Descriptive Epidemiology of Scrub Typhus in Nepal,2017

Government of Nepal

Nepal Health Research Council

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October, 2017

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Nepal Health Research Council (NHRC)
Kathmandu, Nepal

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Recommended Citation

Karki KB, Acharya BP, Dhimal M, Aryal KK, Sharma GN, Khanal P, Ranabhat K, Dumre SP, Jha AK. Descriptive Epidemiology of Scrub Typhus in Nepal. Kathmandu, Nepal, 2017. Nepal Health Research Council (NHRC).

ACKNOWLEDGEMENT

I would like to offer my sincere thanks to my research team who contributed from the conception to completion of this study. I am also thankful to the Epidemiology and Diseases Control Division (EDCD) and National Public Health Laboratory (NPHL) for their cooperation to conduct this study. My special thanks go to Dr. Baburam Marasini, the former EDCD director for the inputs; Mr. Lalan Prasad Shah for his hard work on entomological work of this study and Dr. Dinesh Dharel for his support during data collection. I am grateful to Armed Forces Research Institute of Medical Sciences and Walter-Reed AFRIMS Research Unit Nepal for their technical support for identification and testing of mites. I would like to particularly thank Dr.Wuttikon Rodkvamtook , Dr. Silas A. Davidson, Dr. Piyada Linsuwanon, Mr. Surachai Leepitakrat and Ms. Maneerat Somsri for their support in training on scrub typhus laboratory tests to the Nepalese team.

I am also thankful to administrative and financial coordination efforts made by Mr. Nirbhay Kumar Sharma and Mr. Subodh Kumar Karna of NHRC. At the last but not the least, we are very thankful to all public health offices, district hospitals, district health staffs and referral hospitals of Nepal who provided line listing data for this study.

Prof. Dr. Anjani Kumar Jha
Executive Chairman
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EXECUTIVE SUMMARY

Scrub typhus is a mite borne acute febrile infectious illness that is caused by Orientia tsutsugamushi. Very few attempts were made in Nepal before 2014 to investigate the presence of this disease. A total of 101 confirmed scrub typhus cases were reported from 16 districts in 2015. The magntitude of the outbreak was so disastrous in 2016 that by the end of third week of December, 831 cases of scrub typhus was reported in 47 of the 75 districts and fatalties reaching to 14. A national wide epidemiological study was thus felt necessary to understand the distribution of the disease. This study in addition documents entomological and rodentological evidence to confirm the presence of the outbreak. Line listing data of scrub typhus cases reported to EDCD were compiled and used for analysis. Data was collected from January to October 2016. Chitwan district was selected for rodentological and entomological study based on the severity and prevalence of the disease. Rodents were trapped; mites collected and laboratatory investigation of rodents and chiggers was done to confirm the presence of Orientia tsutsugamushi. Human blood samples were also collected from suspected scrub typhus patients and both IgM Elisa and IFA, the gold standard assay was done to confirm the presence of the parasite.

Although 831 cases were reported in the country during April-December 2016 in Nepal, complete line listing was available for only 401 cases. Around sixty percent (59.4%) of the cases were female while the median age of the cases was 25 years. Majority of the cases belonged to Janajati/Aadhibasi (44.4%) and Brahmin/Chhetri (44.1%) ethnic groups. From Tarai 81.5% of the cases were reported while on the regional basis, the central region (45.6%) had the highest number of reported cases. Chitwan was the most affected district contributing to 34.4% of the total cases. The outbreak peaked during the month of August and September.

Out of 12 rodents trapped, three were positive for chigger mites. Representative human serum samples tested by IFA confirmed the presence of Orientia tsutsugamushi in Nepal. Out of the 61 samples, 29 cases were confirmed scrub typhus positive by IFA. Similalry, two out of nine rodent serum samples were confirmed scrub typhus positive by IFA. One of the three chiggers' samples was also confirmed positive by PCR. Thus, there is clear evidence of circulation of Orientia tsutsugamushi in Nepal. The study hints the potential of scrub typhus outbreak in Nepal and there is a clear need of early preparedness and control measures.

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List of Abbreviations

µl	Microliter
AFRIMS	Armed Forces Research Institute of Medical Sciences
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BPKIHS	B.P. Koirala Institute of Health Sciences
CMC	Chitwan Medical College
DEET	N,N-diethyl-meta-toluamide
EDCD	Epidemiology and Diseases Control Division
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
IACUC	Institutional Animal Care and Use Committee
IFA	Immunofluorescent Assay
IP	Intraperitoneal Injection
NHRC	Nepal Health Research Council
NPHL	National Public Health Laboratory
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
UNICEF	The United Nations Children's Fund
VDC	Village Development Committees
WHO	World Health Organization

CHAPTER I

INTRODUCTION

1.1 Background of study

The disease

Scrub typhus is a mite borne acute febrile infectious illness that is caused by *Orientia tsutsugamushi*. The organism is an obligate intracellular gram-negative bacterium from the Rickettsiaceae family. Scrub typhus is transmitted to humans and rodents by some species of trombiculid mites *(Leptotrombidium deliense and others)*. The size of the mite is 0.2 – 0.4 mm and it can only be seen through a microscope or magnifying glass. Primarily, chigger mites (larval stage of mite) feed on rodents and small mammals. Humans are the accidental hosts. Chigger mite sucks the body fluid, not blood. This mite is both vector and reservoir. Humans get infected through a bite of an infected chigger. The incubation period of the disease is about 5 to 20 days after the initial bite. There is no human to human transmission and it doesn't transmit through a bite of infected rodent.[1]

Historical perspective

Historically, in 313 AD, a clinical manual by Hong Ge called "Zhouhofang" had mentioned the clinical description of disease and there was accurate morphological description of mites. Later, in 1596 AD, well-known Chinese physician Shi-Zen Li described the characteristics of the disease.[2] Similarly, Japanese researcher started research on this disease in 1879. The name *Rickettsia tsutsugamushi* was first used in 1930.[3] The word "*tsutsugamushi*" is derived from a Japanese word tsutsuga meaning something small and dangerous, and mushi, meaning creature, so it was known as small dangerous creature.

During World War II, there were 18,000 recorded scrub typhus cases. At that time, scrub typhus was the third most common infectious disease in Pacific theater after malaria and dengue. During the US involvement in World War II, 337 US army personnel died from scrub typhus.4 Similarly in 1972, the US Armed Forces Epidemiology Board reported that 20-30% of pyrexia of unknown origin (PUO) cases were scrub typhus.[5] Scrub typhus is endemic to a part of the world known as the "tsutsugamushi triangle" which extends from northern Japan and far-eastern Russia in the north, to northern Australia in the south, and to Pakistan in the west.6 Scrub typhus has been reported from many Asian countries. It is also endemic in India (sub-Himalayan belt, from Jammu to Nagaland, Himanchal Pradesh, Sikkim and Darjeeling) and is considered as one of the emerging infectious diseases in India. [7]

Nepalese scenario

There were very few attempts to investigate scrub typhus in Nepal. As early as 1981, a study had revealed the high possibility of scrub typhus in Nepal by showing high antibody titers among healthy adults (10%). [8] Unfortunately, further disease

possibility in the country was not followed up for next 25 years until 2004. A serological investigation of scrub typhus earlier carried out in Patan hospital found a small number of febrile patients (28/876) positive for scrub typhus antibodies.[9] Since the sample analysis was performed by using "multi-test assay" with no further confirmation for scrub typhus by immunofluorescent assay (IFA), it was not conclusive for scrub typhus (instead murine typhus was confirmed). Another report in 2007, also indicated the presence of scrub typhus in Nepal.[10] However, there was no clear evidence of apparent outbreak (and fatality) of scrub typhus in Nepal before 2014 and in fact no systematic investigation/surveillance was conducted by the government. As a consequence, there was no scrub typhus case reported to Epidemiology and Disease Control Division (EDCD) until 2014.

The 2015 scrub typhus outbreak

Three months after the devastating earthquake in Nepal (August 2015), BP Koirala Institute of Health Sciences (BPKIHS), Dharan had alerted EDCD that children with fever and severe respiratory features had not been responded with usual course of treatment. This clinical anomaly was initially hypothesized to be hanta virus infection as a potential etiology. Serum samples were brought to National Public Health Laboratory (NPHL), Kathmandu and tested for a panel of viral diseases which turned out to be negative. Subsequently, scrub typhus specific IgM enzyme-linked immunosorbent assay (ELISA) was performed and it came out to be positive. To further confirm the diagnosis, representative samples were sent to Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. The samples were confirmed with the gold standard IFA at AFRIMS. To this end, the scrub typhus fatal episodes of outbreak magnitude have officially been confirmed in Nepal in 2015. A total of 101 confirmed scrub typhus cases were reported from 16 districts in 2015 (Figure 1). Out of them, eight cases died, accounting for a crude case fatality rate of 8%. By the end of August 2016, more than 500 confirmed cases and six additional deaths were reported from the various districts of the country.

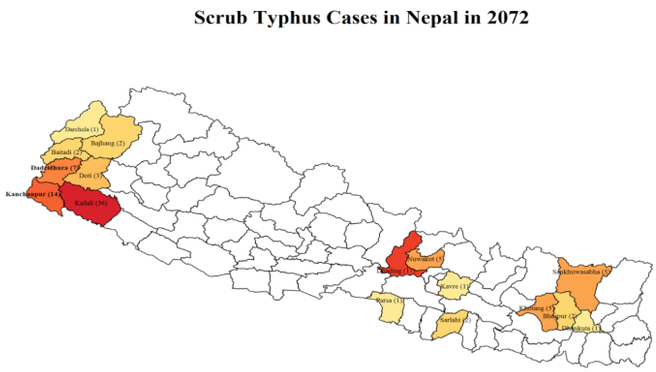

Figure 1: Distribution of scrub typhus in Nepal, 2015

EDCD case definitions

Responding the increasing number of scrub typhus cases with reported fatality, EDCD developed an Interim Guideline on Prevention and Control of Scrub Typhus in September 2015 (and later updated in August 2016).[11] The EDCD guideline outlined the following case definitions for scrub typhus.

Suspected/clinical case: Acute undifferentiated febrile illness of 5 days or more with or without eschar should be suspected as a case of Rickettsial infection (If eschar is present, fever of less than 5 days duration should be considered as scrub typhus).

Probable case: A suspected clinical case with an IgM titer > 1:32 and/or a four-fold increase of titers between acute and convalescent sera confirm a recent infection.

Confirmed case: A scrub typhus case is considered "confirmed" when the Rickettsial DNA is detected in eschar or whole blood samples by polymerase chain reaction (PCR) method; OR, four fold rise in antibody titers on acute and convalescent sera detected by IFA or indirect immunoperoxidase assay (IPA) methods.

Supportive laboratory investigations:

- Total leucocyte count during early stages may be normal but may be elevated to more than 10,000/cu mm later in the course of disease.
- Thrombocytopenia (low platelet count), usually < 1, 50,000/cu mm is seen in majority of patients.
- Elevated liver enzymes (AST, ALT etc.) are also seen in many patients.

Specimen for diagnosis:

- *Heparinized blood:* Cryopreserve at -80°C or liquid nitrogen and then ship in dry ice for cell culture.
- *EDTA blood:* Conserve at 4°C and then ship at room temperature for PCR.
- *Serum:* Conserve at 4°C, then ship at room temperature. Collect two serum specimens at 10 days interval.
- Skin or lymph node biopsy can also give the diagnosis.

Scrub typhus in Nepal — newly emerging or endemic disease

As mentioned earlier, Nepal is within the "tsutsugamushi triangle". Scrub typhus is endemic in India (sub-Himalayan belt, from Jammu to Nagaland, Himanchal Pradesh, Sikkim and Darjeeling). Similarly, scrub typhus is also endemic in Bhutan.[12] High antibody titers were observed among healthy Nepalese adults as early as 1981.[8] Moreover, Patan hospital had already identified serologically positive cases in 2004 and 2007.[9,10] One of the commonest clinical diagnoses of acute febrile illness in Nepal is enteric fever. Because of poor laboratory facilities and limited availability of laboratory services, most enteric fever cases were clinically diagnosed and put on an empirical treatment. We can have some clues of scrub typhus if we look at the historical treatment regimen for enteric fever.

1980s	1990s	2000s	2010s
Chloramphenicol	Fluoroquinolones e.g. Ciprofloxacin	Newer Fluoro-quinolones e.g. Ofloxacin Alternatively third generation Cepha-losporins e.g. Ceftriaxone	Third generation cephalosporin e.g. Ceftriaxone

Chronological trend of enteric fever treatment in Nepal (Observation)

Chloramphenicol and fluoroquinolones are effective against scrub typhus but cephalosporin group of antibiotics are not. With all these facts, most probably scrub typhus was endemic in Nepal since long but it was largely unrecognized in the common clinical settings. Probably, ceftriaxone has major role to unveil the scrub typhus in Nepal. A review article mentioned that "scrub typhus is the single most prevalent, under-recognized, neglected, and severe but easily treatable disease in the world".[13] This statement is also true for Nepal.

Scrub typhus program initiatives in Nepal

Immediately after notification from BPKIHS and serological confirmation at NPHL, EDCD had developed an Interim Guideline on Prevention and Control of Scrub Typhus in Nepal.[11] It was distributed throughout the country through District (Public) Health Offices. All health care workers were made aware on early diagnosis and treatment of scrub typhus since it would be treated with easily available medicines like doxycycline, azithromycin, chloramphenicol and ciprofloxacin. Clinicians , public health experts and paramedics throughout the country were trained on common infectious diseases including scrub typhus. Three control measures (three pillars) recommended by World Health Organization (WHO) were followed by Nepal.

- Case identification
- Public education
- Rodent control and habitat modification

For public education, EDCD developed message for public and disseminated through different channels and media. The message has been made available at: www.edcd,.gov.np

1.2 Statement of the problem and justification

Scrub typhus is an infection that may lead to generalized vasculitis, which may cause multi-organ dysfunction syndrome. Severe complications such as encephalitis, pneumonia, myocarditis, pericarditis, acute renal failure, acute hepatic failure, acute hearing loss, and acute respiratory distress syndrome have been reported in many cases. Some of these complications may lead to death. Although scrub typhus is endemic in Nepal, episodes of outbreak magnitude was reported in few districts in 2015 following the mega-earthquake. In 2015, 101 scrub typhus cases and eight deaths were reported to EDCD while by the end of August 2016, about 500 cases and six additional deaths were reported from more than 25 districts of Nepal indicating the endemicity of diseases throughout the country.[11] Scrub typhus has been increasingly reported in Nepal, and public health authorities are concerned about its increased incidence. Despite the intensity and magnitude of the scrub typhus problem in Nepal, so far there is no clear epidemiological picture, and this study aims to address it.

1.3 Objectives of the study

General Objective

The general objective of the study is to describe epidemiology of scrub typhus in Nepal

Specific Objectives

The specific objectives of the study are:

1. To examine socio-demographic characteristics of scrub typhus cases
2. To examine temporal distribution of scrub typhus cases
3. To describe spatial distribution of scrub typhus cases
4. To document rodentological and entomological evidences of scrub typhus

CHAPTER II

METHODOLOGY

This was a descriptive study using quantitative method of data collection.

2.1 Epidemiological data

In this study, line listing data of all scrub typhus cases reported to EDCD were compiled and used for analysis. Data was collected from January to October 2016.

2.2 Rodentological and entomological data

Chitwan district was selected for rodentological and entomological study based on the severity and prevalence of the disease. Three village development committees (VDCs): Mangalpur, Sharadanagar and Shukranagar which lie to the South-West of Bharatpur, the district headquarters were selected. The death due to scrub typhus occurred in Mangalpur VDC.

2.2.1 Rodent trapping

Rats were trapped by using a single catch type live traps (Sherman rodent traps). A total of 104 such traps were taken to the field from EDCD. All the traps were equipped with different baits (ripen banana and tomato, piece of chicken) and placed at various sites inside and outside houses, cattle sheds, near granaries (Aali) and the nearby fields e.g. kitchen gardens, where live rodents burrow. This was continued for two days. The baits were kept in the traps and placed during evening and then collected the next morning. The trappings were mainly done in the houses of the indicator cases and their neighbors. All the traps were tagged with a unique number so as to easily identify the baits used.

2.2.2 Euthanasia of rats

With universal aseptic precautions using full protective measures, the rodents along with the traps were placed inside a chamber one by one. Carbon dioxide gas was supplied at the rate of 2-3 liters per hour for about 4-5 minutes to anaesthetize the rodent painlessly. Then, the rodents were carefully taken out from the traps, weighed and blood sample was collected by direct heart puncturing method. Collected rodent blood was transferred to the gel tube that was stored in cold box supplied with pre-frozen ice packs at -80°C for > 24 hours at Nepal Red Cross Society, Chitwan.

Body and body parts were measured (in centimeter) for their easy identification to species level. Specifically, the color of fur on dorsal and ventral surfaces, length from snout to distal end of tail, length of bilateral pinnas, number of mammary gland and their position on ventral side of abdomen and color of incisor teeth were observed and noted for scientific identification up to species level.

2.2.3 Mite collection, preservation and identification

Mite and other ecto-parasites were collected by combing the rodents' outer surfaces such as ventral and dorsal surfaces, arm-pits, groins, and areas near pinnas, etc. onto a white clean paper sheet to help locate and identify ecto-parasites easily.

The moving ecto-paracites were picked by using a brush with fine white bristles and transferred into a vial containing 70% ethanol. The vial with ecto-parasite was labeled properly and stored in the cold box supplied with pre-frozen packs as mentioned before. Slides were prepared, mounted and transferred to the laboratory of Walter Reed / AFRIMS Research Unit Nepal (WARUN) for further identification.

2.2.4 Laboratory investigation of non-human samples (rodents and chiggers)

Rodents: The rodent sera were tested for the presence of IgG antibody against O. tsutsugamushi by IFA test with a panel of O. tsutsugamushi antigens. The IFA assay was considered positive when the IgG antibody titer was >50.

After identifying the rodent species, lung and liver tissues were homogenized. The extracted DNA from the homogenate was used for PCR. PCR amplification of O. tsutsugamushi specific gene (56-kDa protein-encoding gene) fragment was carried out with gene specific primers. The DNA fragment was resolved by agarose gel electrophoresis and UV system.

Chiggers: Each chigger mite was placed in a tiny drop of phosphate buffered saline (PBS) under a dissecting microscope. The exoskeleton and internal tissue contents were separated by puncture and squeeze method. The chigger exoskeleton was mounted on a slide and used for species identification, while the internal contents was homogenized and used for PCR identification of O. tsutsugamushi. O. tsutsugamushi gene specific PCR was performed as described above.

2.3 Human sample collection, preservation and laboratory investigation

Blood samples were collected from the suspected scrub typhus cases at NPHL, Kathmandu , Chitwan Medical College (CMC) hospital and Bharatpur hospital, Chitwan. Samples collected at Chitwan were transferred to NPHL maintaining cold chain. Serum samples were tested by O. tsutsugamushi specific IgM ELISA (Srub Typhus Detect IgM ELISA kit, Inbios, USA) at NPHL and interpreted as per the manufacturer's instructions. The remaining quantity of samples was cryopreserved at -80°C. Some of the representative positive and negative serum samples (earlier confirmed by IgM ELISA) were transferred to AFRIMS, Thailand for further confirmation by IFA (gold standard assay) using a panel of O. tsutsugamushi specific antigens. The IFA assay was considered positive if antibody titers were >50 for IgM, or if seroconversion was demonstrated.

2.4 Data analysis

Data were entered in Microsoft excel and analyzed in IBM SPSS version 20.0.

2.5 Ethical consideration

Ethical approval for this study was granted by the Ethical Review Board of Nepal Health Research Council (NHRC). All patient records were anonymized prior to analysis. The study was done in conformity with National Ethical Guidelines for Health Research in Nepal and Standard Operating Procedures 2011 and Ethical Guidelines for the Care and Use of Animals in Health Research in Nepal 2005.

CHAPTER III

FINDINGS

3.1 Socio-demographic findings

In 2016, a total of 831 cases with 14 deaths (CFR = 1.68%) were reported from 47 districts out of 75 districts of Nepal. The distribution of scrub typhus cases in 2016 has been shown in figure 2.

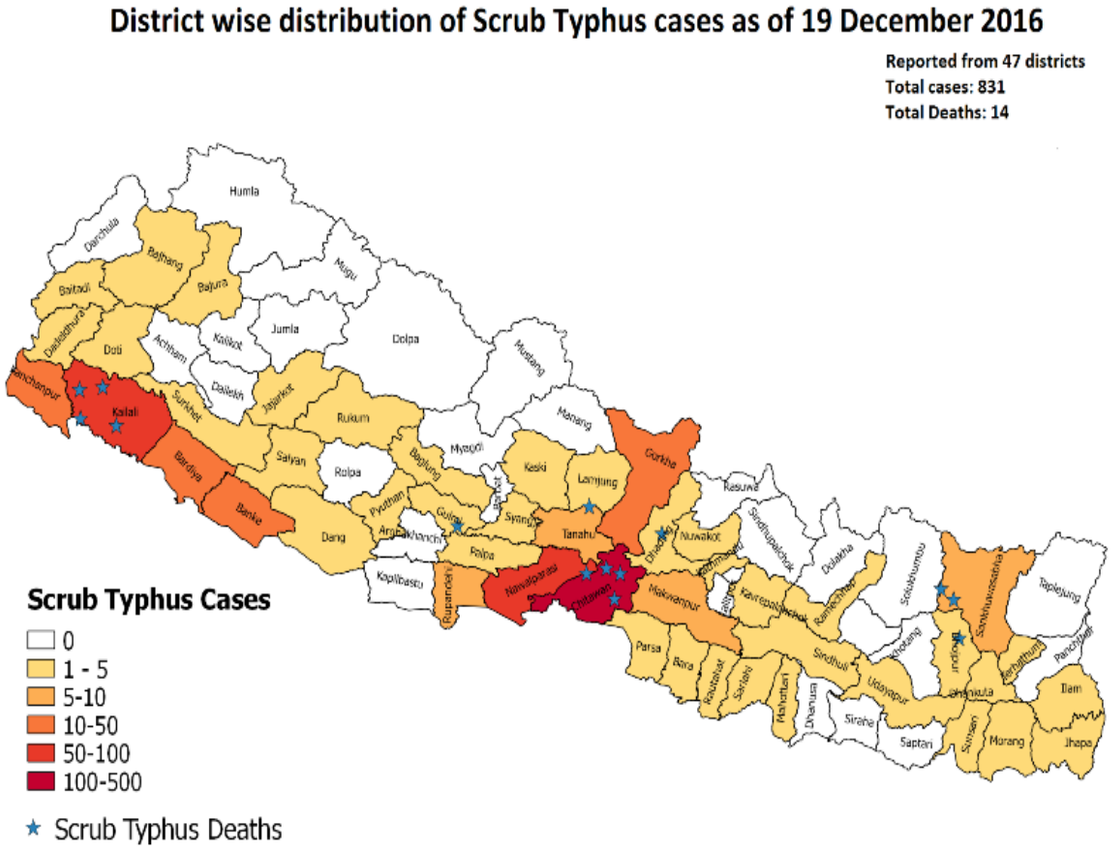

Figure 2: Distribution with of scrub typhus cases in Nepal, 2016

Although 831 cases were reported in the country during April - December 2016 in Nepal, complete line listing was available for only 401 cases (male = 163; 40.6%) from EDCD. The median age of the cases was 25 years. More than half of the cases (57.1%) were below 30 years of age, of that 14.5% were children below 10 years of age (Table 1). Majority of the cases belonged to Janajati/Aadhibasi (44.4%) and Brahmin/Chhetri (44.1%) ethnic groups.

Table 1: Socio-demographic profile of scrub typhus cases in Nepal, 2016 (n=401)

Socio-demographic characteristics	Frequency (percentage)
Age in years	
0-10	58 (14.5)
10-19	88 (21.9)
20-29	83 (20.7)
30-39	60 (15.0)
40-49	32 (8.0)
50-59	53 (13.2)
60 and above	27 (6.7)
Sex	
Male	163 (40.6)
Female	238 (59.4)
Ethnicity	
Janajati/Aadibashi	178 (44.4)
Brahmin/Chhetri	177 (44.1)
Dalit	27 (6.8)
Madheshi	10 (2.5)
Muslim	3 (0.7)
Others	6 (1.5)

3.2 Geographical distribution of scrub typhus cases

Majority (81.3%) of the reported scrub typhus cases were from Tarai, the low-land ecology of southern Nepal and similarly on the regional basis, the central region (45.4%) had the highest number of reported cases.

Table 2: Geographical distribution of scrub typhus cases

Geographical parameters	Frequency (percentage)
Ecological region	
Mountain	10 (2.5)
Hill	65 (16.2)
Tarai	326 (81.3)
Administrative region	
Eastern	18 (4.5)
Central	182 (45.4)
Western	95 (23.7)
Mid-western	4 (1.0)
Far-western	102 (25.4)

3.3 District wise distribution of scrub typhus cases

Chitwan was the most affected district, contributing to 34.4% of the total cases. Six districts namely Chitwan (n = 138), Kailali (n = 63), Nawalparasi (n = 55), Kanchanpur (n = 26), Gorkha (n = 15) and Tanahun (n = 10) reported at least 10 cases each.

Table 3: Distribution of scrub typhus cases by district in Nepal, 2016

Districts	Frequency (percentage)
Chitwan	138 (34.4)
Kailali	63 (15.7)
Nawalparasi	55 (13.7)
Kanchanpur	26 (6.5)
Gorkha	15 (3.7)
Tanahun	10 (2.5)
Makwanpur	9 (2.2)
Sankhuwasava	8 (2.0)
Bara	6 (1.5)
Rupandehi	6 (1.5)
Dadeldhura	5(1.2)
Dhading	5(1.2)
Sarlahi	5(1.2)
Kavrepalanchok	4 (1.0)
Sindhuli	4 (1.0)
Baitadi	3 (0.7)
Dhankuta	3 (0.7)
Nuwakot	3 (0.7)
Rautahat	3 (0.7)
Baglung	2 (0.5)
Bajhang	2 (0.5)
Doti	2 (0.5)
Lamjung	2 (0.5)
Parsa	2 (0.5)
Pyuthan	2 (0.5)
Rukum	2 (0.5)
Bajura	1(0.2)
Bhojpur	1(0.2)
Gulmi	1(0.2)
Illam	1(0.2)
Jhapa	1(0.2)
Kapilvastu	1(0.2)
Kaski	1(0.2)
Kathmandu	1(0.2)
Mahottari	1(0.2)
Morang	1(0.2)
Palpa	1(0.2)
Ramechhap	1(0.2)
Sunsari	1(0.2)
Syanjga	1(0.2)
Terathum	1(0.2)

Udaypur	1(0.2)
Total	**401 (100)**

3.4 Diagnosis and outcome of scrub typhus cases

Chitwan Medical College (CMC), a private medical college in central Nepal reported 61.3% cases while the rest 34.7% were verified at NPHL. Regarding the prognosis of the disease, 12 cases (3%) died while the rest recovered after treatment.

Table 4: Diagnosis and outcome of scrub typhus cases in Nepal, 2016

Case verification and clinical outcome	Frequency(percentage)
Place of verification	
Chitwan Medical College, Chitwan	246 (61.3)
National Public Health Laboratory, Kathmandu	139 (34.7)
Narayani Sub-regional Hospital, Birgunj	7(1.8)
Bharatpur Hospital, Chitwan	4(1.0)
Siddhartha Hospital, Kailali	3(0.7)
Missing	2(0.5)
Clinical outcome	
Improved	389 (97.0)
Death	12 (3.0)

3.5 Temporal variation of scrub typhus cases

The outbreak of scrub typhus peaked during the month of August (n = 164; 40.9%) and September (n = 105; 26.2%) in 2016 (table 5).

Table 5: Temporal variation of scrub typhus cases in Nepal, 2016

Month	Frequency (percentage)
April	1(0.2)
June	17(4.2)
July	56(14.0)
August	164(40.9)
September	105(26.2)
October	58(14.5)

3.6 Distribution of scrub typhus cases by sex

Scrub typhus among males were more predominant in age group of 0-9 years (22.8%) and 10-19 years (22.8%) while among females, more cases were from age group of 20-29 years (27.1%) followed by 10-19 years (21.2%). Majority of the scrub typhus cases in both males and females belonged to Brahmin/Chhetri (44.1%) and Janajati/ Aadhibasi (44.4%) ethinic group and resided in central development region (45.4%) and Tarai region (81.3%) of Nepal (Table 6). The number of deaths due to scrub typhus was equal (six each) in both sexes.

Table 6: Epidemiological characteristics of scrub typhus cases in Nepal, 2016

Variables	Male n (%); n = 163	Female n (%); n = 238	Total n (%); n = 401	p value (X^2 test)
Age, years				
Below 10	37 (22.7)	21 (8.8)	58 (14.5)	0.01
10-19	37 (22.7)	51 (21.4)	88 (21.9)	
20-29	19 (11.7)	64 (26.9)	83 (20.7)	
30-39	16 (9.8)	44 (18.5)	60 (15.0)	
40-49	11 (6.7)	21 (8.8)	32 (8.0)	
50-59	28 (17.2)	25 (10.5)	53 (13.2)	
60 and above	15 (9.2)	12 (5.0)	27 (6.7)	
Ethnicity				
Brahmin/ Chhetri	72 (44.2)	105 (44.1)	177 (44.1)	0.87
Janajati/ Aadhibasi	74 (45.4)	104 (43.7)	178 (44.4)	
Madheshi	5 (3.1)	5 (2.1)	10 (2.5)	
Dalit	9 (5.5)	18 (7.6)	27 (6.7)	
Others	3 (1.8)	6 (2.5)	9 (2.2)	
Month of diagnosis				
June	12 (7.4)	5 (2.1)	17 (4.2)	0.01
July	27 (16.6)	29 (12.2)	56 (14.0)	
August	71 (43.6)	93 (39.2)	164 (41.0)	
September	36 (22.1)	69 (29.1)	105 (26.2)	
October	17 (10.4)	41 (17.3)	58 (14.5)	
Ecological region				
Mountain	8 (4.9)	2 (0.8)	10 (2.5)	0.03
Hill	28 (17.2)	37 (15.5)	65 (16.2)	
Terai	127 (77.9)	199 (83.6)	326 (81.3)	
Administrative region				
Eastern	12 (7.4)	6 (2.5)	18 (4.5)	0.08
Central	75 (46.0)	107 (45.0)	182 (45.4)	
Western	39 (23.9)	56 (23.5)	95 (23.7)	
Mid-West and Far West	37 (22.7)	69 (29.0)	106 (26.4)	
Clinical outcome				
Improved	157 (96.3)	232 (97.5)	389 (97.0)	0.50
Death	6 (3.7)	6 (2.5)	12 (3.0)	

3.7 Rodentological and entomological findings

Of the total 104 traps used, 12 live rodents were successfully trapped. Due to the rainy days, the number of rodents trapped was less than expected. Out of 12 rodents, three were positive for chigger mites. Three rodents each had 4, 3 and 3 mites respectively. The chigger index was 0.92. The details on the rodentological and entomological findings are given in table 7.

Table 7: Rodentological and entomological findings of scrub typhus study in Nepal, 2016

ID	Species	Wt (g)	Sex	Mammae	Length (cm)			Colour		Tail Patterns	Fur	
					Head and body	Tail	Ear	Teeth	Hind foot		Dorsum	Ventrum
1	Rattus rattus	117	F	2+3	17	21	2	Grey	Brown	Straight and brown	Dark brown	Brown
2	R. rattus	92	M		17	19	2	Grey	Brown	Straight and brown	Dark brown	Brown
3	R. rattus	146.6	M		19	12*	2	Grey	Brown	Straight and brown	Dark brown	Grey
4	R. rattus	84.3	M		16	19	2	Grey	Brown	Straight and brown	Dark brown	Grey
5	R. rattus	126.3	F	2+3	18	13*	2.2	Grey	Brown	Straight and brown	Dark brown	Grey
6	S. aquaticus	21.2	F		12	5.5	0.5	Sharp and white	Brown	Straight and brown	Dark grey	Grey
7	R. rattus	171	M		20	18*	2.2	Grey	Brown	Straight and brown	Dark brown	Grey
8	S. aquaticus	20.7	F		11	6	0.5	Sharp and white	Brown	Straight and brown	Dark grey	Grey
9	R. rattus	128.9	F	2+3	17.5	21	2.3	Grey	Brown	Straight and brown	Dark brown	Grey
10	R. rattus	90	M		17	19	2	Grey	Brown	Straight and brown	Dark brown	Grey
11	R. rattus	125	F	2+3	18	16	2.1	Grey	Brown	Straight and brown	Dark brown	Grey
12	R. rattus	105.6	F	2+3	17	18.5	2	Grey	Brown	Straight and brown	Dark brown	Grey

Wt: weight; g: gram; cm: centimeters; M: male; F = female; *part of tail was cut in the trap so, only remaining tail was measured.

3. 8 Confirmation of the ongoing transmission of O. tsutsugamushi in Nepal

Results of human samples:

Representative serum samples (n = 61; 50 from NPHL and 11 from Chitwan) tested by IFA confirmed the presence of O. tsutsugamushi infection in Nepal. Out of 33 samples positive by ELISA and/or RDT in Nepal, 28 were also found positive by IFA. Similarly, of the rest 28 samples considered negative by ELISA and/or RDT in Nepal, one turned out to be positive by IFA. In total, 29 cases were confirmed scrub typhus positive by IFA.

Results of human (rodents, chiggers) samples:

Rodents: Two out of nine rodent serum samples were confirmed scrub typhus positive by IFA (IgG titer > 50). However, all the 24 rodent tissue samples (12 lungs, 12 livers) were found O. tsutsugamushi negative by PCR.
Chiggers: One of the three chiggers' samples was also confirmed O. tsutsugamushi positive by PCR.This is clear evidence of circulation of O. tsutsugamushi (evidence from human, rodent and chigger samples) in Nepal with the potential of any outbreak magnitude.

Table 8: Laboratory results of samples

Sample collection sites	Total samples	Positive by RDT (%)	Positive by IgM ELISA (%)	Positive by IFA (%)
NPHL	50	-	30 (60.0)	26(52.0)
Bharatpur	11	3(27.3)	-	3 (27.3)

CHAPTER IV

DISCUSSION

This study has provided the clear evidence of circulation of O. tsutsugamushi (evidence from human, rodent and chigger samples) in Nepal with the potential of any outbreak magnitude. Scrub typus is a re-emerging health problem in Nepal. Following the devastating earthquake in 2015, outbreaks of scrub typhus have been reported in Nepal which is linked to the diagnostic dilemma of resource-limited country, and outlines a plan for empirical treatment for undifferentiated fever.[14] Scrub typhus is one of the neglected tropical diseases in Nepal.[15] There is an urgent need of reliable and affordable diagnostic tests at all level of health facilities of Nepal to diagnose any patients with acute febrile illness for scrub typhus.[15] Surveillance and public health awareness about the disease transmission and preventive measures needs to be initiated as the disease is re-emerging and physicians are not well aware about it. Recent reports of scrub typhus from various parts of Nepal might be a true reflection of the ecological niche and epidemiologic behavior of the vector due to the altered environmental factors that could have occurred after the recent earthquake. The outbreaks could have been triggered as a result of intimate contact between human beings and rats that might have come out of their usual underground habitat after the collapse of many houses.[16] Overcrowding and unsanitary conditions could augment the linkage between vector, pathogen and man.

In this study majority of cases (>80%) were reported from lowland terai and in less than 40 years old. Majority of cases were reported in female which shows gender difference. Cases have been observed in all ethnic groups and both in urban and rural areas. Study from Korea and China also shows higher incidence of Scrub typhus among female.[17, 18] In this study, the peak months of scrub typhus are recorded in August and September. Similar findings are reported from Darjeeling of West Bangal, India.[19] The age and sex distribution of cases in our study is similar to the study from Darjeeling indicating similar pattern in Asia.[19]

Rapid expansion of cases and probably increased incidence is observed in our study. Such type of rapid expansion and increase in incidence is reported in a study from Mainland of China[18] and India[20] indicating geographical expansion of cases in neighboring countries too.

Our study has some limitations. This study was a descriptive study and thus we were not able to find out risk factors. Also the gold standard testing by IFA was limited to representative samples, not all. Moreover, the provided line list information from EDCD may not represent whole country situation and asymptomatic cases from community are underreported.

CHAPTER V

CONCLUSION AND RECOMMENDATION

Conclusion

The presence of O. tsutsugamushi in human serum, rodent serum and chigger mites was confirmed in this study, thereby indicating the ongoing transmission cycle of scrub typhus in Nepal.

Recommendation

Not all cases could be analyzed indicating that record linkages need to be made and information system needs to be strengthened to detect early outbreaks and initiate immediate response. Similarly, diagnostic services need to be made available along with training to health workers at referral and community levels throughout the country so that early identification and response can be done.

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Annex I: Data collection tool

S.N	Test Date	Month of infection	Patients No.	Age	Gender	Address	District	Con-firmed By	Patient Admitted in	Outcome	Entomologi-cal evidenc-es if any

Annex II: EDCD interim Guideline on Prevention and Control of Scrub Typhus September 2015 (Updated in August 2016)

Introduction:

Scrub typhus is an acute, febrile, infectious disease that is caused by Orientia (formerly Rickettsia) tsutsugamushi. It is also known as tsutsugamushi disease. It is an obligate intracellular gram-negative bacterium from the Rickettsiaceae family. Last year (2015) total of eighty two confirmed (IgM Elisa) cases were reported to EDCD from August to October. Among them, eight cases with fever, rash and with severe ARDS died in September 2015. Out of which 6 cases were from Eastern parts of Nepal and two cases were from Far Western region. This year, total of 92 cases were reported from April to mid- August and three died.

Clinical features:

- Fever is high grade (>1040F) and usually lasts 14 days.
- Maculopapular rash is seen over trunk, which is transient, and is seen around day 7 of fever
- Severe headache
- Profuse sweating
- Conjunctival injection
- The site of insect bite is usually painless and a black eschar (scab) is seen in 40% of cases (see image)
- Lymphadenopathy

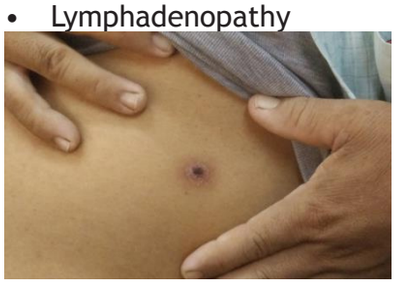

Figure 1: Typical Eschar

The most common signs are similar to a variety of other infectious diseases (typhoid fever, malaria, murine typhus, leptospirosis and dengue fever, meningococcal infection, etc.) which should be taken into consideration.

Complications:

- Interstitial pneumonia- X-ray evidence of pneumonitis are common and may progress to ARDS
- Pulmonary edema
- Congestive heart failure
- Circulatory collapse
- Diarrhea and features of acute gastroenteritis is also possible ,sometimes GI Bleeding can occur
- Neurological findings may suggest meningo-encephalitis.
- Multi-organ failure
- Death may occur as a result of these complications
- Spontaneous abortion may occur during pregnancy if infected

Case Definition

Suspected/clinical case: Acute undifferentiated febrile illness (UFI) of 5 days or more with or without eschar should be suspected as a case of Rickettsial infection. (If eschar

is present, fever of less than 5 days duration should be considered as scrub typhus.)
Probable case: A suspected clinical case with an IgM titer > 1:32 and/or a four-fold increase of titers between two sera confirm a recent infection.

Confirmed case: The one in which:

- Rickettsial DNA is detected in eschar samples or whole blood by PCR OR,
- Rising antibody titers on acute and convalescent sera detected by Indirect ImmuneFluorescence Assay (IFA) or Indirect Immunoperoxidase Assay (IPA)Supportive laboratory investigations:
- Total Leucocytes Count during early stages may be normal but may be elevated to more than10,000/cu mm later in the course of disease.
- Thrombocytopenia (low platelet count), usually <1,50,000/cu mm is seen in majority of patients.
- Elevated liver transaminases (AST, ALT) is also seen in many patients.

Specimen for diagnosis:

- Heparinized blood: Conserve at -80°C and then ship in dry ice for culture.
- EDTA blood: Conserve at +4°C and then ship at room temperature for PCR.
- Serum: Conserve at +4°C, then ship at room temperature. Collect two serum specimens 10 days apart.
- Skin or lymph node biopsy can also give the diagnosis.

The sample collected at the site should be sent to National Public Health Laboratory (NPHL), Teku, and Kathmandu through courier/WHO surveillance mechanism following IATA guidelines (triple packing and biosafety). The information on the sample shipment should be intimated to NPHL (Focal point), EDCD (Focal point).
(Contact details of all three are available at the end of this document.)

Transmission/Reservoir:

Humans acquire the disease from the bite of an infected trombiculid mite (chigger). The mites are both the vector and reservoir of the disease. The mite is very small (0.2 –0.4mm) and can only be seen through a microscope or magnifying glass. The larva is the only stage that can transmit the disease to humans and other vertebrates. There is no human to human transmission.
Incubation period: About 5 to 20 days (mean, 10-12 days) after the initial bite
Risk groups: Agricultural workers, people living in houses with shrubs/ bush nearby, and travelers in areas with potential exposure to mice and mites, for e.g. camping, rafting, or trekking and people staying in the temporary shelter following earthquake where there is mouse infestation.

Treatment:

- Pediatric treatment: Azithromycin for less than 8 years: 10mg/kg orally single dose

Chigger mite

For more than 8 years: Doxycycline 2.2mg/kg orally twice daily for 3 days after resolution of fever (usually 5-10 day course)

- Adult treatment: Azithromycin 500 mg orally single dose; OR Doxycycline 100 mg orally twice daily for 5 to 10 days.

- Pregnant women: Azithromycin 500 mg orally single dose

Alternatives:

- Ciprofloxacin 10 mg/kg twice daily for 5-10 days
- Chloramphenicol 25 mg/kg/dose 6 hourly for 5-10 days

Supportive treatment for management of complications.

Since diagnostic facilities for scrub typhus and other common Undifferentiated Febrile Illnesses (UFIs) like typhoid and leptospirosis are generally unavailable or have a poor yield (such as blood culture in typhoid fever), it may be best to use both doxycycline (for example for scrub typhus and leptospirosis) and azithromycin or ceftriaxone (for typhoid fever) in adequate dosage.
Timely reporting of any suspected or confirmed case should be done to EDCD (see contact details at the end of this document).

Prophylaxis:

Single oral dose of chloramphenicol or tetracycline given every five days for a total of 35 days, with 5- day non-treatment intervals (for endemic regions). No vaccine is available for scrub typhus.

Prevention/Control/Precautions:

Early case detection by healthcare workers is needed.

Other strategies are to make public aware and give preventive information like:

- Wear protective clothing including boots
- Insect repellents containing benzyl benzoate can be applied to the skin and clothing to prevent chigger bites.
- Do not sit or lie on bare ground or grass; use a suitable ground sheet or other ground cover
- Clear vegetation spray insecticides on the soil to break up the cycle of transmission

Sources in information:

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Annex III: SOP for Euthanasia of Field-Collected & Wild-Caught Rodents

1. Purpose- This Standard Operating Procedure (SOP) describes the procedures for euthanasia of field-collected and wild-caught rodents.

2. Materials and Equipment-

2.1. Anesthetic agent [i.e. Fatal plus® (380 mg pentobarbital/ml)]
2.2. Balance or weigh scale
2.3. Detergent or disinfectant [i.e., Quatricide PV®, Envirocide®, CLOROX® (containing ~ 5% sodium hypochlorite)]
2.4. Euthanasia chamber (a regular polycarbonate rat cage)
2.5. Compressed CO2 gas in a cylinder
2.6. Spectromed Cylinder pressure regulator FM 41-S1
2.7. Infectious waste bags and sharp containers
2.8. Insect repellents (i.e., Permanone, DEET or equivalent)
2.9. Masking tape for wrapping up pants
2.10. 20 -25 gauge, ½ - ¾ inch needle
2.11. Personal Protective Equipment
2.11.1. Filter or respirator masks
2.11.2. Head cover
2.11.3. Heavy (i.e., leather) gloves
2.11.4. Latex or nitrile gloves
2.11.5. Protective eyewear and/or goggles
2.11.6. Steel-toe and/or FirstMed-Barrier waterproof boots and rubber boots
2.11.7. Scrub uniforms, disposable lab coats/coveralls (i.e., Tyvek®), rain suits and/or chest & hip waders
2.12. 1 - 3 ml syringe

3. Definitions/Abbreviations-

3.1. CO2 – carbon dioxide
3.2. DEET – N,N-diethyl-meta-toluamide
3.3. IACUC – Institutional Animal Care and Use Committee
3.4. gm – gram
3.5. IP – intraperitoneal injection
3.6. kg – kilogram
3.7. mg – milligram
3.8. psi – pounds per square inch
3.9. PPE – personal protective equipment

4. Safety and Precautions-

4.1. All personnel who handle animals should wear the appropriate PPE: heavy gloves, masks, head cover, rubber boots, goggles or eyewear protection, scrub uniforms and/or disposable lab coats/coveralls. Long pants will be tucked into their shoes and taped with masking tape. It is preferable to wear a long sleeve shirt that is cuffed and taped at all times. Scrub uniforms and/or disposable Tyvek® lab coat should be impregnated with insect repellents (Permanone, DEET) at the beginning of the field trip. If clothes become wet or washed, insect repellent must be reapplied.
4.2. Workers will wash hands and other skin surfaces contaminated with blood and other potentially infectious fluids immediately and thoroughly. Hands will be washed imme diately after removing gloves.

4.3. Perform CO2 euthanasia in adequate ventilation area since CO2 is toxic in higher concentrations, 1% will make some people feel drowsy. Concentration 7-10% cause dizziness, headache, visual and hearing dysfunction and unconsciousness.

5. Procedure

5.1. Euthanasia- will be performed only at the sites of capture for field/wild-caught animals. The person performing euthanasia must be technically proficient, use humane handling methods, understand the reasons for euthanasia, and be trained on the method of euthanasia being employed. Gentle restraint minimizes animal distress, including fear and anxiety.

5.2. Carbon Dioxide (CO_2) Euthanasia- This is a routine method of euthanasia for wild-caught rodents on active research protocols unless the protocol requires an alternate method.

5.2.1. Slide the lid off the chamber rather than lifting it up. Place field/wild-caught rodent in a wire live trap directly inside the euthanasia chamber (1 rodent / chamber). Slide the lid to close the chamber.

5.2.2. Filling the euthanasia chamber: When filling the euthanasia chamber, slowly turn the valve on top of the CO2 cylinder. The flow regulator is designed to automatically adjust the pressure to approximately 15 psi. The goal is to obtain a displacement rate from 10% to 30% of the chamber volume/minute. In order to do this, the pressure regulator is utilized to achieve a flow rate based on cage size, as follows:

5.2.2.1. Mouse cage (6" x 10" x 5"): Use pressure regulator to set flow rate at 1 L/min (at 15 psi CO2).

5.2.2.2. Mouse cage (7" x 12" x 7.75"): Use pressure regulator to set flow rate at 2 L/min (at 15 psi CO2).

5.2.2.3. Rat cage (8.5" x 17" x 8"): Use pressure regulator to set flow rate at 4 L/min (at 15 psi CO2).

5.2.3. Slide the lid to close the chamber. Gas flow should be maintained for at least 5 minutes after the animals stop breathing.

5.2.4. Ensure that the animal is dead by the absence of a heartbeat before removing it from the chamber. If any of the animals have a detectable heartbeat, continue the gas flow for anm additional 5 minutes, then verify the absence of a heart beat. If the animal is cyanotic and has a complete absence of heart beat, respiratory efforts, and movement, the animal is verified to be dead.

5.2.5. Slide off the lid and remove dead animals from the chamber. The dead animals are placed in double plastic bags prior to disposal.

5.2.6. The chamber must be cleaned to minimize odors that might distress animals subsequently euthanized. It is always cleaned with detergent and/or disinfect antsolution after each use. The assigned technician is responsible for the safe storage and appropriate number of CO2 tanks.

5.2.7. While the potential for intoxication of personnel performing CO2 euthanasia is small, this method should only be performed where adequate ventilation exists. Personnel will work in pairs with one worker and one observer who is unexposed. If at any time worker begins to feel faint or light headed or develop a headache, that person should shut off the CO2 valve and seek help from observer.

5.2.8. Dead animals will be placed in double plastic bags before disposal. At the end of each day, the dead animals will be disposed by proper burial at the field sites,

approximately 300-500 meters from the outskirt of the village.

5.2.9. Injectable Anesthetic Agent- The only injectable anesthetic agent to be used alone for euthanasia is Fatal plus® (380 mg pentobarbital/ml). This is a controlled drug, and usage must be properly recorded for each time use.

5.2.9.1. Pre-Anesthetic Preparation

5.2.9.1.1. Weigh rodents prior to anesthesia.

5.2.9.1.2. Calculate the dosage used in mg/kg or for 1000 gm animal weight.

5.2.9.1.2.1. Suggested dosage is 100 mg pentobarbital euthanasia solution per kg body weight.

5.2.9.1.2.2. For rodents and small animals, using a 1-3 ml syringe with a 20-25 gauge, ½ to ¾ inch needle.

5.2.9.1.2.3. The volume to be inoculated intraperitoneally is prepared according to the calculated drug dosages.

5.2.9.2. Intraperitoneal Injection

5.2.9.2.1. Draw the appropriate volume of inoculum into the syringe and remove the air bubbles. Verify the volume with the animal collection #.

5.2.9.2.2. Restrain the animal to be injected manually.

5.2.9.2.3. Restrain the mouse by the scruff method.

Hold or place the animal to allow access to the caudal part of the abdomen. For small rodents, let its head down. Swab the area to be injected with 70% ethanol.

5.2.9.2.4. Insert the needle into the lower right quadrant of the animal's abdomen slightly off midline. The needle should be directed cranially at a 30-40 degree angle.

5.2.9.2.5. Pull back on the plunger of the syringe to check for correct placement of the needle hub. If there are no intestinal contents, blood, or urine appearing in the hub, injects the inoculum.

Note: If there are intestinal contents, blood, or urine appearing in the hub, do not inject the contents but remove the needle and discard it in a sharp container. Restart the procedure with a new syringe unit.

5.2.9.2.6. Dispose of used needles and syringe units in a sharps container.

5.2.9.2.7. The complete absence of a heartbeat is confirmed by the technician after 5 minutes after the injection. If the animal is cyanotic and has a Complete absence of heart beat, respiratory efforts, and movement, the animal is verified to be dead.

5.2.9.3. Dead animals will be placed in double plastic bags before disposal. At the end of each day, the dead animals will be disposed by proper burial at the field sites, approximately 300-500 meters from the outskirt of the village.

5.2.10. Exsanguination-This technique can be used as an adjunctive method to ensure death in unconscious animals. It is not used as a sole means of euthanasia. Animals may be exsanguinated to obtain blood, but only when they are sedated, unconscious or anesthetized.

5.2.10.1. Severe blood loss (exsanguination) is usually done in rodents by cardiac puncture using ½ (size #26) – 1 inch (size#23) needle and 1 or 3 ml syringe.

5.2.10.2. After maximum blood collection (for a mouse, about 200-300 µL of blood will be collected; larger volumes of blood will be collected in larger species), the animal subsequently stops breathing and dies. The complete absence of a heart beat is confirmed. If the animal is not dead within a few minutes, CO_2 euthanasia is performed.

5.2.10.3. Dead animals will be placed in double plastic bags and will be disposed by proper burial at the field sites, wherever appropriate on the same day.

5.3. Data/Records/Reporting Results

5.3.1. Record the animal information, sex, weight, etc. on appropriate logbook

5.3.2. Record the date, user, quantity received, quantity used and quantity balance of controlled substance on Controlled Substance Log/Record Form.

Annex IV: SOP for Field Rodent Trapping

Purpose- This Standard Operating Procedure (SOP) describes practices and procedures for field animal trapping including setting up animal traps as well as removing animal from trap.

2. Materials and Equipment

2.1. Animal traps (e.g., small wire live-trap, rodent snap-trap, Sherman trap)
2.2. Baits (e.g., banana, sticky rice, dried fish)
2.3. Detergent or disinfectant (e.g., Quatricide PV®, Envirocide®, CLOROX®)
2.4. Insect repellents (e.g., Permanone, DEET or equivalent)
2.5. Masking tape for wrapping up pants
2.6. Personal Protective Equipment
2.6.1. Filter, respirator or face masks
2.6.2. Head cover
2.6.3. Heavy (e.g., leather) gloves
2.6.4. Latex or nitrile gloves
2.6.5. Protective eyewear and/or goggles
2.6.6. Steel-toe and/or First Med-Barrier waterproof boots and rubber boots
2.6.7. Scrub uniforms, disposable lab coats/coveralls (e.g., Tyvek®), rainsuits and/or chest & hip waders

3. Definitions/Abbreviations

3.1. CITES – Convention on International Trade in Endangered Species of Wild Flora and Fauna
3.2. DEET – N,N-diethyl-meta-toluamide
3.3. IACUC – Institutional Animal Care and Use Committee
3.4. PPE – Personal Protective Equipment

4. Procedure

4.1. Trapping
4.1.1. Setting Up Animal Traps
4.1.1.1. Specific types of animal traps will be used for specific animal.
4.1.1.1.1. Small wire live-trap for large size rodents (300-500 gm)
4.1.1.1.2. Rodent snap-trap for medium size rodents (100-300 gm)
4.1.1.1.3. Sherman trap for small size rodents (10-150 gm)
4.1.1.2. All traps will be set up in the afternoon, under vegetation for shade whenever possible, and left at a site for 1-3 consecutive nights.
4.1.1.3. Traps will be baited with appropriate baits, e.g., bananas, sticky rice, or dried fish.
4.1.2. Removing Animal from Trap
4.1.2.1. Traps will be checked every morning and trapped animals will be removed from the traps.
4.1.2.2. Each trapped animal (small rodent) will be given an animal collection number assigned by Section Chief, e.g., A-B-XXXX. A indicates the first letter of the last

name of department chief and B indicates the first letter of the first name of chief of Mite and Rodent Support Section. XXXX is a 4-digit number.

Note: Any endangered animal species as listed by CITES, the Wild Animals Preservation and Protection Act, and the National Parks Act, which is live caught, will be released at the site of capture.

4.2. Data/Records/Reporting Results- Record the date, month, year, animal collection #, host information, location, habitat, collector, etc. on appropriate logbook

Annex V: SOP for Rodent Necropsy

1. Purpose

This Standard Operating Procedure (SOP) describes and outlines the proper procedures for performing necropsy and post mortem examination of field-collected and wild-caught rodents.

2. Responsibility

2.1. Laboratory supervisor ensures that designated laboratory personnel for performing this method are properly trained and receive documented training on this SOP.
2.2. Laboratory personnel performing this method ensure that the procedure is properly carried out according to this SOP.

3. Materials and equipment

3.1.Detergent or disinfectant [i.e., Quatricide PV®, Envirocide®, CLOROX® (containing ~5% sodium hypochlorite)]
3.2. Dry Ice
3.3. Examination table
3.4. Infectious waste bags and sharp containers
3.5. Insect repellents (i.e., Permanone, DEET or equivalent)
3.6. Masking tape for wrapping up pants
3.7. Necropsy equipment
3.7.1. Blood collection tube contains polymer barrier material & clot activator (i.e., Vacutainer® tube with SST® Gel & Clot Activator)
3.7.2. 70% Ethyl alcohol
3.7.3. Dissecting board
3.7.4. ½ inch (size #23)-1 inch (size#26) needles
3.7.5. Syringe 1, 3 ml
3.7.6. Scissors & forceps
3.7.7. Vials: cryogenic vials for collecting tissue samples & 1.5 ml snap-cap-plastic vials (one with EDTA, another without EDTA)
3.8. Personal Protective Equipment
3.8.1. Filter, respirator, face masks, face shield
3.8.2. Head cover
3.8.3. Heavy (i.e., leather) gloves
3.8.4. Latex or nitrile gloves
3.8.5. Protective eyewear and/or goggles
3.8.6. Steel-toe and/or First Med-Barrier waterproof boots and rubber boots
3.8.7. Scrub uniforms, lab coats/coveralls (i.e., Tyvek®), rain suits and/or chest & hip waders
3.9. Sponge surgery gauzes and paper towels

4. Definitions/Abbreviations

4.1. DEET – N,N-diethyl-meta-toluamide
4.2. EDTA – Ethylenediaminetetraacetic acid
4.3. IACUC – Institutional Animal Care and Use Committee
4.4. PPE – Personal Protective Equipment

4.5. PI – Principal Investigator
4.6. gm – gram
4.7. ml – milliliter
4.8. in situ – means to examine the phenomenon exactly in place where it occurs
4.9. µl – microliter

5. Safety and Precautions

5.1. All personnel who perform animal necropsy and handle animals should wear the appropriate PPE: heavy gloves, masks, head cover, rubber boots, goggles or eye wear protection, scrub uniforms and/or disposable lab coats/coveralls. Long pants will be tucked into their shoes and taped with masking tape. It is preferable to wear a long sleeve shirt that is cuffed and taped at all times. Scrub uniforms and/or disposable Tyvek® lab coat should be impregnated with insect repellents (Permanone, DEET) at the beginning of the field trip. If clothes become wet or washed, insect repellent must be reapplied.

5.2. Workers will wash hands and other skin surfaces contaminated with blood and other potentially infectious fluids immediately and thoroughly. Hands will be washed immediately after removing gloves.

5.3. All specimens (tissue, blood and other body fluids) collected from wild/field captured- animals should be considered infective whether they are from the infected or uninfected rodent hosts. All specimens will be held in a well-constructed container with a secure lid to prevent leakage or escape during transport. Care will be taken when collecting each specimen to avoid contaminating the outside of the container.

6. Procedure

6.1. Pre-Necropsy Activities

6.1.1. Prepare all the equipment, including labels and tissue containers/vials, to be ready and available prior to the necropsy.

6.1.2. Be familiar with the animal use protocol specific for the necropsy.

6.1.3. Prepare appropriate logbook

6.1.4. Prior to performing the necropsy, the animal's identity will be confirmed using their animal collection.

6.1.5. All body surfaces and orifices will be observed carefully. All abnormalities will be recorded in the appropriate logbook

6.2. Animal necropsy procedure is performed at the site of capture. The task will be performed by properly trained technicians.

6.2.1. Animals will be humanely euthanized.

6.2.2. Euthanized animals are placed on an examination table. A midline incision is then made extending from the pelvis to the xiphoid cartilage, exposing the abdominal cavity.

6.2.3. Blood is collected by heart puncture.

6.2.3.1. Two sets of whole blood samples (approximately 50-100 µl) will be drawn from the captured-animal [via Cardiac (Heart)-puncture], transferred into two (1.5 ml) snap-cap-plastic vials (one with EDTA, another without EDTA) and stored in dry ice for further use. For serum samples, about 1-2 ml of whole blood will be drawn from the captured-animal [via Cardiac (Heart)-puncture], transferred into a blood collection tube (containing polymer barrier material & clot activator).

6.2.4. Dissection

6.2.4.1. Tissues and/or organs required for field collection will be examined in situ, then dissected from the carcass and reexamined, including cut surfaces.

6.2.4.2. All gross lesions will be recorded. A section of grossly visible lesions will also be removed for later microscopic examination.

6.2.4.3. Removal of organs – Examine organs and tissues in situ before dissecting or collecting tissues and record any abnormalities. Animal tissue samples, liver, spleen, kidney, about 1-2 gm each, will be dissected out of the captured animals and deposited in screw cap plastic vials (i.e., 2 ml Corning®), stored on dry ice and later in -70°C freezer for future diagnostic evaluations.

6.2.4.4. Tissue disposition- As organs are removed, place them in vials with or without fixative.

6.2.4.5. Examination table will be decontaminated with appropriate disinfectant when work activities are completed and/or after spill of blood or other body fluids.

6.3. Post Necropsy Activities – All tissue samples, whether collected for submission to a laboratory for analysis or for archival purposes, must be labeled as to the PI, IACUC #, animal collection #, date of collection, the tissue or sample collected when appropriate, and the fixative or specific storage requirements when necessary.

6.4. Data/Records/Reporting Results- Record the date, animal collection#, animal tissue collection on the appropriate logbook

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