use.

REF	EL0405	BUF	WASH
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To prepare a 1X wash buffer solution, mix 100 mL 10X Wash Buffer with 900 mL distilled (or deionized) water and rinse out any crystals. Use only the highest grade purified water for reconstitution of the wash buffer. It has been observed that some sources of deionized water contain materials, which can interfere in the assay. Swirl until well mixed and all crystals are dissolved.

**Stop Reagent, 16 mL**

1 vial of 1M sulfuric acid

REF	EL0105	SOLN	STOP
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**Substrate Reagent, 16 mL**

1 vial of tetramethylbenzidine (TMB) and hydrogen peroxide in buffer. A dark blue color indicates contamination with peroxidase; and, if this occurs, use a fresh bottle.

REF	EL0009	SUBS	TMB
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**Sealing Tape**

2 sheets of sealing tape.

**MATERIALS REQUIRED, BUT NOT SUPPLIED**

1. Distilled water
2. 250 or 500 mL wash bottle
3. 1 L graduated cylinder
4. 12 x 75 mm borosilicate glass test tubes or equivalent
5. 10 µL pipettors with disposable tips
6. 100 µL pipettors with disposable tips (100 µL 8- or 12-channel pipettor recommended for runs over 48 wells)
7. 1 mL pipet or dispenser
8. 5 mL pipet
9. Timer
10. Paper towels or absorbent paper
11. Sink
12. Vortex mixer or equivalent
13. ELISA plate spectrophotometer, wavelength = 450 nm

**SHELF LIFE AND HANDLING**

1. Kits and kit reagents are stable through the end of the month indicated by the label expiration date when stored at 2 to 8°C.
2. Do not use test kit or reagents beyond their expiration dates.
3. Do not expose reagents to strong light during storage or incubation.
4. Allow reagents to warm to room temperature before use.

**WARNINGS AND PRECAUTIONS**

1. This package insert is for export only and not for distribution in the United States. Outside of the United States this kit is for *in vitro* diagnostic use.
2. All blood products should be treated as potentially infectious. Source materials from which this product (including controls) was derived have been screened for Hepatitis B surface antigen, Hepatitis C antibody and HIV-1/2 (AIDS) antibody by FDA-approved methods and found to be negative. However, as no known test methods can offer 100% assurance that products derived from human blood will not transmit these or other infectious agents, all controls, serum specimens and equipment coming into contact with these specimens should be considered potentially infectious and decontaminated or disposed of with proper biohazard precautions. CDC and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2.<sup>6,7</sup>
3. The Hantavirus antigen plates are produced with recombinant antigens; however, the plates should be considered potentially infectious and handled accordingly.
4. Sodium azide at a concentration of 0.1% has been added to the controls as an antibacterial agent. To prevent buildup of explosive metal azides in lead and copper plumbing, controls should be discarded into sewerage only if diluted and flushed with large volumes of water. Use copper-free and lead-free drain systems where possible. Occasionally decontaminate the drains with 10% sodium hydroxide (CAUTION: caustic), allow to stand for 10 minutes, then flush with large volumes of water.
5. The stop reagent contains sulfuric acid. Do not allow to contact skin or eyes. If exposed, flush with copious amounts of water.
6. Do not substitute or mix reagents from different kit lots or from other manufacturers.
7. Use only protocols described in this insert. Incubation times or temperatures other than those specified may give erroneous results.
8. Cross-contamination of patient specimens can cause erroneous results. Add patient specimens and handle strips carefully to avoid mixing of sera from adjoining wells. Avoid contamination of the substrate reagent with traces of the enzyme conjugate.
9. Bacterial contamination of serum specimens or reagents can produce erroneous results. Use aseptic techniques to avoid microbial contamination.
10. Perform the assay at room temperature (approximate range 20 to 25°C).
11. Use proper pipetting techniques, maintaining the pipetting pattern throughout the procedure to ensure optimal and reproducible values.

**SPECIMEN COLLECTION AND PREPARATION**

Serum is the preferred specimen source. No attempt has been made to assess the assay's compatibility with other specimens. Hyperlipemic, heat-inactivated, hemolyzed, or contaminated sera may cause erroneous results; therefore, their use should be avoided.

**Specimen Collection and Handling**

Collect blood samples aseptically using approved venipuncture techniques by qualified personnel. Allow blood samples to clot at room temperature prior to centrifugation. Aseptically transfer serum to a tightly closing sterile container for storage at 2 to 8°C. If testing is to be delayed longer than 5 days, the sample should be frozen at -20°C or colder. Thaw and mix samples well prior to use.





#### Specimen, Controls and Calibrator Preparation

Dilute each specimen, control and calibrator 1:101 as follows: label tubes and dispense 1000 µL of Sample Diluent into each labeled tube. Add 10 µL of specimen, control or calibrator to each appropriate tube containing the 1000 µL IgG Sample Diluent and mix well by vortex mixing.

#### TEST PROCEDURE

1. Bring all reagents to room temperature before use. Remove the Antigen Well packet from cold storage. To avoid condensation, allow micro-well strips to reach room temperature before opening the foil packet. If less than a full plate is to be used, return unused strips to the foil packet with desiccant and reseal completely. Store unused antigen wells at 2 to 8°C. (Note: At the end of the assay, retain the frame for use with the remaining strips.)
2. Fill wells with 1X Wash Buffer solution (see MATERIALS SUPPLIED, above) and allow to soak for 5 minutes. Decant (or aspirate) the antigen wells and tap vigorously to remove Wash Buffer. Blot the emptied Antigen Wells face down on clean paper towels or absorbent paper to remove residual Wash Buffer.
3. Dispense 100 µL of the Sample Diluent into the “blank” wells and 100 µL of each diluted specimen, control or calibrator (see Specimen, Controls, and Calibrator Preparation, above) into the appropriate wells. (Note: For runs with more than 48 wells it is recommended that 250 µL of each diluted sample first be added to a blank microtiter plate in the location corresponding to that in the ELISA wells. The samples can then be efficiently transferred into the Antigen Wells with a 100 µL 8- or 12-channel pipettor.)
4. Cover plates with sealing tape (or place in a humid chamber), and incubate for 60 ± 1 minutes at room temperature (20 to 25°C).
5. Remove sealing tape (or remove wells from the humid chamber), and empty the contents of the wells into a sink or a discard basin.
6. Fill each well with a gentle stream of 1X Wash Buffer solution from a wash bottle, then empty contents into a sink or a discard basin.
7. Repeat wash (step 6) an additional 2 times.
8. Tap the antigen wells vigorously to remove 1X Wash Buffer. Blot the emptied Antigen Wells face down on clean paper towels or absorbent paper to remove residual 1X Wash Buffer.
9. Dispense 100 µL Conjugate to all wells, using a 100 µL 8- or 12-channel pipettor.
10. Cover plates with sealing tape (or place in a humid chamber) and incubate for 30 ± 1 minutes at room temperature (20 to 25°C).
11. Repeat wash steps 5 through 8.
12. Pipet 100 µL of Substrate Reagent to all wells, using a 100 µL 8- or 12-channel pipettor. Begin incubation timing with the addition of Substrate Reagent to the first well. (Note: Never pour the substrate reagent into the same trough as was used for the conjugate.)
13. Incubate for 10 ± 1 minutes at room temperature (20 to 25°C).
14. Stop the reaction by adding 100 µL of Stop Reagent to all wells using a 100 µL 8- or 12-channel pipettor. Add the Stop Reagent in the same sequence and at the same pace as the Substrate was added. In antibody-positive wells, color should change from blue to yellow.
15. Gently blot the outside bottom of wells with a paper towel to remove droplets that may interfere with reading by the spectrophotometer. Do not rub with the paper towel as it may scratch the optical surface of the well. (Note: Large bubbles on the surface of the liquid may affect the OD readings.)
16. Measure the absorbance of each well within 1 hour of stopping the assay. Set the microwell spectrophotometer at a wavelength of 450 nm. Zero the instrument on the blank wells.

#### QUALITY CONTROL

Each plate run (or strips or wells from a single plate) must include the Cut-off Calibrator and 2 controls. If multiple plates are run, include the Cut-off Calibrator and both controls on each plate. It is recommended that until the user becomes familiar with the kit performance, all specimens, controls and the Cutoff Calibrator should be run in duplicate with the Cut-off Calibrator run twice for a total of 4 wells. If single wells are used, the Cut-off Calibrator should be run in triplicate. Include a minimum of 1 blank well (containing sample diluent only) for instrument calibration purposes.

The Cut-off Calibrator has been formulated to give the optimum differentiation between negative and positive sera. Although the absorbance value may vary between runs and between laboratories, the mean value for the Cut-off Calibrator wells must be within 0.100 to 0.700 OD units. All replicate Cut-off Calibrator ODs should be within 0.10 absorbance units from the mean value.

Report results as index values relative to the Cut-off Calibrator. To calculate index values, divide specimen optical density (OD) values by the mean of the Cutoff Calibrator absorbance values.

1. The **Detectable Control** index value should be between 1.5 and 3.0.
2. The **Non-Detectable Control** index value should be less than 0.8.

If the Calibrator or controls are not within these parameters, patient test results should be considered invalid and the assay repeated.

#### INTERPRETATION OF TEST RESULTS

Report all patient results as index values relative to the Cut-off Calibrator: to calculate index values, divide specimen optical density (OD) values by the mean of the Cut-off Calibrator absorbance values.

>1.10	<b>Positive.</b> An index value of > 1.10 is presumptive for the presence of IgG antibodies to Hantavirus.
≥0.90 and ≤1.10	<b>Equivocal.</b> An index value of ≥ 0.90 but ≤ 1.10 is considered an equivocal result. These samples should be retested. If, on retesting, the result remains equivocal, a second sample should be drawn several weeks later and tested to identify a rise in IgG antibody titer. If the second sample is either negative or equivocal, report results as negative. Alternatively, the specimen may be tested using a different methodology such as IFA or Western Blot.
<0.90	<b>Negative.</b> An index value of < 0.90 indicates no IgG antibodies to Hantavirus were detected.

#### LIMITATIONS

1. All results from this and other serologies must be correlated with clinical history, epidemiological data, and other data available to the attending physician in making the diagnosis of Hantavirus infection.
2. Patients with early Hantavirus infection may test negative for IgG antibodies, since the IgG response may be undetectable until 5 weeks post-onset. Therefore, testing for IgM class antibody is recommended. If a negative test result is reported on a patient with signs and symptoms of Hantavirus infection, repeat testing for IgG and IgM antibodies on a second sample obtained 2 to 4 weeks later is recommended.

#### PERFORMANCE CHARACTERISTICS

For customers outside of the United States, the product performance characteristics are supplied as a separate sheet.

# REFERENCES

1. Clement, J., McKenna, P., et al. Epidemiology and laboratory diagnosis of hantavirus (HTV) infections. *Acta Clinica Belgica* (1995), 50:9 – 19.
2. Lundkvist, A., Hukic, M., Horling, J., Gilljam, M., Nichol, S., Niklasson, B.. Puumala and Dobrava viruses cause hemorrhagic fever with renal syndrome in Bosnia-Herzegovina: Evidence of highly cross-neutralizing antibody responses in early patient sera. *J Med Virol* (1997) 53:51.
3. Elgh, F., Linderholm, M., Wadell, G., Tarnvik, A., Juto, P. Development of humoral cross-reactivity to the nucleocapsid protein of heterologous hantaviruses in nephropathia epidemica. *FEMS Immunol Med Microb* (1998) 22:309.
4. Rossi, C.A., Schmaljohn, C.S. Meegan, J.M., LeDuc, J.W. Diagnostic potential of a baculovirus-expressed nucleocapsid protein for hantaviruses. *Arch Virol* (1990)[Suppl 1]: 19-28.
5. Schuldt, C., Zoller, L., et al. Baculovirus expression of the nucleocapsid protein of a Puumala serotype hantavirus. *Virus Genes* (1994), 8: 143 – 149.
6. NCCLS. Procedures for the Handling and Processing of Blood Specimens; Approved Guideline (NCCLS H18-A2). 2<sup>nd</sup> ed. (1999).
7. CDC-NIH Manual. (1999) Biosafety in Microbiological and Biomedical Laboratories. 4<sup>th</sup> ed. And National Committee for Clinical Laboratory Standards (NCCLS). Protection of Laboratory Workers from Instruments, Biohazards and Infectious Disease Transmitted by Blood, Body Fluids and Tissue (NCCLS M29-A).

*This package insert is available in French, German, Italian, and Spanish at [www.focusdx.com](http://www.focusdx.com), and may be available in other languages from your local distributor.*

## AUTHORIZED REPRESENTATIVE

mdi Europa GmbH, Langenhagener Str. 71, 30855 Langenhagen-Hannover, Germany

## ORDERING INFORMATION

Telephone: (562) 240-6500 (International)  
Fax: (562) 240-6510

## TECHNICAL ASSISTANCE

Telephone: (562) 240-6500 (International)  
Fax: (562) 240-6526

Visit our web site at: [www.focusdx.com](http://www.focusdx.com)



PIEL1600G.OUS

Rev. P

Date written: 02 July 2012



Cypress, California 90630, U.S.A



# Hantavirus IgM DxSelect™ (OUS)

**REF** EL1600M

Rev. O

Enzyme-linked immunosorbent assay (ELISA) for the qualitative detection of human IgM class antibodies to hantavirus

This package insert is for export only and not for distribution in the United States.

Outside of the United States:  
For *in vitro* Diagnostic Use.



## INTENDED USE

Focus Diagnostics' Hantavirus IgM DxSelect™ test is intended for the qualitative detection of human IgM class antibodies to Hantavirus in human sera.

## SUMMARY AND EXPLANATION OF TEST

Hantavirus (HTV) is a novel genus in the *Bunyaviridae* family, in which it stands as the only non-arthropod-borne human pathogen. Transmission to man occurs via inhalation of viral particles from aerosolized excreta from HTV-infected rodents. Phylogeny and epidemiology of HTV's are closely linked to those of their respective rodent reservoirs. To date, up to 30 different HTV strains have been isolated or characterized, of which at least 13 have pathogenic significance for man. The clinically most important strains are murine Hantaan (HTN) and Seoul (SEO) in Asia, Puumala (PUU) and Dobrava (DOB) in Europe, and Sin Nombre (SNV) in the Americas.<sup>1</sup> Whereas HTN, PUU, SEO, and DOB all have the kidney as the main target organ in man, SNV and SNV-like agents primarily affect the lung. The HTV strains typically have regional distribution; however, geographic overlap between strains does exist.

Over 150,000 cases of hemorrhagic fever with renal syndrome (HFRS) occur world wide each year.<sup>2</sup> HFRS is characterized by fever, renal failure and, at times hemorrhagic manifestations. The clinical manifestations of HFRS are more severe when caused by HTN and less severe when due to SEO and PUU. In central Europe HFRS due to DOB may also cause a severe form of disease with mortality of up to 20%.<sup>3</sup>

Hantavirus pulmonary syndrome (HPS) is a disease of rapid onset characterized by fever and severe pulmonary dysfunction with mortality approaching 50%. SNV in North America and the Andes virus in South America have been the causative agents of HPS<sup>3</sup> to date.

The HTV are enveloped and have a tripartite, negative-stranded RNA which encodes several proteins including an RNA-dependent RNA polymerase, 2 envelope glycoproteins and a nucleocapsid protein (NP). The small (S) segment of the genome encodes for the NP and the medium (M) segment encodes for 2 envelope proteins, G1 and G2.<sup>4,5</sup> The NP is the predominant target of the antibody response; however, antibodies directed to the NP are highly cross-reactive between the HTV species. The envelope proteins tend to induce a lower antibody response, but the antibody is species specific. Previously Rossi, et al.<sup>6</sup> found that the HTN NP was a potential candidate for the target antigen in an ELISA-based format to detect IgG.

Recently Elgh, et al.<sup>4</sup> studied the kinetics of the antibody response to recombinant NP (rNP) in nephropathia epidemica patients caused by Puumala virus. They found that IgG was detected within 2 to 8 days of disease onset, remained high for 2 to 5 months and gradually declined over 2 to 3 years. Nearly all patients remained positive for IgG after 2 to 3 years. The IgM was detectable in most cases within 2 to 8 days of disease onset with nearly all patients positive at 5 to 15 days. The IgM titers declined rapidly and most patients are negative at 2 to 5 months after disease onset.

As is true with other viral diseases, it is difficult to diagnose HTV infection based on clinical grounds alone; thus, serologic methods to detect HTV have evolved as the test of choice for aiding the diagnosis of HTV infection. The Focus Diagnostics Hantavirus DxSelect™ kit uses a cocktail of baculovirus-derived recombinant NP of HTV strains. Using a rNP cocktail allows for detecting antibodies to a broad range of pathogenic species of HTV. The Focus Diagnostics Hantavirus DxSelect™ will detect antibodies to the most clinically relevant pathogenic strains of Hantaviruses, i.e., SEO, HTN, PUU, DOB, and SNV.

## TEST PRINCIPLE

In the Focus Diagnostics Hantavirus IgM DxSelect™ assay, the polystyrene microwells are coated with Hantavirus antigens. Patient sera and controls are diluted in a solution containing hyper-immune anti-human IgG precipitating immunoglobulin to remove both free and complexed IgG from the sample. The diluted serum samples and controls are incubated in the wells to allow any specific antibody present in the samples to react with the antigen. Nonspecific reactants are removed by washing and peroxidase-conjugated anti-human IgM is added to react with the IgM present. Excess conjugate is removed by washing. Enzyme substrate and chromogen are added, and the color is allowed to develop. After adding the Stop Reagent, the resultant color change is quantified by a spectrophotometric reading of optical density (OD), which is directly proportional to the amount of antigen-specific IgM present in the sample. Sample OD readings are compared with reference cut-off OD readings to determine results.

## MATERIALS SUPPLIED

The Focus Diagnostics Hantavirus IgM DxSelect™ Test kit contains sufficient materials to perform 96 determinations. Allow the supplied reagents to warm to room temperature before use. All un-opened materials are stable at 2 to 8°C until the expiration date stated on the reagent label.

### Antigen Wells, 96 wells

12 eight-well polystyrene microwell strips on a frame. Each well is coated with a blend of recombinant Hantavirus antigens. Each strip may be broken down into individual wells for cost effective use. To avoid condensation, allow the antigen strips to warm to room temperature before opening the sealed packets.

<b>REF</b>	<b>EL1601</b>	<b>Ag</b>
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### IgM Conjugate, 16 mL

1 vial of affinity-purified and peroxidase-conjugated goat anti-human IgM (μ chain specific). Contains protein, buffer, and preservatives.

<b>REF</b>	<b>EL1602</b>	<b>CONJ</b>	<b>IgM</b>
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### IgM Detectable Control, 0.30 mL

1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

<b>REF</b>	<b>EL1615</b>	<b>CONTROL</b>	<b>&gt;</b>
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### Non-Detectable Control, 0.30 mL

1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

<b>REF</b>	<b>EL1612</b>	<b>CONTROL</b>	<b>&lt;</b>
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### IgM Cut Off Calibrator, 0.30 mL

1 vial of human serum. Contains 0.1% sodium azide as a preservative. Requires dilution before use (see Specimen, Controls and Calibrator Preparation, below).

<b>REF</b>	<b>EL1603</b>	<b>CONTROL</b>	<b>CAL</b>
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**IgM Sample Diluent, 100 mL**

1 vial of goat anti-human IgG antibody, protein, surfactant, food coloring, and preservatives in PBS.

REF

EL1613

DIL

IgM

**10X Wash Buffer, 100 mL**

1 vial of surfactant in PBS with preservatives.

REF

EL0405

BUF

WASH

To prepare a 1X wash buffer solution, mix 100 mL 10X Wash Buffer with 900 mL distilled (or deionized) water and rinse out any crystals. Use only the highest grade purified water for reconstitution of the wash buffer. It has been observed that some sources of deionized water contain materials, which can interfere in the assay. Swirl until well mixed and all crystals are dissolved.

**Substrate Reagent, 16 mL**

1 vial of tetramethylbenzidine (TMB) and hydrogen peroxide in buffer. A dark blue color indicates contamination with peroxidase; and, if this occurs, use a fresh bottle.

REF

EL0009

SUBS

TMB

**Stop Reagent, 16 mL**

One vial of 1 M sulfuric acid.

REF

EL0105

SOLN

STOP

**Sealing Tape**

2 sheets of sealing tape.

**MATERIALS REQUIRED, BUT NOT SUPPLIED**

1. Distilled water
2. 250 or 500 mL wash bottle
3. 1 L graduated cylinder
4. 12 x 75 mm borosilicate glass test tubes or equivalent
5. 10 µL pipettors with disposable tips
6. 100 µL pipettors with disposable tips (100 µL 8- or 12-channel pipettor recommended for runs over 48 wells)
7. 1 mL pipet or dispenser
8. 5 mL pipet
9. Timer
10. Paper towels or absorbent paper
11. Sink
12. Vortex mixer or equivalent
13. ELISA plate spectrophotometer, wavelength = 450 nm

**SHELF LIFE AND HANDLING**

1. Kits and kit reagents are stable through the end of the month indicated in the expiration date when stored at 2 to 8°C.
2. Do not use test kit or reagents beyond their expiration dates.
3. Do not expose reagents to strong light during storage or incubation.
4. Allow reagents to warm to room temperature before use.

**WARNINGS AND PRECAUTIONS**

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2. All blood products should be treated as potentially infectious. Source materials from which this product (including controls) was derived have been screened for Hepatitis B surface antigen, Hepatitis C antibody and HIV-1/2 (AIDS) antibody by FDA-approved methods and found to be negative. However, as no known test methods can offer 100% assurance that products derived from human blood will not transmit these or other infectious agents, all controls, serum specimens and equipment coming into contact with these specimens should be considered potentially infectious and decontaminated or disposed of with proper biohazard precautions. CDC and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2.<sup>6,7</sup>
3. The Hantavirus antigen plates are produced with recombinant Hantavirus antigens; however, the plates should be considered potentially infectious and handled accordingly.
4. Sodium azide at a concentration of 0.1% has been added to the controls as an antibacterial agent. To prevent buildup of explosive metal azides in lead and copper plumbing, controls should be discarded into sewerage only if diluted and flushed with large volumes of water. Use copper-free and lead-free drain systems where possible. Occasionally decontaminate the drains with 10% sodium hydroxide (CAUTION: caustic), allow to stand for 10 minutes, then flush with large volumes of water.
5. The stop reagent contains sulfuric acid. Do not allow to contact skin or eyes. If exposed, flush with copious amounts of water.
6. Do not substitute or mix reagents from different kit lots or from other manufacturers.
7. Use only protocols described in this insert. Incubation times or temperatures other than those specified may give erroneous results.
8. Cross-contamination of patient specimens can cause erroneous results. Add patient specimens and handle strips carefully to avoid mixing of sera from adjoining wells. Avoid contamination of the substrate reagent with traces of the enzyme conjugate.
9. Bacterial contamination of serum specimens or reagents can produce erroneous results. Use aseptic techniques to avoid microbial contamination.
10. Perform the assay at room temperature (approximate range 20 to 25°C).
11. Use proper pipetting techniques, maintaining the pipetting pattern throughout the procedure to ensure optimal and reproducible values.

**SPECIMEN COLLECTION AND PREPARATION**

Serum is the preferred specimen source. No attempt has been made to assess the assay's compatibility with other specimens. Hyperlipemic, heat-inactivated, hemolyzed, or contaminated sera may cause erroneous results; therefore, their use should be avoided.

**Specimen Collection and Handling**

Collect blood samples aseptically using approved venipuncture techniques by qualified personnel. Allow blood samples to clot at room temperature prior to centrifugation. Aseptically transfer serum to a tightly closing sterile container for storage at 2 to 8°C. If testing is to be delayed longer than 5 days, the sample should be frozen at -20°C or colder. Thaw and mix samples well prior to use.

**Specimen, Controls and Calibrator Preparation**

Dilute each specimen, control and calibrator 1:101 as follows: label tubes and dispense 1000 µL of IgM Sample Diluent into each labeled tube. Add 10 µL of specimen, control or calibrator to each appropriate tube containing the 1000 µL IgM Sample Diluent and mix well by vortex mixing. Wait 10 minutes during which time a fine precipitate will form in the tubes, sequestering IgG into an immune complex and preventing its interference in the IgM assay. The precipitate will not interfere with the assay.



#### TEST PROCEDURE

1. Bring all reagents to room temperature before use. Remove the Antigen Well packet from cold storage. To avoid condensation, allow micro-well strips to reach room temperature before opening the foil packet. If less than a full plate is to be used, return unused strips to the foil packet with desiccant and reseal completely. Store unused antigen wells at 2 to 8°C. (Note: At the end of the assay, retain the frame for use with the remaining strips.)
2. Fill wells with 1X Wash Buffer solution (see MATERIALS SUPPLIED, above) and allow to soak for 5 minutes. Decant (or aspirate) the antigen wells and tap vigorously to remove Wash Buffer. Blot the emptied Antigen Wells face down on clean paper towels or absorbent paper to remove residual Wash Buffer.
3. Dispense 100 µL of the IgM Sample Diluent into the "blank" wells and 100 µL of each diluted specimen, control or calibrator (see Specimen, Controls, and Calibrator Preparation, above) into the appropriate wells. (Note: For runs with more than 48 wells it is recommended that 250 µL of each diluted sample first be added to a blank microtiter plate in the location corresponding to that in the ELISA wells. The samples can then be efficiently transferred into the Antigen Wells with a 100 µL 8- or 12-channel pipettor.)
4. Cover plates with sealing tape (or place in a humid chamber), and incubate for 60 ± 1 minutes at room temperature (20 to 25°C).
5. Remove sealing tape (or remove wells from the humid chamber), and empty the contents of the wells into a sink or a discard basin.
6. Fill each well with a gentle stream of 1X Wash Buffer solution from a wash bottle, then empty contents into a sink or a discard basin.
7. Repeat wash (step 6) an additional 2 times.
8. Tap the antigen wells vigorously to remove 1X Wash Buffer. Blot the emptied Antigen Wells face down on clean paper towels or absorbent paper to remove residual 1X Wash Buffer.
9. Dispense 100 µL Conjugate to all wells, using a 100 µL 8- or 12-channel pipettor.
10. Cover plates with sealing tape (or place in a humid chamber) and incubate for 30 ± 1 minutes at room temperature (20 to 25°C).
11. Repeat wash steps 5 through 8.
12. Pipet 100 µL of Substrate Reagent to all wells, using a 100 µL 8- or 12-channel pipettor. Begin incubation timing with the addition of Substrate Reagent to the first well. (Note: Never pour the substrate reagent into the same trough as was used for the conjugate.)
13. Incubate for 10 ± 1 minutes at room temperature (20 to 25°C).
14. Stop the reaction by adding 100 µL of Stop Reagent to all wells using a 100 µL 8- or 12-channel pipettor. Add the Stop Reagent in the same sequence and at the same pace as the Substrate was added. In antibody-positive wells, color should change from blue to yellow.
15. Gently blot the outside bottom of wells with a paper towel to remove droplets that may interfere with reading by the spectrophotometer. Do not rub with the paper towel as it may scratch the optical surface of the well. (Note: Large bubbles on the surface of the liquid may affect the OD readings.)
16. Measure the absorbance of each well within 1 hour of stopping the assay. Set the microwell spectrophotometer at a wavelength of 450 nm. Zero the instrument on the blank wells.

#### QUALITY CONTROL

Each plate run (or strips or wells from a single plate) must include the Cut-off Calibrator and 2 controls. It is recommended that until the user becomes familiar with the kit performance, all specimens, controls and the Cut-off Calibrator should be run in duplicate with the Cut-off Calibrator run twice for a total of 4 wells. If single wells are used, the Cut-off Calibrator should be run in triplicate. Include a minimum of 1 blank well (containing sample diluent only) for instrument calibration purposes.

The Cut-off Calibrator has been formulated to give the optimum differentiation between negative and positive sera. Although the absorbance value may vary between runs and between laboratories, the mean value for the Cut-off Calibrator wells must be within the range of 0.100 to 0.700 OD units. All replicate Cutoff Calibrator ODs should be within 0.10 absorbance units from the mean value.

Report results as index values relative to the Cut-off Calibrator. To calculate index values, divide specimen optical density (OD) values by the mean of the Cutoff Calibrator absorbance values.

1. The **Detectable Control** index value should be between 1.5 and 3.0.
2. The **Non-Detectable Control** index value should be less than 0.8.

If the Calibrator or controls are not within these parameters, patient test results should be considered invalid and the assay repeated.

#### INTERPRETATION OF TEST RESULTS

Report all patient results as index values relative to the Cut-off Calibrator: to calculate index values, divide specimen optical density (OD) values by the mean of the Cut-off Calibrator absorbance values.

> 1.10	<b>Positive.</b> Patient specimens, which exhibit an index value of > 1.10 indicate the presence of IgM antibodies to Hantavirus. This is indicative of an early response to infection.
≥ 0.90 and ≤ 1.10	<b>Equivocal.</b> Patient specimens which exhibit an index value of ≥ 0.90 but ≤ 1.10 are considered doubtful or equivocal results. All equivocal results should be retested. If, on retesting, the first sample remains equivocal, a second sample should be drawn several weeks later to identify a rise in IgM antibody titer. If the second sample is either negative or equivocal, this indicates no IgM antibody to Hantavirus are detectable.
< 0.90	<b>Negative.</b> Patient specimens which exhibit an index value of < 0.90 indicate no detectable IgM antibodies to Hantavirus.

#### LIMITATIONS

1. All results from this and other serologies must be correlated with the clinical history, epidemiological data, and other data available to the attending physician in making the diagnosis of Hantavirus infection.
2. Timing of the sample is critical in the serological demonstration of an IgM response. Patients with early Hantavirus may test IgM-negative. A negative result does not rule out Hantavirus. If a negative test is obtained on a patient with signs and symptoms of Hantavirus, repeat testing on a second sample 2 weeks later.
3. Specimens from patients with IgM antibodies to cytomegalovirus, influenza virus, and mycoplasma may give positive or equivocal results. If these diseases are suspected, specific serological testing should be carried out.

#### PERFORMANCE CHARACTERISTICS

For customers outside of the United States, the product performance characteristics are supplied as a separate sheet.

**REFERENCES**

1. Clement, J., McKenna, P., et al. Epidemiology and laboratory diagnosis of hantavirus (HTV) infections. Acta Clinica Belgica (1995), 50:9 – 19.
2. Lundkvist, A., Hukic, M., Horling, J., Gilljam, M., Nichol, S., Niklasson, B. Puumala and Dobrava viruses cause hemorrhagic fever with renal syndrome in Bosnia-Herzegovina: Evidence of highly cross-neutralizing antibody responses in early patient sera. J Med Virol (1997) 53:51.
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**AUTHORIZED REPRESENTATIVE**

mdi Europa GmbH, Langenhagener Str. 71, 30855 Langenhagen-Hannover, Germany

**ORDERING INFORMATION**

Telephone: (562) 240-6500 (International)  
Fax: (562) 240-6510

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Cypress, California 90630, U.S.A

## Appendix II: Leptospira ELISA Kit Instructions

### **SERION ELISA *classic* Leptospira IgG/IgM**

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# SERION ELISA *classic* Leptospira IgG/IgM

## ENZYME IMMUNOASSAY FOR DETECTION OF HUMAN ANTIBODIES (IGG/IGM)

For sale in the U.S. for Research Use Only. Not for use in diagnostic procedures.

IgG-Kit (quantitative)    order number:    ESR125G  
IgM-Kit (quantitative)    order number:    ESR125M

**Tests evaluated: Dade Behring BEP ® III / BEP ® 2000, DSX, manually**

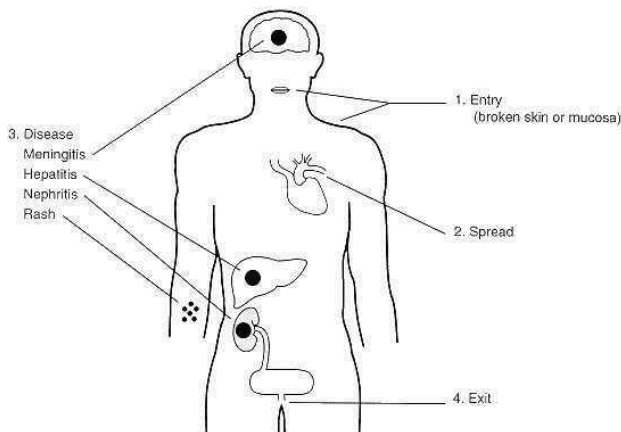
### 1. INTENDED USE

**SERION ELISA CLASSIC LEPTOSPIRA IGG/IGM** ARE QUANTITATIVE AND QUALITATIVE TESTS FOR DETECTION OF GENUS-SPECIFIC HUMAN ANTIBODIES AGAINST LEPTOSPIRA IN SERUM OR PLASMA. FOR SALE IN THE U.S. FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

### 2. BACKGROUND

LEPTOSPIRES ARE SPIRAL-SHAPED, GRAM-NEGATIVE, OBLIGATE AEROBIC SPIROCHETES WITH INTERNAL FLAGELLA. THE GENUS IS DIVIDED INTO TWO SPECIES, THE PATHOGENIC LEPTOSPIRA INTERROGANS AND THE FREE-LIVING NONPATHOGENIC LEPTOSPIRA BIFLEXA. LEPTOSPIRA INTERROGANS HAS ABOUT 200 DIFFERENT SEROVARS BASED ON THE VARIABILITY OF SURFACE ANTIGENS. LEPTOSPIRES AFFECT MAMMALS (WILD AND DOMESTIC ANIMALS), REPTILES AND AMPHIBIANS; THEY MAY BE SHED IN THE URINE LIFELONG. RATS AND OTHER RODENTS ARE PRIMARY RESERVOIRS FOR HUMAN INFECTION. INFECTION IS TRANSMITTED BY URINE-CONTAMINATED SOIL OR WATER, RAT BITES OR ANIMAL TISSUE. ESPECIALLY OCCUPATIONAL GROUPS LIKE AGRICULTURISTS, PLUMBERS, MINE WORKERS, FISHERMEN AND MEAT- INDUSTRY WORKERS ARE AT GREAT RISK OF EXPOSURE.

MUCOSA AND SKIN LESIONS ARE THE MOST LIKELY SITES OF ENTRY FOR LEPTOSPIRES (FIG. 1). BACTERIA MAINLY PROLIFERATE IN THE CENTRAL NERVOUS SYSTEM, KIDNEYS AND LIVER.



**Figure 1.**



THE IMMUNE SYSTEM REMOVES ORGANISMS FROM BLOOD AND ORGANS WITHIN 4-7 DAYS BY COMPLEMENT AND HUMORAL IMMUNITY. CELL-MEDIATED IMMUNITY DOES NOT APPEAR TO BE IMPORTANT. LEPTOSPIRES WITHIN THE CONVOLUTED TUBULES OF THE KIDNEYS MAY SURVIVE DUE TO THE INEFFICIENCY OF THE IMMUNE SYSTEM, THE COMPLEMENT SYSTEM IN PARTICULAR. FOR THIS REASON INFECTIOUS LEPTOSPIRES ARE SHED IN URINE.

MICRO-AGGLUTINATION TESTS, ELISAS AND INDIRECT FLUORESCENCE-ANTIBODY TESTS ARE MOST FREQUENTLY USED FOR SERODIAGNOSIS.

### 3. SERION ELISA *classic* - TEST PRINCIPLE

Microtest plates are coated with **antigens**. This constitutes the **solid phase**. Sample is added to the plates and any antibodies specific for the antigen present will bind to the solid phase. After removal of unbound material, anti-human **IgG, IgA or IgM conjugated** to an enzyme (**alkaline phosphatase**) is allowed to react with the immune complex. After removal of excess conjugate by washing, an appropriate **substrate (para-nitrophenylphosphate)** is added, with which the conjugated enzyme reacts producing a **colored derivative of the substrate**. The color intensity is proportional to the level of specific antibody bound and can be quantified photometrically.

#### 4. COMPONENTS OF THE KIT

Test components	amount / volume
<b>Break apart microtiter test strips each with 8 antigen coated single wells (altogether 96),</b> 1 frame the coating material is inactivated	12
<b>Standard serum (ready-to-use)</b> Human serum in phosphate buffer with protein; negative for anti-HIV-Ab, anti-HBs-Ag (Hepatitis B-Virus-surface antigen) and anti-HCV-Ab; preservative: < 0.1 % sodium azide coloring: Amaranth O	2 x 2 ml
<b>Negative control serum (ready-to-use)</b> Human serum in phosphate buffer with protein; negative for anti-HIV, anti-HBs (Hepatitis B-Virus-surface antigen) and anti-HCV; preservative: < 0.1 % sodium azide coloring: Lissamin green V	2 ml
<b>Anti-human-IgG-, IgA-, IgM-conjugate (ready-to-use)</b> Anti-human-IgG, -IgA, -IgM from goat (polyclonal), conjugated to alkaline phosphatase, stabilized with protein stabilization solution preservative: 0.01 % methylisothiazolone, 0.01 % bromnitrodioxane	13 ml
<b>Washing solution concentrate (sufficient for 1 litre)</b> Sodium chloride solution with Tween 20, 30 mM Tris preservative: < 0.1 % sodium azide	33.3 ml
<b>Dilution buffer</b> Phosphate buffer with protein and Tween 20; preservative: < 0.1 % sodium azide 0.01 g/l Bromphenol blue sodium salt	2 x 50 ml
<b>Stopping solution</b> 1.2 N sodium hydroxide	15 ml
<b>Substrate (ready-to-use)</b> Para-nitrophenylphosphate, solvent free buffer preservative: < 0.1 % sodium azide (Substrate in unopened bottle may have a slightly yellow color. This does not reduce the quality of the product!)	13 ml
<b>Quality control certificate with standard curve and evaluation table</b> (quantification of antibodies in IU/ml or U/ml)	1

## 5. MATERIAL REQUIRED BUT NOT SUPPLIED

- common laboratory equipment
- for the IgM-ELISA: SERION Rf-Absorbent (Order no. Z200/20ml)
- photometer for microtiter plates with filter, wavelength 405 nm, recommended reference wavelength 620 nm - 690 nm (e.g. 650nm)
- incubator 37°C
- moist chamber
- distilled water

## 6. STORAGE AND STABILITY

Reagent	Storage	Stability
microtiter strips (antigen)	after opening at 2-8°C in closed aluminum bag with desiccant  <i>Strips which are not used must be stored in the press-seal bag of aluminum compound foil under dry and airtight conditions!</i>	4 weeks
control sera / standard sera	after opening at 2-8°C	until expiry date; 24 months from date of production
conjugate	ready-to-use solution, at 2-8°C  <i>Avoid contamination (sterile tips!)</i>	until expiry date 28 months from date of production
dilution buffer	after opening at 2-8°C <i>Discard cloudy solutions!</i>  unopened	24 months  until expiry date; 36 months from date of production
washing solution	concentrate after opening at 2-8°C working dilution at 2-8°C working dilution at room temperature  <i>Bottles used for the working dilution should be cleaned regularly, discard cloudy solutions.</i>	until expiry date 2 weeks 1 week
substrate	ready-to-use solution at 2-8°C, protected from light!  <i>Avoid contamination (sterile tips!) Discard when solution turns yellow (extinction against distilled water &gt; 0.25).</i>	until expiry date 24 months from date of production
stopping solution	after opening at room temperature	until expiry date

## 7. TEST PROCEDURE SERION ELISA *classic*

### 7.1 Evidence of deterioration

ONLY USE SERION ELISA CLASSIC REAGENTS FOR TEST PROCEDURE, SINCE ALL REAGENTS ARE MATCHED. IN PARTICULAR STANDARD AND CONTROL SERA ARE DEFINED EXCLUSIVELY FOR THE TEST KIT TO BE USED. DO NOT USE THEM IN OTHER LOTS. DILUTION BUFFER, WASHING SOLUTION AND SUBSTRATE SOLUTION CAN BE USED FOR ALL SERION ELISA CLASSIC KITS IRRESPECTIVE OF THE LOT AND THE TEST.

THERE ARE THREE DIFFERENT CONJUGATE CONCENTRATIONS FOR EACH IMMUNOGLOBULIN CLASS: LOW, MEDIUM, HIGH

THE CLASSIFICATION IS WRITTEN ON EACH LABEL AS FOLLOWS:

E.G.                      IGG + LOWLY CONCENTRATED IGG  
                                 CONJUGATE IGG ++ MEDIUM  
                                 CONCENTRATED IGG CONJUGATE  
                                 IGG +++ HIGHLY CONCENTRATED  
                                 IGG CONJUGATE

IN RARE CASES THE USE OF SPECIAL CONJUGATE IS NECESSARY TO GUARANTEE CONSISTENT QUALITY FOR OUR PRODUCTS. SPECIAL CONJUGATES ARE PRODUCED IN A SEPARATE LOT AND DO NOT WEAR THE “+” SIGN. THEREFORE, SPECIAL CONJUGATES ARE NOT EXCHANGEABLE WITH OTHER CONJUGATES.

**Please pay close attention to notifications on labels!**

UNOPENED, ALL COMPONENTS OF THE SERION ELISA CLASSIC KITS MAY BE USED UP TO THE DATES GIVEN ON THE LABELS, IF STORED AT +2°C TO +8°C. COMPLETE STABILITY AND STORAGE DATA ARE DESCRIBED UNDER “6. STORAGE AND STABILITY”.

EACH REAGENT HAS BEEN CALIBRATED AND OPTIMIZED FOR THE TEST. DILUTION OR ALTERATION OF THESE REAGENTS MAY RESULT IN A LOSS OF SENSITIVITY.

AVOID EXPOSURE OF REAGENTS TO STRONG LIGHT DURING STORAGE AND INCUBATION. REAGENTS MUST BE TIGHTLY CLOSED TO AVOID EVAPORATION AND CONTAMINATION WITH MICROORGANISMS SINCE INCORRECT TEST RESULTS COULD OCCUR DUE TO INTERFERENCE FROM PROTEOLYTIC ENZYMES.

TO OPEN THE PRESS-SEAL BAG PLEASE CUT OFF THE TOP OF THE MARKED SIDE, ONLY. DO NOT USE THE STRIPS IF THE ALUMINUM BAG IS DAMAGED

OR IF THE PRESS-SEAL BAG WITH REMAINING STRIPS AND DESICCANT WAS NOT PROPERLY CLOSED.

BRING ALL REAGENTS TO ROOM TEMPERATURE BEFORE TESTING.

USE ASEPTIC TECHNIQUES FOR REMOVING ALIQUOTS FROM THE REAGENT TUBES TO AVOID CONTAMINATION. TO AVOID FALSE POSITIVE RESULTS ENSURE NOT TO CONTACT OR SPRINKLE THE TOP-WALLS OF WELLS WHILE PIPETTING CONJUGATE. BE CAREFUL NOT TO MIX THE CAPS OF THE BOTTLES AND/OR VIALS. REPRODUCIBILITY DEPENDS ON THOROUGH MIXING OF THE REAGENTS. SHAKE THE FLASKS CONTAINING CONTROL SERA BEFORE USE AND ALSO ALL SAMPLES AFTER DILUTION (E.G. BY USING A MONOMIXER).

BE SURE TO PIPETTE CAREFULLY AND COMPLY WITH THE GIVEN INCUBATION TIMES AND TEMPERATURES. SIGNIFICANT TIME DIFFERENCES BETWEEN PIPETTING THE FIRST AND LAST WELL OF THE MICROTITER PLATE WHEN FILLING SAMPLES/CONTROL SERA, CONJUGATE OR SUBSTRATE MAY RESULT IN DIFFERENT

„PRE INCUBATION“ TIMES, WHICH MAY INFLUENCE THE PRECISION AND REPRODUCIBILITY OF THE RESULTS.

OPTIMUM RESULTS CAN ONLY BE ACHIEVED IF SERION ELISA *CLASSIC* INSTRUCTIONS ARE FOLLOWED STRICTLY.

THE TEST IS NOT VALID, IF THE LOT-SPECIFIC VALIDATION CRITERIA ON THE QUALITY CONTROL CERTIFICATE ARE NOT FULFILLED.

INADEQUATE WASHING WILL AFFECT THE TEST RESULTS:

THE WASHING PROCEDURE SHOULD BE CARRIED OUT CAREFULLY. IF THE WASHING PROCEDURE IS CARRIED OUT AUTOMATICALLY FOLLOW THE INSTRUCTION MANUAL OF THE RESPECTIVE WASHER. FLAT BOTTOM WELLS ARE USED FOR SERION ELISA *CLASSIC*. ALL WELLS SHOULD BE FILLED WITH EQUAL VOLUMES OF WASHING BUFFER. AT THE END OF THE PROCEDURE ENSURE THAT THE WELLS ARE FREE OF ALL WASHING BUFFER BY TAPPING THE INVERTED MICROTTEST PLATE ON A PAPER TOWEL. AVOID FOAM! DO NOT SCRATCH COATED WELLS DURING WASHING AND ASPIRATION. IF USING AN AUTOMATED WASHER, ENSURE IT IS OPERATING CORRECTLY.

## **7.2 Sample preparation and storage**

LIPAEMIC, HEMOLYTIC OR ICTERIC SAMPLES SHOULD ONLY BE TESTED WITH RESERVATIONS ALTHOUGH IN OUR TESTING NO NEGATIVE INFLUENCE HAS BEEN FOUND. OBVIOUSLY CONTAMINATED SAMPLES (SERUM OR PLASMA) SHOULD NOT BE TESTED DUE TO THE RISK OF WRONG RESULTS.

SERUM OR PLASMA (EDTA, CITRATE, HEPARIN) COLLECTED ACCORDING TO STANDARD LABORATORY METHODS ARE SUITABLE SAMPLES.

**Samples must not be thermally inactivated.**

### **7.2.1 Sample preparation**

BEFORE RUNNING THE TEST, SAMPLES MUST BE DILUTED IN DILUTION BUFFER ( $V_1 + V_2$ ) AS FOLLOWS:

**SERION ELISA *classic* Leptospira IgG**

$V_1 + V_2 = 1+100$	add	10 µl	sample
	each to	1000 µl	dilution buffer

AFTER DILUTION AND BEFORE PIPETTING INTO THE MICROTITER PLATE THE  
SAMPLES MUST BE MIXED THOROUGHLY TO PREPARE A HOMOGENOUS  
SOLUTION.

## SERION ELISA *classic* Leptospira IgM

### RHEUMATOID FACTOR-INTERFERENCE:

RHEUMATOID FACTORS ARE **AUTOANTIBODIES MAINLY OF THE IGM-CLASS**, WHICH PREFERABLY BIND TO IGG-IMMUNE-COMPLEXES. THE PRESENCE OF NON-SPECIFIC IGM-ANTIBODIES (RHEUMATOID FACTORS) CAN LEAD TO **FALSE-POSITIVE** RESULTS IN THE IGM-ASSAY. FURTHERMORE, THE POSSIBILITY EXISTS, THAT WEAK-BINDING PATHOGEN-SPECIFIC IGM-ANTIBODIES ARE DISPLACED BY STRONGER-BINDING IGG-ANTIBODIES. IN THIS CASE, IGM-DETECTION CAN LEAD TO **FALSE-NEGATIVE** RESULTS. THEREFORE IT IS NECESSARY TO PRETREAT SAMPLES WITH RHEUMATOID FACTOR-ABSORBENS PRIOR TO IGM DETECTION (SERION RHEUMATOID FACTOR-ABSORBENT Z200 (20 ML/100 TESTS)).

BEFORE RUNNING THE TEST, RHEUMATOID FACTOR-ABSORBENT (V<sub>1</sub>) MUST BE DILUTED 1+4 IN DILUTION BUFFER (V<sub>2</sub>).

V <sub>1</sub> + V <sub>2</sub> = 1 + 4	add	200 µl	Rf-absorbent
		each to 800 µl	dilution buffer

SAMPLES (V<sub>4</sub>) MUST BE DILUTED IN THIS RF-DILUTION BUFFER (V<sub>3</sub>)

V <sub>4</sub> + V <sub>3</sub> = 1+100	add	10 µl	sample
	each to	1000 µl	Rf-dilution buffer

### 7.2.2 Sample storage

THE STOPPERED SAMPLES CAN BE STORED IN A REFRIGERATOR UP TO 7 DAYS AT 2-8°C. EXTENDED STORAGE IS POSSIBLE AT ≤ -20°C.

AVOID REPEATED FREEZING AND THAWING OF SAMPLES.

**Diluted samples can be stored at 2-8°C for one week.**



### 7.3. Preparation of kit reagents

#### 7.3.1 Microtest strips

MICROTEST STRIPS IN FRAME ARE PACKED WITH DESICCANT IN AN ALUMINUM BAG. TAKE UNREQUIRED CAVITIES OUT OF THE FRAME AND PUT THEM BACK INTO THE PRESS-SEAL BAG. CLOSE PRESS-SEAL BAG CAREFULLY TO ENSURE AIRTIGHT CONDITIONS.

#### 7.3.2 Control sera / standard sera

CONTROL AND STANDARD SERA ARE READY-TO-USE AND MUST NOT BE DILUTED ANY FURTHER. THEY CAN BE USED DIRECTLY FOR THE TEST RUN.

FOR EACH TEST RUN AND FOR EACH TEST SYSTEM - INDEPENDENT OF THE NUMBER OF MICROTEST STRIPS TO BE USED - CONTROL AND STANDARD SERA MUST BE INCLUDED. THE CUT-OFF-CONTROL SHOULD BE SET UP IN DUPLICATE. WITH THE QUANTITATIVE TESTS THE STANDARD SERUM SHOULD ALSO BE SET UP IN DUPLICATE.

DO NOT TREAT CONTROL SERA WITH RF-ABSORBENT.

#### 7.3.3 Anti-human-IgG-, IgM- or IgA-AP-conjugate (ready-to-use)

PLEASE DO NOT MIX UP CONJUGATES FROM DIFFERENT KITS. THEY ARE OPTIMIZED FOR EACH LOT. CONJUGATES ARE EXCHANGEABLE AS DESCRIBED IN 7.1.

AVOID CONTAMINATION OF READY-TO-USE CONJUGATES (PLEASE POUR SUFFICIENT FOR TEST INTO A SECONDARY CONTAINER TO AVOID REPEATEDLY PIPETTING FROM THE ORIGINAL BOTTLE).

#### 7.3.4 Washing solution

DILUTE WASHING BUFFER CONCENTRATE ( $V_1$ ) 1:30 WITH DISTILLED WATER TO A FINAL VOLUME OF  $V_2$ .

EXAMPLE:

buffer concentrate ( $V_1$ )	final volume ( $V_2$ )
33.3 ml	1000 ml
1 ml	30 ml

#### 7.3.5 Dilution buffer for samples (ready-to-use)

#### 7.3.6 Substrate (ready-to-use)

TO AVOID CONTAMINATION USE GLOVES. FOR PIPETTING SUBSTRATE SOLUTION USE STERILE TIPS ONLY!

#### 7.3.7 Stopping solution (ready-to-use)

#### 7.4. Overview - test procedure

##### **Leptospira IgG/IgM quantitative**

IN CASE OF IGM-DETECTION ABSORPTION OF  
RHEUMATOID FACTOR! SAMPLE DILUTION

1 + 100

PIPETTE DILUTED SAMPLES AND READY-TO-  
USE CONTROL SERA / STANDARD SERA INTO  
THE MICROTEST WELLS (100 µL)

INCUBATION 60  
MIN./37°C

WASH

PIPETTE CONJUGATE SOLUTION (100 µL)

INCUBATION 30  
MIN./37°C

WASH

PIPETTE SUBSTRATE SOLUTION (100 µL)

INCUBATION 30  
MIN./37°C

PIPETTE STOPPING SOLUTION (100 µL)

READ EXTINCTION AT 405 NM

## 7.5 Test procedure

1. Place the required number of cavities in the frame and prepare a protocol sheet.
2. Add each **100 µl of diluted sample or ready-to-use controls** into the appropriate wells of microtest strips. Spare one well for substrate blank, e.g.:

IgG/IgM quantitative	
well A1	substrate blank
well B1	negative control
well C1	standard serum
well D1	standard serum
well E1	sample 1....

3. **Sample incubation for 60 minutes (+/- 5 min) at 37°C (+/- 1°C) in moist chamber**
4. After incubation **wash** all wells with washing solution (by automated washer or manually):
  - aspirate or shake out the incubation solution
  - fill each well with 300 µl washing solution
  - aspirate or shake out the washing buffer
  - repeat the washing procedure 3 times (altogether 4 times!)
  - dry by tapping the microtest plate on a paper towel
5. **Addition of conjugate**  
ADD 100 µL OF IGG-/IGM-/IGA-CONJUGATE (READY-TO-USE) TO THE APPROPRIATE WELL (EXCEPT SUBSTRATE BLANK)
6. **Conjugate incubation for 30 minutes (+/- 1 min) \*at 37°C (+/- 1°C) in moist chamber.**
7. After incubation **wash** all wells with washing solution (see above)
8. **Addition of substrate**  
ADD 100 µL SUBSTRATE SOLUTION (READY-TO-USE) TO EACH WELL (INCLUDING WELL FOR SUBSTRATE BLANK!)
9. **Substrate incubation for 30 minutes (+/- 1 min) \*at 37°C (+/- 1°C) in moist chamber.**
10. **Stopping of the reaction**  
ADD 100 µL STOPPING SOLUTION TO EACH WELL, SHAKE MICROTEST PLATE GENTLY TO MIX.
11. **Read optical density**  
READ OD WITHIN 60 MINUTES AT 405 NM AGAINST SUBSTRATE BLANK, REFERENCE WAVE LENGTH BETWEEN 620 NM AND 690 NM (E.G. 650 NM).

**\* Please note, that under special working-conditions internal laboratory adaptations of the incubation times could be necessary.**

## 8. TEST EVALUATION

### SERION ELISA *classic* Leptospira IgG/IgM (quantitative)

#### 8.1 Single-point quantification with the 4PL method

OPTIMIZED ASSIGNMENT OF EXTINCTION SIGNALS TO QUANTITATIVE VALUES IS GUARANTEED BY USING NON-LINEAR FUNCTIONS, WHICH ADJUST A SIGMOIDE CURVE WITHOUT ANY FURTHER TRANSFORMATION TO OD-VALUES.

Determination of antibody concentrations with the SERION ELISA *classic* is carried out by the **logistic-log-model (4 PL; 4 parameter)** which is ideal for exact curve-fitting. It is based on the formula:

$$OD = A + \frac{D - A}{1 + e^{B(C - \ln \text{conc.})}}$$

THE PARAMETERS A, B, C, AND D ARE REPRESENTATIVE FOR THE EXACT SHAPE OF THE CURVE:

- |                       |               |
|-----------------------|---------------|
| 1. lower asymptote    | ☐ parameter A |
| 2. slope of the curve | ☐ parameter B |
| 3. turning point      | ☐ parameter C |
| 4. upper asymptote    | ☐ parameter D |

FOR EACH LOT THE STANDARD CURVE IS EVALUATED BY INSTITUT VIRION\SERION GMBH (WÜRZBURG, GERMANY) IN SEVERAL REPEATED TEST RUNS UNDER OPTIMAL CONDITIONS. TIME CONSUMING AND COST INTENSIVE CONSTRUCTION OF THE STANDARD CURVE BY THE USER IS NOT NECESSARY.

FOR EVALUATION OF ANTIBODY CONCENTRATIONS A LOT SPECIFIC STANDARD CURVE AS WELL AS A LOT SPECIFIC EVALUATION TABLE IS INCLUDED WITH EACH TEST KIT. APPROPRIATE EVALUATION SOFTWARE IS AVAILABLE ON REQUEST.

TO COMPENSATE FOR NORMAL TEST VARIATIONS AND ALSO FOR TEST RUN CONTROL A STANDARD SERUM IS USED IN EACH INDIVIDUAL TEST RUN. FOR THIS CONTROL SERUM A "REFERENCE VALUE" WITH A VALIDITY RANGE IS DETERMINED BY THE QUALITY CONTROL OF THE PRODUCER. WITHIN THIS RANGE A CORRECT QUANTIFICATION OF ANTIBODY CONCENTRATION IS ENSURED. SINCE THE STANDARD SERUM IS NOT NECESSARILY A POSITIVE CONTROL, THE VALUE OF THE STANDARD SERUM MAY BE BORDERLINE OR NEGATIVE IN SOME ELISA TESTS.

## 8.2 Criteria of validity

- the substrate blank must be  $OD < 0.25$
- the negative control must be negative
- quantitative ELISA: the mean OD-value of the standard serum must be within the validity range, which is given on the lot specific quality control certificate of the kit (after subtraction of the substrate blank!)
- qualitative ELISA: the mean OD-value of the positive control must be within the validity range, which is given on the lot specific quality control certificate of the kit (after subtraction of the substrate blank!)
- the variation of OD-values may not be higher than 20%.

IF THESE CRITERIA ARE NOT MET, THE TEST IS NOT VALID AND MUST BE REPEATED.

## 8.3 Calculation

**SERION ELISA *classic* Leptospira IgG/IgM (quantitative)**

### 8.3.1 Non-automated evaluation

FOR THE TEST EVALUATION A STANDARD CURVE AND AN EVALUATION TABLE ARE INCLUDED IN THE TEST KIT SO THAT THE OBTAINED OD-VALUES MAY BE ASSIGNED TO THE CORRESPONDING ANTIBODY ACTIVITY. THE REFERENCE VALUE AND THE VALIDITY RANGE OF THE STANDARD SERUM IS GIVEN ON THE EVALUATION TABLE (QUALITY CONTROL CERTIFICATE).

**The blank (A1) must be subtracted from all OD-values prior to the evaluation.**

#### Method 1: Qualitative Evaluation

TO FIX THE CUT-OFF RANGES PLEASE MULTIPLY THE MEAN VALUE OF THE MEASURED STANDARD-OD WITH THE NUMERICAL DATA OF THE CERTIFICATE OF QUALITY CONTROL (SEE SPECIAL CASE FORMULAS), E.G.:  
 $OD = 0.502 \times MW (STD)$  WITH UPPER  
 $CUT-OFF OD = 0.352 \times MW (STD)$   
WITH LOWER CUT-OFF

IF THE MEASURED MEAN ABSORBANCE VALUE OF THE STANDARD SERUM IS 0.64, THE RANGE OF THE CUT- OFF IS IN BETWEEN 0.225-0.321.

**METHOD 2: CONTINUOUS DETERMINATION OF ANTIBODY ACTIVITIES USING THE STANDARD CURVE.**

SO CALLED *INTERASSAY VARIATIONS* (DAY TO DAY DEVIATIONS AND LABORATORY TO LABORATORY DEVIATIONS) ARE COMPENSATED BY MULTIPLICATION OF THE CURRENT MEASURED VALUE OBTAINED WITH A

$$\mathbf{F} = \frac{\text{OD-reference value (of standard serum)}}{\text{OD-current value (of standard serums)}}$$

SAMPLE WITH THE **CORRECTION FACTOR F**. THIS FACTOR IS CALCULATED AS FOLLOWS:

THE PROCEDURE IS NECESSARY TO ADJUST THE CURRENT LEVEL OF THE TEST OF THE USER WITH THE LOT- SPECIFIC STANDARD CURVE.

FIRST, DAILY DEVIATIONS HAVE TO BE CORRECTED BY CALCULATING A FACTOR (CORRECTION FACTOR F):

1. The mean of the two OD-values of the standard serum has to be calculated and checked that it is within the given validity range.
2. Calculation of the factor "F": the given reference value is divided by the mean of the extinction of the standard serum:  
$$F = \text{REFERENCE VALUE EXTINCTION STANDARD SERUM} / \text{MEAN VALUE EXTINCTION STANDARD SERUM}.$$
3. All measured values of samples are multiplied by "F".
4. Antibody activities in IU/ml or U/ml can be determined from the standard curve with the corrected values.

### **8.3.2 Automatic test evaluation with SERION *easy base* 4PL-Software/SERION *evaluate*-Software**

AFTER INPUT OF THE 4 PARAMETERS AND THE REFERENCE VALUE OF THE STANDARD SERUM, ANTIBODY ACTIVITIES ARE CALCULATED ONLINE. IF THE OPTICAL DENSITY OF THE STANDARD IS OUT OF THE VALID RANGE, THE FOLLOWING MESSAGE WILL APPEAR:

**SERION *easy base* 4PL-Software:**

**"Standards are not in tolerance range" and/or "Distance between standards is greater than 20 %."**

**SERION *evaluate*-Software:**

**"Standard values out of ranges in following groups: Group 1-24. Standard value differ more than 20% in following groups: Group 1-24."**

IN THESE CASES THE TEST RUN IS INVALID AND SHOULD BE REPEATED.

PARAMETERS AND REFERENCE VALUE NEED TO BE CHANGED ONLY IF THERE IS A CHANGE OF LOT (EVALUATION TABLE SHOWS PARAMETERS AND REFERENCE VALUES). CORRECT INPUT OF THE LOT SPECIFIC DATA CAN BE CHECKED ON THE BASIS OF THE IU/ML OR U/ML ASSIGNED TO THE STANDARD SERUM. THE CALCULATED MEAN VALUE OF THE UNITS HAS TO CORRESPOND TO THE UNIT VALUE INDICATED ON THE LOT SPECIFIC CERTIFICATE. THERE IS AN AUTOMATIC CORRECTION OF THE MEASURED VALUES. IN THE STANDARD VERSION THE PRINTOUT DISPLAYS THE FOLLOWING:



SAMPLE CODE OD- VALUE IU/ML OR
---

## **9. STATEMENTS OF WARNING**

### **9.1 Statements of warning**

THE SERION ELISA *CLASSIC* IS ONLY DESIGNED FOR QUALIFIED PERSONNEL WHO ARE FAMILIAR WITH GOOD LABORATORY PRACTICE.

ALL KIT REAGENTS AND HUMAN SPECIMEN SHOULD BE HANDLED CAREFULLY, USING ESTABLISHED GOOD LABORATORY PRACTICE.

- This kit contains human blood components. Although all control- and cut-off-sera have been tested and found negative for HBs-Ag-, HCV- and HIV-antibodies, they should be considered potentially infectious.
- Do not pipette by mouth.
- Do not smoke, eat or drink in areas in which specimen or kit reagents are handled.
- Wear disposable gloves, laboratory coat and safety glasses while handling kit reagents or specimen. Wash hands thoroughly afterwards.
- Samples and other potentially infectious material should be decontaminated after the test run.
- Reagents should be stored safely and be inaccessible to unauthorized access e.g. children.
- Stopping solution: corrosive (C); cause acid burn (R34)  
USE SAFETY GLASSES, GLOVES AND LABORATORY COAT WHILE HANDLING!

### **9.2 Disposal**

PLEASE OBSERVE THE RELEVANT STATUTORY REQUIREMENTS!

## **10. BIBLIOGRAPHY**

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3. Ko, A. I., Galvao Reis, M., Ribeiro Dourado, C. M., Johnson, W. D. J., Riley, L. W., and the Salvador Leprositis Study Group Urban epidemic of severe leptospirosis in Brazil. *Lancet* 354: 820-824, 1999.
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### Appendix III: BCM Letter of Support

27 August 2018

**RE: Nicole Delgado**

MPH Student Thesis Project: HSC-SPH-18-0688

"INVESTIGATION OF HANTAVIRUS AND LEPTOSPIROSIS AS POSSIBLE CONTRIBUTING CAUSES OF UNEXPLAINED KIDNEY DISEASE EPIDEMIC IN NICARAGUA"

Reference number: 176220

Dear Committee for the Protection of Human Subjects,

Please accept this letter of support for the thesis work proposed at Baylor College of Medicine by Nicole Delgado. I am the Principal Investigator and Dr. Rebecca Fischer is the thesis Advisor here in our department. Ms. Delgado's work will be of great public health importance and contribute to our overarching investigation into the cause of a mysterious disease causing a major public health crisis in Latin America. Please do not hesitate to contact Dr. Fischer directly at [rebecca.fischer@bcm.edu](mailto:rebecca.fischer@bcm.edu) if anything further is needed on our end.

Sincerely,

Kristy O. Murray, DVM, PhD

Rebecca S.B. Fischer, MPH, PhD

## Appendix IV: BCM IRB Approval Letter

5/24/2018

Human Approval Letter

February 12, 2018

KRISTY ORSBURN MURRAY  
BAYLOR COLLEGE OF MEDICINE  
PEDIATRICS: TROPICAL MEDICINE



Baylor College of Medicine  
Office of Research  
One Baylor Plaza, 600D  
Houston, Texas 77030  
Phone: (713) 798-6970  
Fax: (713) 798-6990  
Email: [irb@bcm.tmc.edu](mailto:irb@bcm.tmc.edu)

### **H-36498 - INVESTIGATION INTO THE ETIOLOGY OF UNEXPLAINED KIDNEY DISEASE IN NICARAGUA**

**APPROVAL VALID FROM 2/12/2018 TO 2/11/2019**

Dear Dr. MURRAY

The Institutional Review Board for Human Subject Research for Baylor College of Medicine and Affiliated Hospitals (BCM IRB) is pleased to inform you that the research protocol named above was reviewed and approved by Expedited procedures on 2/12/2018 by Board 6.

The study may not continue after the approval period without additional IRB review and approval for continuation. You will receive an email renewal reminder notice prior to study expiration; however, it is your responsibility to assure that this study is not conducted beyond the expiration date.

Please be aware that only IRB-approved informed consent forms may be used when written informed consent is required.

Any changes in study or informed consent procedure must receive review and approval prior to implementation unless the change is necessary for the safety of subjects. In addition, you must inform the IRB of adverse events encountered during the study or of any new and significant information that may impact a research participants' safety or willingness to continue in your study.

The BCM IRB is organized, operates, and is registered with the United States Office for Human Research Protections according to the regulations codified in the United States Code of Federal Regulations at 45 CFR 46 and 21 CFR 56. The BCM IRB operates under the BCM Federal Wide Assurance No. 00000286, as well as those of hospitals and institutions affiliated with the College.

Sincerely yours,

A handwritten signature in black ink, appearing to read "Flor Munoz-Rivas".

FLOR MUNOZ-RIVAS, M.D.  
Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals





**Committee for the Protection of Human Subjects**

6410 Fannin Street, Suite 1100  
Houston, Texas 77030

**Nicole Delgado**  
**UT-H - SPH - Epidemiology & Disease Control**

**NOTICE OF APPROVAL TO BEGIN RESEARCH**

**September 06, 2018**

**HSC-SPH-18-0688** - INVESTIGATION OF HANTAVIRUS AND LEPTOSPIROSIS AS POSSIBLE CONTRIBUTING CAUSES OF UNEXPLAINED KIDNEY DISEASE EPIDEMIC IN NICARAGUA

**Number of Subjects Approved: Target: 200**

**PROVISIONS:** This approval relates to the research to be conducted under the above referenced title and/or to any associated materials considered by the Committee for the Protection of Human Subjects, e.g. study documents, informed consent, etc.

**APPROVED:** By Expedited Review and Approval

**REVIEW DATE:** September 6, 2018

**APPROVAL DATE:** September 6, 2018

**CHAIRPERSON:** L. Maximilian Buja, MD

A handwritten signature in black ink that reads "L. Maximilian Buja".

Subject to any provisions noted above, you may now begin this research.

**PLEASE NOTE:** Due to revisions to the common rule that went into effect July 19, 2018, this study that was approved under expedited approval no longer needs to submit for continuing review. Changes to the study, adverse events, protocol deviations, personnel changes, and all other types of reporting must still be submitted to CPHS for review and approval. When this study is complete, the PI must submit a study closure report to CPHS.

**CHANGES:** The principal investigator (PI) must receive approval from the CPHS before initiating any changes, including those required by the sponsor, which would affect human subjects, e.g. changes in methods or procedures, numbers or kinds of human subjects, or revisions to the informed consent document or procedures. The addition of co-investigators must also receive approval from the CPHS. **ALL PROTOCOL REVISIONS MUST BE SUBMITTED TO THE SPONSOR OF THE RESEARCH.**

**INFORMED CONSENT DETERMINATION:**

Waiver of Consent Granted

**INFORMED CONSENT:** When Informed consent is required, it must be obtained by the PI or designee(s), using the format and procedures approved by the CPHS. The PI is responsible to instruct the designee in the methods approved by the CPHS for the consent process. The individual obtaining informed consent must also sign the consent document. Please note that only copies of the stamped approved informed consent form can be used when obtaining consent.

**HEALTH INSURANCE PORTABILITY and ACCOUNTABILITY ACT (HIPAA):**

**HIPAA Authorization required:**

**Waiver for Retrospective Chart Review granted:**

Information to be accessed: City

Information to be retained: City

**UNANTICIPATED RISK OR HARM, OR ADVERSE DRUG REACTIONS:** The PI will immediately inform the CPHS of any unanticipated problems involving risks to subjects or others, of any serious harm to subjects, and of any adverse drug reactions.

**RECORDS:** The PI will maintain adequate records, including signed consent and HIPAA documents if required, in a manner that ensures subject confidentiality.

## Appendix VI: Variable Description Table

Variable Name	Variable Label
sex	Participant Sex
agecat1	Age is 30+ (years)
agecat2	Participant Age
agecat3	Age is 25+ (years)
agecat4	Age is 35+ (years)
city	City of Residence
resid_chichi	Lived in Chichigalpa
drug	Drug Use
drugs	Drugs
drug_ever	Drug Use (Current/Former)
wk_isa_yrs	Total years worked at ISA
wk_isa_cat0	Total years worked at ISA (categorical)
wk_isa_cat1	Total years worked at ISA (<5)
wk_isa_cat3	Total years worked at ISA
wk_isa_cat4	Total years worked at ISA >2 yrs
wk_isa_cat5	Total years worked at ISA >10 yrs
wk_Campo_cat1	Total years in Cane Fields (>5)
wk_Campo_cat0	Total years in Cane Fields (categorical)
wk_Campo_cat4	Total years worked at Field > 2 yrs
wk_Campo_cat5	Total years worked at Field >10yrs
occup_acarreo	Works in Paqueta/Carry
occup_cosecha	Works in Harvest (operario general)
occup_camaron	Works in Camaronera
occup_plagas	Works in Control de Pest
occup_horno	Works in Oven
occup_maleza	Works in Control de Weeds
occup_quema	Works in Burn (Field/Oven)
occup_corte_m	Works in Cut Sugar Cain - Mechanized
occup_siembra	Works in occup_Planting/Reseeding
occup_riego	Works in Irrigation/Drainage
occup_rymma	Automechanic
occup_fabrica	Works in Factory
worksoil	works with soil
Campoworker	Works in field (either soil and/or water)
worksoil_road	Type of Working Soil - Road
worksoil_ditch	Type of Working Soil - Ditch
worksoil_aero	Type of Working Soil - Aerosolizado
worksoil_dry	Type of Working Soil - Dry
worksoil_wet	Type of Working Soil - Wet
worksoil_Campo	Type of Working Soil - Field
worksoil_other	Type of Working Soil or Other_Spec
Variable Name	Variable Label
rats_Casa	Ever sees Rodents in Home
rats_Casa_feces	Ever sees Rodent Feces in Home 61



rats_Casa_daily	Sees Rodents in Home Daily
rats_Casa_freq	Sees Rodents in Home, Frequency
rats_Casa_freq2	Sees Rodents in Home, Frequency
handwash_Casa	Handwash <a href="#">agudo</a> Water from My House
handwash_foun	Handwash <a href="#">agudo</a> Fountain
handwash_ditch	Handwash <a href="#">agudo</a> Drain
handwash_drain	Handwash <a href="#">agudo</a> Drainage Tube
handwash_well	Handwash Well
handwash_truck	Handwash Truck
handwash_other	Handwash Other
handwash_always	Handwash Always
handwash_usualy	Handwash Usually
handwash_rarely	Handwash Rarely
handwash_never	Handwash Never
handwash_cat	Handwash <a href="#">Catagorical</a>
drink_etoh_freq	Drink etoh Frequently
drink_etoh_otro	drink_etoh_Other
drink_etoh_ot	drink_etoh_Other1
drink_etoh_ce	drink_etoh_
drink_bottle	Drink from Bottle
drink_fountain	in the field drinks water from fountain in the field drinks water from ISA
drink_truck	drink_truck
drink_well	in the field drinks water from a drink_well in the field drinks water from the irrigation
drink_ditch	ditch in the field drinks water from
drink_drain	drink_drainage pipe drinks water from the ditch or drainage
drink_drain ditch	tube
wellwater_drink	Drinks Well Water at Home or Work
ppe_gloves	Used Gloves - Always/Occasionally/Never
ppe_gloves_yn	Used Gloves - Yes/No
ppe_glasses	Used Protective Glasses
ppe_glasses_yn	Used Protective Glasses - Yes/No
ppe_mask	Used Mask
ppe_mask_yn	Used Mask - Yes/No
ppe_nose	Used Protective Nose Covering
ppe_nose_yn	Used Protective Nose Covering - Yes/No
ppe_pants	Used Long Pants
ppe_pants_yn	Used Long Pants - Yes/No

# Appendix VII: Previous Study Acute Case Interview Form

## SECTION 1: Acute Case

I'm going to ask you about what happened when you were sick on:

How long were you sick before going to the hospital?

☐ the same day ☐ \_\_\_ days ☐ \_\_\_ weeks

☐ I Don't

What time did your symptoms start? \_\_\_\_:

☐ Morning

☐ Afternoon

☐ Night

☐ I Don't

Know

Were you working at the time you got sick?

☐ Yes

☐ No

☐ I Don't Know

If you answer is Yes

What job were you currently working when you got sick? \_\_\_\_\_

How many hours of the day had you been working when your symptoms began? \_\_\_\_\_

Did you feel very hot immediately when your symptoms began? ☐ Yes ☐ No ☐ I Don't Know

Please tell me if you had any of these symptoms during your illness (this episode, including the week before):

<u>Symptoms during the acute disease</u>		<u>Description or notes</u>
Fever	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	Temp: ____ °C, duration: ____ days
Headache	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Abdominal pain	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Low back pain/Back pain	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Cervicalgia/Neck pain	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Arthralgia	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	Which joint:
Myalgia	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Chest pain	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Nausea	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Vomiting	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Diarrhea	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Muscle weakness	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Cold/Chills	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Cough	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Dyspnea/Difficulty breathing	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Fatigue/Discomfort	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Jaundice	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Tremors	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Rash	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	Where:
Difficulty or Pain with Urination	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Blurry vision	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Edema/Swelling	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	Where:
Cramps	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Paresthesia (ex. Burning/Prickling)	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Dizziness	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Confusion	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Red eyes	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Other symptoms:		

How many nights did you spend in the hospital? ☐ \_\_\_ night(s) ☐ I Don't Know

How long did the symptoms last (in total)? ☐ \_\_\_ days ☐ \_\_\_ weeks ☐ I Don't Know

## Occupation

I want to know about the job you were working on:

What was your job/occupation?

☐ Hauler/loader

☐ Shrimp farm worker

☐ Pest control

☐ Weed control

☐ Sugarcane cutting, mechanical

☐ Sugarcane cutting, manual

☐ Seed cutting

☐ Factory

Acute Case Date (from Form A):

\_\_\_\_/\_\_\_\_/\_\_\_\_

☐ Sugar Cane Operator

☐ Fertilizer Operator

☐ Oven Operator

☐ Sugarcane burning

☐ Sowing/Reseeding

☐ Irrigation/Drainage

☐ Auto mechanic

☐ Other: \_\_\_\_\_

**What is the schedule that you normally worked?**

☐ 6am ☐ 7am ☐ 8am ☐ 9am ☐ 10am ☐ 11am ☐ 12md ☐ 1pm ☐ 2pm ☐ 3pm ☐ 4pm ☐ 5pm  
☐ 6pm ☐ 7pm ☐ 8pm ☐ 9pm ☐ 10pm ☐ 11pm ☐ 12mn ☐ 1am ☐ 2am ☐ 3am ☐ 4am ☐ 5am

*If you work shift, the second shift:*

☐ 6am ☐ 7am ☐ 8am ☐ 9am ☐ 10am ☐ 11am ☐ 12md ☐ 1pm ☐ 2pm ☐ 3pm ☐ 4pm ☐ 5pm  
☐ 6pm ☐ 7pm ☐ 8pm ☐ 9pm ☐ 10pm ☐ 11pm ☐ 12mn ☐ 1am ☐ 2am ☐ 3am ☐ 4am ☐ 5am

**How many days a week?** ☐ ≤3 days ☐ 4 days ☐ 5 days ☐ 6 days ☐ 7 days ☐ other \_\_\_\_\_

**Which months did you work during this season?**

☐ Jan ☐ Feb ☐ Mar ☐ April ☐ May ☐ Jun ☐ Jul ☐ Aug ☐ Sep ☐ Oct ☐ Nov ☐ Dec

**When did you start working this season?** \_\_\_\_/\_\_\_\_/\_\_\_\_

**What other work were you doing during the 3 weeks before you got sick?**

☐ Only the same as above

☐ Other: \_\_\_\_\_

**Did your work imply that you are working on water contact (standing or digging in water)?** ☐ Yes ☐ No ☐ I Don't Know

*If your answer is Yes: What type of water? (Check all that apply)*

☐ Irrigation ☐ Drainage ☐ Water source ☐ Rain ☐ Wet floor  
☐ Standing water ☐ Shrimp water ☐ Other: \_\_\_\_\_

**Did your work imply that you are working in contact with soil? (ex. contact with skin or respiration)** ☐ Yes ☐ No ☐ I Don't Know

*If your answer is Yes: What type of soil? (Check all that apply)*

☐ Dry soil ☐ Wet soil ☐ Field soil ☐ Drainage soil ☐ Aerosolized soil  
☐ Ground on the Road ☐ Other: \_\_\_\_\_

**Did your work imply that you are working in contact with the burned cane?** ☐ Yes ☐ No ☐ I Don't Know

*If your answer is Yes: What type of contact? (Check all that apply)*

☐ Burning the sugar cane ☐ Pick up the burnt cane ☐ Oven work  
☐ Loading the burned sugar cane ☐ Other: \_\_\_\_\_

**Does your work imply that you are working in contact with chemicals?** ☐ Yes ☐ No ☐ I Don't Know

*If your answer is Yes: What type of chemicals? (Check all that apply)*

☐ pesticide ☐ herbicide ☐ fertilizer ☐ factory chemical(s) ☐ Other: \_\_\_\_\_

**Do you know specifically which chemical(s)?** \_\_\_\_\_

**What type of contact? (Check all that apply)**

☐ Application ☐ Fabric ☐ Skin contact ☐ Ingestion ☐ Dust/Inhalation  
☐ Contact with eyes ☐ Other: \_\_\_\_\_

**Where did you get drinking water in the field? (Check all that apply)**

☐ Water from home ☐ Fountain ☐ Irrigation ☐ Drainage tube ☐ Water well ☐ Water truck  
☐ Bottled water ☐ Other: \_\_\_\_\_

*If you answer is Water from home: What water do you drink if you have finished the water you brought?*

**Do you wash your hands before you eat in the field?**

☐ Yes, Always ☐ Usually ☐ Rarely ☐ Other \_\_\_\_\_ ☐ No, Never ☐ I Don't Know

*If your answer is Yes: Where do you wash your hands? (Check all that apply)*

☐ Water from home ☐ Fountain ☐ Irrigation ☐ Drainage tube ☐ Water well  
☐ Water truck ☐ Bottled water ☐ Other: \_\_\_\_\_

**Which of the following do you use when you are working?**

Gloves	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Glasses or sunglasses	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Mouth cover (mask)	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Nose cap	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Long pants	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Long sleeved shirt	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Boots	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Waterproof boots	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Hat or cap	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know

**Did you work any other job(s) at the time? (ex. mani, plantains, extraction or others jobs)** ☐ Yes ☐ No ☐ I Don't Know

*If your answer is Yes: Describe the other job(s)* \_\_\_\_\_

**Have you ever seen rats while working?**      ☐Yes      ☐No      ☐I Don't Know

**Have you ever had the heat stress while working?**      ☐Yes      ☐No      ☐I Don't Know

*If your answer is Yes:*

**Have you ever had to stop working because you had the heat stress?**      ☐Yes      ☐No      ☐I Don't Know

**Have you ever had dehydration while working?**      ☐Yes      ☐No      ☐I Don't Know

*If your answer is Yes:*

**Have you ever had to stop working because of dehydration?**      ☐Yes      ☐No      ☐I Don't Know

**SECTION 2:**

Are you accustomed to taking pills or medications, for example, when you have a pain or a fever? Please think about all the prescriptions, self-treatment, natural treatments that you normally use. ☐ Yes ☐ No ☐ I Don't Know

Please tell me if you have ever taken any of the following medications. (Ask about all)

	How often do you have an episode while taking these pain pills?	How many days do you take during each occurrence?	How much time has passed since the last time you took pills?
<input type="checkbox"/> antibiotic	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> amoxicillin	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> ciprofloxacin	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> gentamicin	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> penicillin	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> trimethoprim	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> acetaminophen/ paracetamol	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> ibuprofen	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> naproxen	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> metamizole	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> diclofenac	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> diclofenac gel	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> ranitidine (zantac)	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/> omeprazole (Prilosec)	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/>	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		
<input type="checkbox"/>	<input type="checkbox"/> every: ____ days ____ weeks ____ months <input type="checkbox"/> less than 1/year <input type="checkbox"/> never		

**Which one did you use in the week before you became sick (the acute case date)? (Check all that apply)**

☐None    ☐antibiotic    ☐acetaminophen/paracetamol    ☐ibuprofen    ☐omeprazole (Prilosec)  
☐ranitidine (zantac)    ☐Other \_\_\_\_\_ ☐I Don't Know

**Which type do you use to treat your symptoms/disease during the occurrence? (Check all that apply)**

☐None    ☐antibiotic    ☐acetaminophen/paracetamol    ☐ibuprofen    ☐omeprazole (Prilosec)  
☐ranitidine (zantac)    ☐Other \_\_\_\_\_ ☐I Don't Know

**Has there been a time a doctor told you that you have elevated creatine or any kidney problem?** ☐Yes ☐No ☐I Don't Know

*If the answer is Yes: When? \_\_\_\_\_ (Age, Date, or Time since then)*

**Has a doctor ever told you that you have... (Please ask about all)**

<u>Health condition</u>		<b>When?</b> <u>(Date or age)</u>	<u>Description and notes</u>
Asthma	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Anemia	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Diabetes	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Gout	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Heart disease	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Hepatitis	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Hepatic disease	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Hypertension	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Kidney stones	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Cystitis	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Other problem/renal disease	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Sexually transmitted disease	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Frequent urinary tract infections (UTIs)	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Pancreatitis	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Azotemia	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Leptospirosis	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Malaria	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Chagas	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Dengue	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Chikungunya	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
Zika	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know		
<input type="checkbox"/> Other health condition			
<input type="checkbox"/> Other health condition			

**Have any of your parents or grandparents have...**

<i>Health condition</i>		<b>Who?</b>
Diabetes	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Gout	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Heart disease	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Hepatic disease	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Hypertension/High blood pressure	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Kidney stones	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Cystitis	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Other problem/kidney disease	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Frequent urinary tract infections (UTIs)	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Pancreatitis	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Azotemia	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	

Do you smoke or have smoked at any time in your life?

- ☐ Yes  
☐ Yes, but only in the past  
☐ No, never  
☐ I don't know

Did you drink or have you drank alcohol?

- ☐ Yes  
☐ Yes, but only in the past  
☐ No, never  
☐ I don't know

Did you take or have you taken Lija?

- ☐ Yes  
☐ Yes, but only in the past  
☐ No, never  
☐ I don't know

Did you take or have you taken drugs?

- ☐ Yes → What class? \_\_\_\_\_  
 How often?  
☐ Daily ☐ 3-4/week ☐ 1-2/week ☐ 1-2/month ☐ Rarely  
☐ Other: \_\_\_\_\_  
☐ Yes, but only in the past → What class? \_\_\_\_\_  
☐ No, never  
☐ I don't know

Do you use or have you used traditional medicine?

- ☐ Yes → Which? ☐ Red radish ☐ Green radish ☐ Basil ☐ Noni  
☐ Horse tail ☐ Malago ☐ Chamomile ☐ Other(s): \_\_\_\_\_  
 How often?  
☐ Daily ☐ 3-4/week ☐ 1-2/week ☐ 1-2/moth ☐ Rarely  
☐ Other: \_\_\_\_\_  
☐ Yes, but only in the past → Which? ☐ Red radish ☐ Green radish ☐ Basil ☐ Noni  
☐ Horse tail ☐ Malago ☐ Chamomile ☐ Other: \_\_\_\_\_  
☐ No, never  
☐ I don't know

**Have you ever been bitten by any insects, rats, or other animal?** ☐Yes ☐No ☐I Don't Know

*If the answer is Yes: Which ones? (Check all that apply)*

- ☐ Mosquitoes ☐ Scorpion ☐ Spider ☐ Snake  
☐ Rat ☐ Dog ☐ Cat ☐ Other \_\_\_\_\_

**SECTION 3: Subjective Case**

In the past, have you ever thought the it is the same type of disease before the episode of

☐ Yes ☐ No ☐ I Don't Know**Acute Case Date**

(from Case Report, Form

A): \_\_\_\_/\_\_\_\_/\_\_\_\_

?

\*If the answer = "No" or if the date is within two weeks of the Acute Case Date—

When was the first time it started? What day?

"Subjective Date":

or How long ago did it start?

If the Date is less than a week before the Acute Ca

Please tell me if you had any of the following sym

t moment:

<u>Symptoms during the acute disease</u>		<u>Description or notes</u>
Fever	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	Temp: _____. ____ °C, duration: ____ days
Headache	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Abdominal pain	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Low back pain/Back pain	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Cervicalgia/Neck pain	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Arthralgia	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	Which joint:
Myalgia	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Chest pain	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Nausea	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Vomiting	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Diarrhea	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Muscle weakness	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Cold/Chills	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Cough	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Dyspnea/Difficulty breathing	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Fatigue/Discomfort	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Jaundice	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Tremors	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Rash	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	Where:
Difficulty or Pain with Urination	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Blurry vision	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Edema/Swelling	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	Where:
Cramps	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Paresthesia (ex. Burning/Prickling)	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Dizziness	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Confusion	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Red eyes	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
Other symptoms:		



### Occupation

#### **What is your current job/occupation at ISA?**

- ☐ The same as above → *MOVE TO SECTION 4\**
- ☐ Hauler/loader
- ☐ Shrimp farm worker
- ☐ Pest control
- ☐ Weed control
- ☐ Sugarcane cutting, mechanical
- ☐ Sugarcane cutting, manual
- ☐ Seed cutting
- ☐ Factory

- ☐ General Crop Operator
- ☐ Fertilizer Operator
- ☐ Oven Operator
- ☐ Sugarcane burning
- ☐ Sowing/Reseeding
- ☐ Irrigation/Drainage
- ☐ Auto mechanic
- ☐ Other: \_\_\_\_\_

#### **What is the schedule that you normally worked?**

- ☐ 6am   ☐ 7am   ☐ 8am   ☐ 9am   ☐ 10am   ☐ 11am   ☐ 12md   ☐ 1pm   ☐ 2pm   ☐ 3pm
- ☐ 4pm   ☐ 5pm
- ☐ 6pm   ☐ 7pm   ☐ 8pm   ☐ 9pm   ☐ 10pm   ☐ 11pm   ☐ 12mn   ☐ 1am   ☐ 2am   ☐ 3am
- ☐ 4am   ☐ 5am

*If you work shift, the second shift:*

- ☐ 6am   ☐ 7am   ☐ 8am   ☐ 9am   ☐ 10am   ☐ 11am   ☐ 12md   ☐ 1pm   ☐ 2pm   ☐ 3pm
- ☐ 4pm   ☐ 5pm
- ☐ 6pm   ☐ 7pm   ☐ 8pm   ☐ 9pm   ☐ 10pm   ☐ 11pm   ☐ 12mn   ☐ 1am   ☐ 2am   ☐ 3am
- ☐ 4am   ☐ 5am

**How many days a week?**   ☐ ≤3 days   ☐ 4 days   ☐ 5 days   ☐ 6 days   ☐ 7 days   ☐ other

#### **Which months did you work during this season?**

- ☐ Jan   ☐ Feb   ☐ Mar   ☐ April   ☐ May   ☐ Jun   ☐ Jul   ☐ Aug   ☐ Sep   ☐ Oct
- ☐ Nov   ☐ Dec

**Did your work imply that you are working on water contact (standing or digging in water)?**   ☐ Yes   ☐ No   ☐ I Don't Know

*If your answer is Yes: What type of water? (Check all that apply)*

- ☐ Irrigation   ☐ Drainage   ☐ Water source   ☐ Rain   ☐ Wet floor
- ☐ Standing water   ☐ Shrimp water   ☐ Other: \_\_\_\_\_

**Did your work imply that you are working in contact with soil? (ex. contact with skin or respiration)**   ☐ Yes

☐ No   ☐ I Don't Know

*If your answer is Yes: What type of soil? (Check all that apply)*

- ☐ Dry soil   ☐ Wet soil   ☐ Field soil   ☐ Drainage soil   ☐ Aerosolized soil
- ☐ Ground on the Road   ☐ Other: \_\_\_\_\_

**Did your work imply that you are working in contact with the burned cane?**   ☐ Yes   ☐ No   ☐ I Don't Know

*If your answer is Yes: What type of contact? (Check all that apply)*

- ☐ Burning the sugar cane   ☐ Pick up the burnt cane   ☐ Oven work
- ☐ Loading the burned sugar cane   ☐ Other: \_\_\_\_\_

**Did your work imply that you are working in contact with chemicals?**   ☐ Yes   ☐ No   ☐ I Don't Know

*If your answer is Yes: What type of chemicals? (Check all that apply)*

- ☐ Pesticide   ☐ Herbicide   ☐ Fertilizer   ☐ Factory chemicals
- ☐ Other: \_\_\_\_\_

**Do you know specifically which chemical(s)?**

**What type of contact? (Check all that apply)**

- ☐ Applied   ☐ Fabric   ☐ Skin contact   ☐ Ingestion
- ☐ Inhalation   ☐ Into the eyes   ☐ Other: \_\_\_\_\_

**Which of the following do you use when you are working?**

Gloves	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Glasses or sunglasses	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Mouth cover (mask)	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Nose cap	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Long pants	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Long sleeved shirt	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Boots	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Waterproof boots	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know
Hat or cap	<input type="checkbox"/> Yes, Always	<input type="checkbox"/> Usually	<input type="checkbox"/> Rarely	<input type="checkbox"/> No, Never	<input type="checkbox"/> I Don't Know

**Are you working any other jobs at the moment?** (ex. peanuts, plantains, extraction or other jobs) ☐ Yes ☐ No ☐ I don't know

If the answer is Yes: Describe all other jobs \_\_\_\_\_

**2. Work History**

**What have been your other jobs/occupations in your work life?**

<u>Activity</u>	<u>Location (Country, Department)</u>	<u>Start Year</u>	<u>Number of Years</u>
<input type="checkbox"/> Laborer			
<input type="checkbox"/> Merchant			
<input type="checkbox"/> Farmer			
<input type="checkbox"/> Rancher			
<input type="checkbox"/> Miner			
<input type="checkbox"/> Ship worker			
<input type="checkbox"/> Brick manufacturer			
<input type="checkbox"/> Technical worker			
<input type="checkbox"/> Other(s):			

**What agricultural activities have you done in your life?**

<u>Activity</u>	<u>Location (Country, Department)</u>	<u>Start Year</u>	<u>Number of Years</u>
<input type="checkbox"/> Rice			
<input type="checkbox"/> Sesame			
<input type="checkbox"/> Sugar			
<input type="checkbox"/> Peanut			
<input type="checkbox"/> Banana			
<input type="checkbox"/> Corn			
<input type="checkbox"/> Coffee			
<input type="checkbox"/> Beans			
<input type="checkbox"/> Vegetables			
<input type="checkbox"/> Other(s):			

**What position do you take part in that plant?**

<u>Activity</u>	<u>Start Year</u>	<u>Number of Years</u>
<input type="checkbox"/> Haulage		

<input type="checkbox"/> Shrimp farmer		
<input type="checkbox"/> Pest control		
<input type="checkbox"/> Weed control		
<input type="checkbox"/> Sugar cane cutter, mechanical		
<input type="checkbox"/> Sugar cane cutter, manual		
<input type="checkbox"/> General Crop Operator		
<input type="checkbox"/> Fertilizer Operator		
<input type="checkbox"/> Oven Operator		
<input type="checkbox"/> Burn the cane		
<input type="checkbox"/> Sowing/Reseeding		
<input type="checkbox"/> Irrigation/Drainage		
<input type="checkbox"/> Auto mechanic		
<input type="checkbox"/> Other(s):		

#### **SECTION 4:**

**Do you have any family members, now or in the past, with kidney disease, such as father, mother, brother, or uncle?**

☐ Yes

☐ No

☐ I Don't Know

Relation	Age when you became sick	Sex	Occupation when you became sick	Was it ever in agriculture?	Did you ever work at ISA? <i>If the answer is Yes,</i> What work and for how long?	How is he/she doing? (Ex. death/dialysis)
				<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
				<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
				<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	
				<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> I Don't Know	

**What do you think is causing kidney disease in your community?**

**What is the last level of the school that you completed?**

☐ Primary → Grade: \_\_\_\_\_

☐ Secondary → Grade: \_\_\_\_\_

☐ Superior → Technical school? ☐ Yes ☐ No

☐ I Don't Know

☐ University → Bachelor's? ☐ Yes ☐ No ☐ I Don't Know

☐ None

**What is your marital status?**

☐ Single ☐ Married ☐ In a relationship ☐ Widowed

☐ Divorced

☐ Other: \_\_\_\_\_

**Where do you live currently?**

Address: \_\_\_\_\_

Neighborhood/Region: \_\_\_\_\_

Municipality: \_\_\_\_\_ Department: \_\_\_\_\_

**Do you work in the field/garden/farm where you currently live?** ☐ Yes ☐ No ☐ I Don't Know

*If the answer is Yes: Have you ever seen rats or mice while working there?* ☐Yes ☐No ☐I Don't Know

¿ Where did you live when you got sick in 

**Acute Case Date**  
(from Case Report, Form A): \_\_\_\_/\_\_\_\_/\_\_\_\_

 ?

- ☐ The same house where I currently live  
☐ Other house:

*If it is a different house:*

Address: \_\_\_\_\_

Neighborhood/Region: \_\_\_\_\_

Municipality: \_\_\_\_\_

Department: \_\_\_\_\_

☐Rural ☐Urban ☐Peri-urban ☐I don't know

**Did you work in the field/garden/farm where you lived?** ☐Yes ☐No ☐I Don't Know

*If the answer is Yes: Have you ever seen rats or mice while working there?* ☐Yes

☐No ☐I Don't Know

\*If Subjective Date = Acute Case Date →MOVE on to the END\*

¿ Where did you live when you got sick in 

**"Subjective Date":**  
(from page 4): \_\_\_\_/\_\_\_\_/\_\_\_\_

 ?

- ☐ The same house where I currently live  
☐ The same house where I lived when I was a child (above)  
☐ Another house:

*If the house is different:*

Address: \_\_\_\_\_

Neighborhood/Region: \_\_\_\_\_

Municipality: \_\_\_\_\_

Department: \_\_\_\_\_

☐Rural ☐Urban ☐Peri-urban ☐I don't know

**Did you work in the field/garden/farm where you lived?** ☐Yes ☐No ☐I Don't Know

*If the answer is Yes: Have you ever seen rats or mice while working*

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