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SPEAK India

Toolkit for Surveillance of Visceral Leishmaniasis

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Introduction

1 Introduction

1.1 THE SPEAK INDIA CONSORTIUM

The Setting the Post-Elimination Agenda for Kala Azar in India (SPEAK India) consortium provides a shared, accessible platform for researchers, implementers and stakeholders working on visceral leishmaniasis (VL) in the Indian subcontinent to exchange experiences, synchronise activities, and identify synergies in their work to ensure optimal use of resources. In doing so, SPEAK India aims to identify and define the key questions, knowledge gaps and data needs that should be addressed for the VL elimination goal to be achieved, and sustained into the future.

Funded by the Bill & Melinda Gates Foundation and facilitated by the London School of Hygiene and Tropical Medicine, SPEAK India was led by the Indian Council of Medical Research (ICMR), and has strong support from India's National Center for Vector Borne Disease Control (NCVBDC, formerly the National Vector Borne Disease Control Programme, NVBDCP), Rajendra Memorial Research Institute for Medical Sciences (RMRIMS), Vector Control Research Centre (VCRC), Banaras Hindu University, the Institute of Public Health Bengaluru (IPH),

WHO South-East Asia Office, WHO Geneva, CARE India, PATH, The Union, Liverpool School of Tropical Medicine and DNDi.

1.2 CHALLENGES IDENTIFIED BY SPEAK INDIA

The critical challenges identified by the SPEAK India consortium during its first assembly meeting (November 2016), and in subsequent interactions between the Steering Committee and SPEAK members, include:

- As the number of VL cases gets lower, and stays low, we will need alternative streams of information about transmission to improve surveillance – by directly assessing sand fly infection, and by monitoring human population for signs of current (PKDL and asymptomatic) infection.

- As progress is made towards elimination there will be increased financial and political pressure to relax the current, intensive programme; in particular, active case detection, treatment and IRS. We need to avoid

Participants at the Launch of SPEAK India in 2016.

this threat by ensuring that a strengthened health system engages with the national programme and responds to change.

- Reducing transmission and exposure to VL will mean that an increasing proportion of the population will be susceptible to infection, if they are exposed to a new case migrating to the area, creating the circumstances in which epidemics are more likely.

1.3 THEMES ADDRESSED THROUGH OPERATIONAL RESEARCH PROJECTS

Four cross-cutting themes were identified to guide the development and implementation of research:

i.   **Surveillance of human infection**

ii. **Molecular Xenomonitoring of vector infection**

iii. **Mathematical Modelling**

iv. **Strengthening Health Systems**

Operational research projects addressing each of the themes were presented for peer-review during the second assembly meeting (April 2017) and were approved by the Leadership group of SPEAK India (including the Director General, DG, of ICMR, the Director of NCVBDC and the World Health Organization's South-East Asia Regional Office) prior to their implementation in 2018.

1.4 SCOPE OF THE TOOLKIT

The main objective of the toolkit is to describe practical tools that were developed during the SPEAK India projects to: improve case-based and population-based surveillance of VL, carry out molecular xenomonitoring in vectors of VL and other vector-borne diseases, and to make short-term and long-term predictions of VL cases at the block level. Importantly, standard operating procedures (SOPs) are provided, as appendices, which give full, easy-to-follow guidance on how the different tools were used by researchers and primary healthcare centre staff working on SPEAK India projects. In addition, suggestions on how the tools may be re-purposed and adapted according to different needs and in different contexts are made. We believe that, if implemented, the tools may play a useful role in surveillance of not only VL in the Indian Subcontinent, but also for other diseases with low levels of transmission in different settings and for strengthening disease elimination programmes.

Caption: Credit

2

Tools to improve case-based and population-based surveillance of VL

2 Tools to improve case-based and population-based surveillance of VL

2.1 BACKGROUND: SURVEILLANCE OF VISCERAL LEISHMANIASIS IN THE PERI-ELIMINATION ERA

Over the past 15 years, case notification rates of visceral leishmaniasis (VL) in India have steadily declined. A similar trend has been observed in neighboring VL endemic countries Nepal and Bangladesh.[2] At the same time the epidemiological pattern appears to have changed. Whereas in the years of high incidence we observed large outbreaks that lasted for some years before shifting to other hamlets, the current pattern is one of sporadic cases spread out across many villages.[3] In 2019, the last pre-Covid19 pandemic year, 284 VL cases were reported in the KAMIS database for Muzaffarpur district of Bihar, a district that used to be highly endemic. Of those, 130 were single cases at village level, another 60 were part of clusters of 2 cases, 42 were part of clusters of 3 cases, there were only 2 clusters of more than 10 cases. This is a sharp contrast to what was observed in the same district 10-15 years ago, when large outbreaks occurred in many villages.[4] A surveillance system in the peri- and post-elimination era will have to be able to also pick up such sporadic cases. At the same time, sporadic cases of VL are now also reported from places that were previously considered non-endemic.[5]

At times of decreasing incidence there are several questions that become increasingly pertinent:

1. Is the decline in case notification rates an actual reflection of decline in incidence or are cases being missed due to a lack of attention?

2. Are the cases detected, based on the existing diagnostic algorithm, still true cases or are there many false positives?

3. How can we ensure that cases of post kala azar dermal leishmaniasis (PKDL) are detected?

4. How can we maintain a sensitive surveillance mechanism that is affordable, even at times of low incidence?

These are the questions the surveillance group in SPEAK has tried to answer. We will distinguish between case-based surveillance for VL and PKDL and serological surveillance at population level.

2.2 TOOL 1: CASE-BASED SURVEILLANCE

Detection of new VL cases

For case-based surveillance we worked with six primary health care centres (PHC) in (previously) VL endemic districts. These PHC facilities perform presumptive diagnosis of VL based on the combination of symptoms, in particular fever of more than two weeks duration with splenomegaly, and a positive rK39 rapid diagnostic test (RDT). We requested the PHC staff whenever they diagnosed a VL case, to take a venous blood sample in an EDTA tube before starting treatment, keep the sample refrigerated, and contact the study coordinator to organize a pickup (Appendix 1). In the meantime the patient was treated, in accordance with the existing guidelines.[6] Samples collected were then tested at the laboratory of Banaras Hindu University (BHU) with a quantitative PCR (qPCR). Out of 78 VL cases identified, 74 gave a sample. Out of those, 70 (94.6%, 95% CI 86.7-98.5%) tested positive. Thus we were able to confirm that even at times of low prevalence, the proportion of falsely diagnosed VL cases is very low (5.4%); the diagnostic algorithm is therefore still adequate.[7]

The rK39 RDT is cheap and readily available. A case-based surveillance system making use of RDTs would serve two purposes; 1) cases are detected and treated, and 2) the disease is kept under surveillance. Continuous monitoring of the rate of PCR confirmed cases among all RDT positive patients treated will ensure that as VL incidence decreases even further, the diagnostic algorithm is still adequate. In addition, monitoring of the numbers of RDTs performed per PHC and the positivity rate of RDTs used will serve as indicators on whether or not the surveillance mechanism is still functional.

The serological surveys we performed, which we will discuss in more detail at a later stage in this document,

provided one crucial piece of information for case-based surveillance using the rK39 RDT: the RDT has an extremely high specificity. Out of 15,426 asymptomatic individuals screened, only 39 tested positive. This is equivalent to a specificity of at least 99.75 % (95% CI 99.65 – 99.82%). This explains why even at times of low prevalence, with a pre-test probability among clinical suspects in the order of 5-10%, the positive predictive value of the diagnostic algorithm is still above 90%. Figure 1 below shows the relation between the positive predictive value and the pre-test probability, based on the observed specificity of 99.75% and a sensitivity of 90%. Given the excellent specificity of the rK39 RDT in these settings in Bihar, India, the diagnostic algorithm based on symptoms plus a positive RDT remains valid even when the pre-test probability further declines. It would even be worthwhile investigating what would be the effect of relaxing the criteria for identifying VL suspect cases, e.g. 1 week of fever instead of 2 weeks of fever.
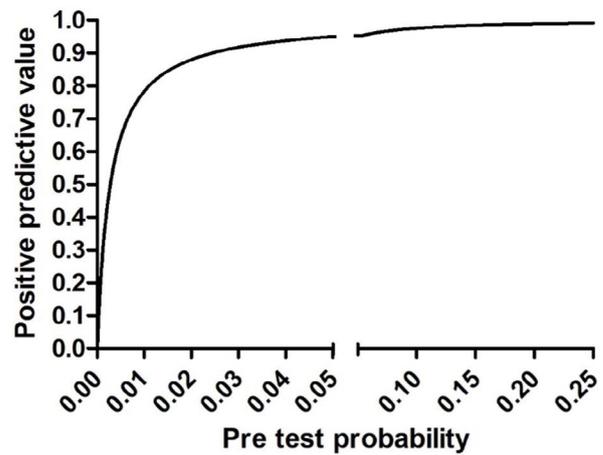
Figure 1.   Association between pre-test probability and positive predictive value of the rK39 RDT.

Project staff taking a finger prick blood sample from a consenting volunteer.

PKDL case detection

For PKDL case detection we worked with the same PHCs. They were provided smartphones with an application in ODK Collect, a free data collection tool, that allows to record findings offline and upload them to a remote server later. PHC staff were requested to not only pay home visits for contact screening of newly detected VL cases but also to revisit all their VL cases registered 1, 3 and 5 years ago to screen them for PKDL. During these home visits they also screened all household members for VL, PKDL and leprosy. In addition, geographic coordinates were recorded of each household visited (Appendices 2-4).

Results of mapping are shown in Figure 2 below. By using a color code by year it becomes clear that there is a strong degree of spatio-temporal clustering of VL cases. The 2021 cases (red dots) are all clustered in one small area. This allows program managers to decide on which areas to prioritize for population screening or other interventions such as indoor residual spraying (IRS).

In total in the six PHCs, 1084 current or former VL-patients were visited and 1056 were actually examined. In addition, 4550 household members were screened. This resulted in the detection of 3 new VL cases, 25 confirmed PKDL cases and 1 leprosy case. Moreover, there were 53 persons who had a history of PKDL (n=46) or were currently on treatment for PKDL (n=7). Thus, in total 78 PKDL cases were identified among 5607 individuals screened, of whom 1338 reported a history of VL, translating into a cumulative incidence of PKDL among former VL patients of 5.8%. Time between VL diagnosis and PKDL diagnosis varied between 0 and 10 years, with a median interval of 2 years (IQR 1 – 3 years) and only two PKDL cases were diagnosed more than five years after their VL episode.

The methodology used only targeted former VL patients reported in the past five years and their household members, which has certainly introduced a selection bias. Still the fact that all PKDL cases identified were former VL cases does confirm the importance of active follow-up of ex VL-cases. In addition, we were able to show that this is feasible within the context of a PHC. Table 1 below shows more detailed results.
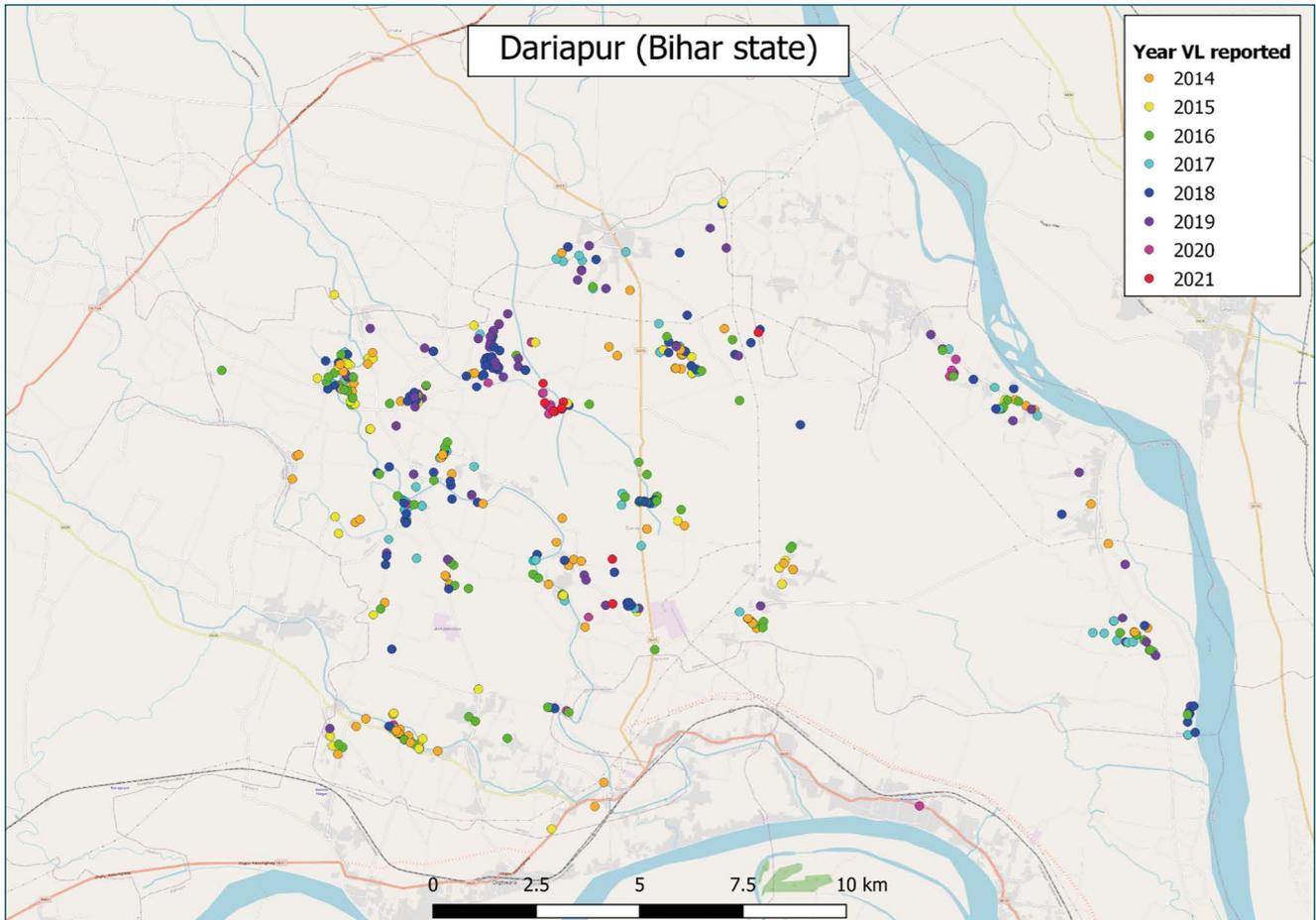
Figure 2.   Mapping of ex-VL cases in Dariapur, Bihar.

Table 1.   Results of active screening for VL, PKDL and leprosy

	Kanti	Dariapur	Bairia	Bansdih	Garhha	Sonepur	Total (pats/hh cont.)
HH members	374	4029	148	145	1662	1099	7457 (1084/6373)
Examined	186	3086	119	100	1260	856	5606 (1056/4550)
New diagnoses							
VL	0	3	0	0	0	0	3
PKDL	0	10	0	0	7	8	25*
Leprosy	0	1	0	0	0	0	1

* Plus 7 on PKDL treatment and 46 with history of PKDL

2.3 TOOL 2: POPULATION-BASED SURVEILLANCE

The other surveillance mechanism that we explored is serological surveys, for which we selected three types of villages: currently endemic, previously endemic and non-endemic (Appendix 5). For the previously endemic villages we had data available of serosurveys done 10 years earlier to compare with. We used three tests: Direct Agglutination Test (DAT), rK39 ELISA, and the earlier mentioned rK39 RDT. Figure 3 below shows results of seroprevalence broken down by age group for the three tests and by category of village.
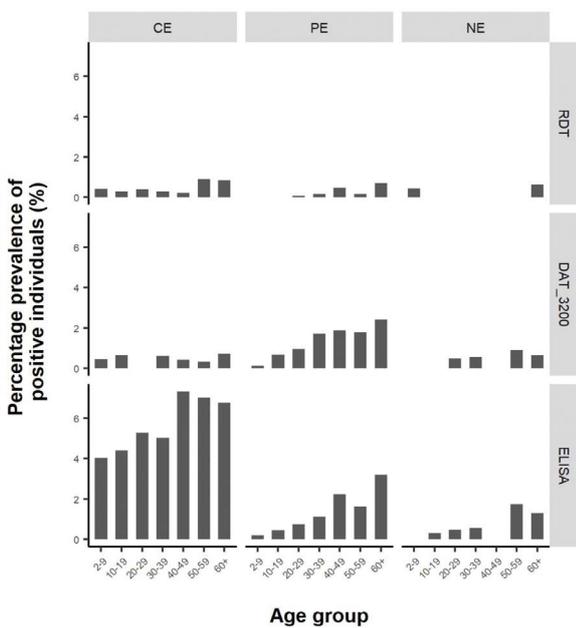
Figure 3. Seroprevalence by age group and epidemiological category (CE = currently endemic, PE = previously endemic, NE = non-endemic).

The clearest distinction between the different categories of endemicity status was seen with the rK39 ELISA, in particular among the youngest age group (2-9 years). This is further highlighted in Figure 4 above right.

Comparison with a sero-survey done 10 years earlier in a subset of villages in the framework of another study (KALANET) confirms the potential for sero-surveys as a tool to monitor elimination of transmission of *L. donovani*. During this earlier study, only the DAT had been used as a marker of infection, therefore we are not able to compare results for the rK39 ELISA. As illustrated in Figure 5, in both villages it is clear that the seroprevalence has very much decreased among all age groups, and that among the younger age group (< 10 years), infection
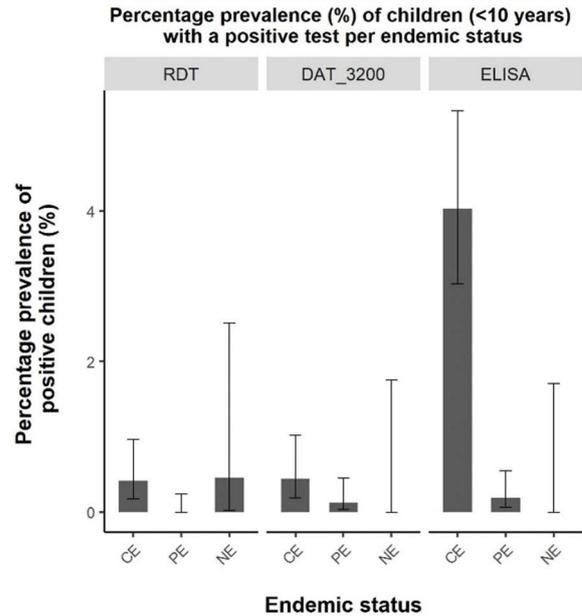
Figure 4. Seroprevalence among children below 10 years of age by test and by epidemiological category (CE = currently endemic, PE = previously endemic, NE = non-endemic).
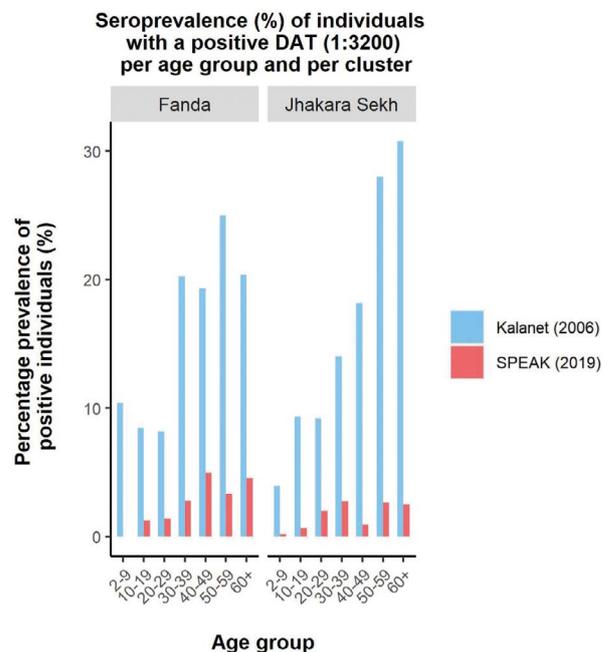
Figure 5. Evolution over time of DAT seroprevalence.

with *L. donovani* has become a rare event. Serial sero-surveys may thus be an additional tool to assess whether a decrease in case notification is due to a decrease in incidence or just an artifact due to under-detection or under-reporting.

2.4 REPURPOSING THE TOOLS FOR OTHER CONTEXTS

We have explored different options of surveillance for the peri- and post-elimination era. Case-based surveillance making use of the rK39 RDT is probably the most cost-effective approach and serves a dual purpose of early case detection. Monitoring numbers of rK39 tests performed, positivity rates and proportions of positive RDT results that can be confirmed by qPCR is feasible and will provide a solid tool to monitor elimination of transmission of *L. donovani.*

In our survey, approximately 7.5% of former VL patients developed PKDL at some time. With dwindling numbers of VL cases, it is far from impossible for PHC staff to revisit each of their former patients on three occasions over a 5-year period. This will allow to keep a close watch on PKDL, which has been demonstrated in some areas of India to have been the inter-epidemic reservoir.[8] In addition, paying home visits to (ex) VL patients allows geolocating their homes and thus facilitating the identification of potential areas of ongoing transmission for leishmaniasis. If done for other diseases as well, this could greatly facilitate the identification of overlapping hotspots for different diseases to guide integrated disease control measures. At the same time, the household visits provide an opportunity for awareness raising and active case detection, not just for VL or PKDL, but for other endemic diseases as well.

Serological surveys may provide an additional assurance that an observed decrease in case notification rates is not an artifact due to neglect, but the result of a true decline in transmission.[9] The rK39 ELISA appears to be a good marker, though obviously this needs to be independently confirmed. The younger age group seems to provide the clearest reflection of the difference of transmission intensity between different endemic settings as well as of the decrease in transmission intensity over time. However, conducting serological surveys just for VL may not be very cost effective. Therefore, such surveys could be combined with those already carried out for other NTDs such as lymphatic filariasis (LF). In the LF National Programs, transmission assessment surveys (TAS) among children have already been adopted as a valuable monitoring tool for transmission.[10] In areas co-endemic for VL and LF it would be easy to add on testing for VL. Another potential candidate could be leprosy, in particular making use of Phenolic glycolipid – 1 (PGL-1). PGL-1 is known as a marker of infection of leprosy, in particular for the multibacillary side of the spectrum.[11] At individual level this may not be a useful tool because it misses most of the paucibacillary side of the spectrum and most infections with *M. leprae* will anyhow remain asymptomatic. However, as a tool to monitor transmission of *M. leprae,* PGL-1 seroprevalence could play a role. There are still other pathogens to be considered; whichever combination is screened for would certainly increase cost-effectiveness and therefore sustainability of the surveillance mechanism.

Combining surveillance for multiple diseases or NTDs is also an option for the case-based surveillance. Sample collection, storage and transport facilities would be more sustainable if used for surveillance of multiple pathogens. The same applies to molecular diagnostic tools in referral laboratories. As VL becomes increasingly rare, the key to effective surveillance appears to be combining surveillance for multiple diseases.[2,12] This has also been identified as a strategic priority by WHO in its 'Regional Strategic Framework for accelerating and sustaining elimination of kala-azar in the South-East Asia Region, 2022–2026'.[13]

3

Tools for molecular xenomonitoring of VL and other vector-borne diseases

3 Tools for molecular xenomonitoring of VL and other vector-borne diseases

3.1 BACKGROUND: MOLECULAR XENOMONITORING AS A POTENTIAL STRATEGY FOR INTEGRATED VECTOR-BORNE DISEASE SURVEILLANCE

Molecular xenomonitoring (MX) is the detection of pathogen DNA or RNA in hematophagous insects that can be used as a proxy for human infection in elimination settings, or for screening of emerging infections.[14] MX has been integrated in surveillance strategies for onchocerciasis and lymphatic filariasis (LF) elimination campaigns since the 1990s.[15,16] MX is usually used in vertical programmes and targets competent vectors only (e.g. *Culex quinquefasciatus* to check for LF). However, this approach can be problematic in areas where transmission of a disease is very low, such as VL in elimination settings in Bangladesh, India and Nepal because thousands of competent vectors would need to be collected to find the few infected and confirm ongoing transmission.

Our novel MX approach (Figure 6) has a wider scope and is based on collecting all insects that feed on blood since they act as "biological syringes" that have already sampled the human population. This approach does not target specific vectors, nor does it confirm transmission,
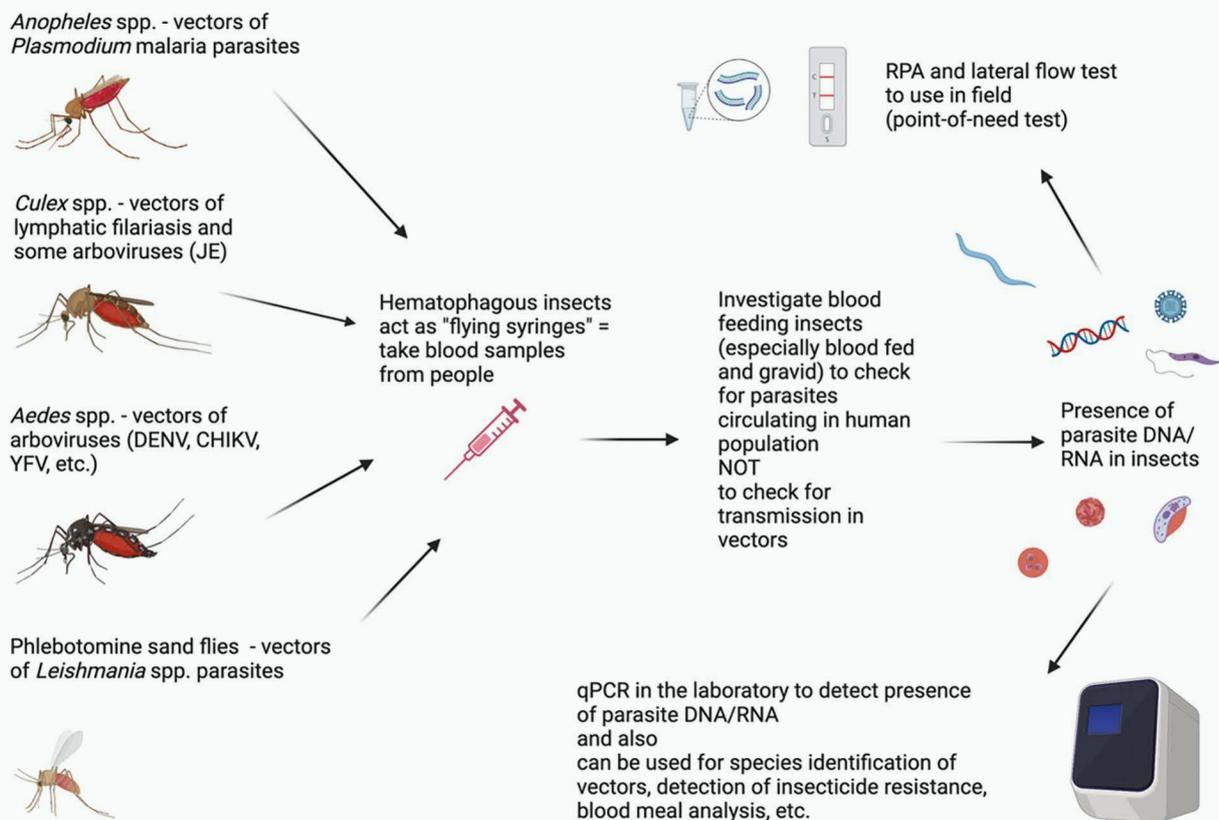
Figure 6.   Principles of MX for integrated VBD surveillance. Figure created with BioRender.com

but it provides information on which pathogens are present in the human population at a given moment in time and can be used to inform disease surveillance and control programmes. It has the potential to be used for surveillance of multiple vector-borne diseases (VBDs), which often co-exist in the same space and time - such as visceral leishmaniasis (VL), LF, malaria, and arboviral infections (e.g. dengue) on the Indian subcontinent. Another advantage of MX is that it can be used for surveillance to monitor the impact of different interventions (e.g. vector control, MDA) on disease transmission.

Insect collection

A variety of traps can be used to maximize the collection of all hematophagous insects rather than specific vectors only. Table 2 shows advantages and disadvantages of the four main collection methods used to collect mosquitoes and sand flies. Standard Operating Procedures (SOPs) for each collection method are provided in Appendix 6 (a-d). The focus should be especially on blood-fed and gravid females, as this guarantees they have fed at least once, thus maximizing the chances of detecting any pathogens circulating in the local human population.

Table 2.   Collection methods for hematophagous insects

Collection Method	Target Population	Advantages	Disadvantages
CDC Light Traps	· Based on insect attraction to light · Host-seeking females especially (gravid females and blood fed females to a lesser extent)	· Can be standardized · Overnight collections · Not overtly disruptive when done indoors · Can also be used outdoors · Good method for subsequent RNA analysis of specimens (e.g. arboviruses) · UV light can be added · Attractants (e.g. CO_2) can be added	· Limited in range of attraction · Poorly attractive to diurnal and crepuscular active insects · Some insect damage · Fewer gravid females collected compared to aspirator collection · Batteries last max one night but can possibly run out sooner · Light bulbs need to be checked regularly · Fan needs to be checked
Aspirator collection – Mouth aspirators – Battery operated aspirators – Large mechanical aspirators (backpack aspirators/ Prokopack)	· Resting population (both male and female) · Good for collection of blood fed females	· Live insects collected · Good for collection of resting sandflies, Anopheles spp and Culex spp adults, and to a certain extent Aedes aegypti · Good for measuring population changes · Specimens collected during a relatively short time · Collects higher proportion of blood fed and gravid females · Broad area of collection · Good method for subsequent RNA analysis of specimens · Morning collections usually more efficient than afternoons or evenings	· Labour intensive · Difficult to standardize · May be disruptive when done indoors · Modifications may be needed to prevent specimen damage · Some of the backpack aspirators can be heavy, two people needed for collections · Torch required to spot adults resting on walls · Mouth aspirator not recommended due to health reasons (inhalation of small particles) but might be required for transfer of specimens

CDC Gravid Traps	· Attract gravid females that fed at least once and need to oviposit	· Mostly used for *Culex* but attractiveness to other insects can be improved by using infusions · Good for arbovirus surveys	· Not great for *Aedes* or for sandflies · Batteries required
BG-Sentinel Traps	· Designed to attract *Aedes aegypti* and *Aedes albopictus* · Can be modified to attract a wider range of insects	· Adding CO_2 source makes the trap attractive to wider range of insects	· Chemical lure required to increase the catch · Run on 12V battery

Pathogen detection

Traditionally, Polymerase chain reaction (PCR) and real-time PCR (rt-PCR) protocols are the most used nucleic acid amplification technologies (NAATs) used for pathogen detection as they are highly sensitive and specific. However, these methodologies can require expensive technology such as a fluorescent-based thermocycler as well as being easily affected by inhibitors which can be present in a given sample, including excess salt, phenol or blood anticoagulants. Other NAATS have been developed including isothermal amplification assays which run at a single temperature, simplifying the deployment of a given methodology in both laboratory and field settings. Loop Mediated Isothermal Amplification (LAMP) and Recombinase Polymerase Amplification (RPA), see Figure 7, are the two most commonly used isothermal techniques.[17,18]
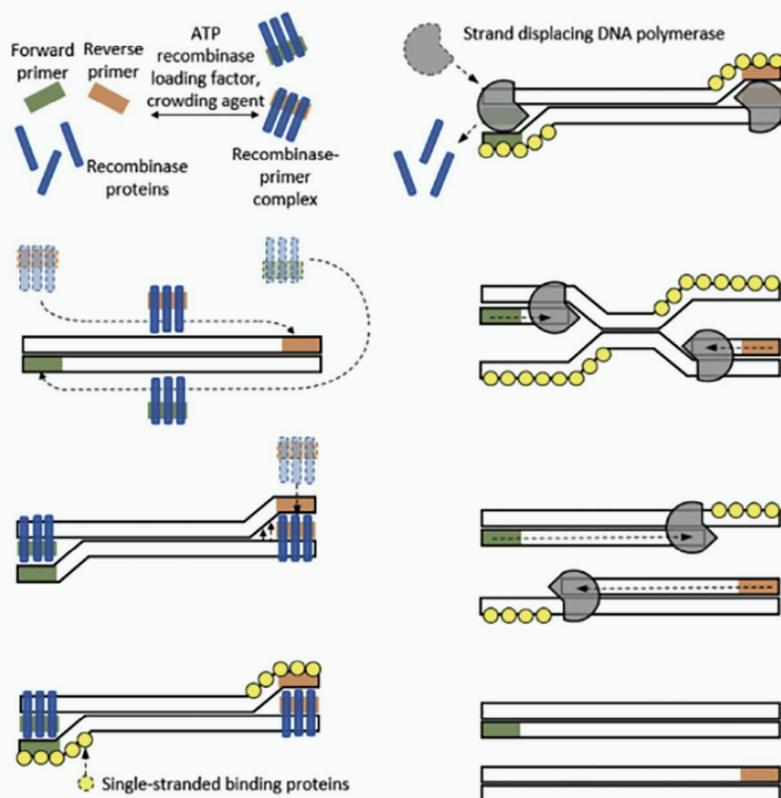
Figure 7.   A diagram highlighting the stages of recombinase polymerase amplification (RPA).[1]

LAMP is performed at 65°C and like PCR has been widely used for pathogen detection in developing regions. However, complex primer design (4-6 oligos required) and the possible generation of false positives from primer-dimer formation are the main limitations of this technique. In comparison, Recombinase Polymerase Amplification (RPA) can be performed between 37-42°C, notably lower than LAMP, thus it can be powered using human body heat, or small portable devices, making this method an excellent candidate to be applied in the field conditions (Table 3). In addition, RPA reagents can be lyophilized making them easy to transport at room temperature. Primer design for RPA is less complex than LAMP with the requirement of 3 oligos (2 primers and 1 probe), limiting the potential for primer-dimer formation. RPA can also be adapted for lateral flow (LF) detection, through the utilisation of labelled primer/probes making RPA outcome easy to determine in field settings. It is also possible to detect RNA by adding a reverse transcriptase enzyme to the RPA reaction, eliminating the need to produce cDNA before detection, also possible when using LAMP. RPA is quite resistant to amplification inhibitors and has successfully been used to detect the DNA/RNA directly from crude extracts, which is challenging when using PCR. RPA had been applied for a range of crude samples such as cultured cells, sera, and animal and plant products (e.g. milk, fruit). For organisms which are difficult to detect, such as bacteria, a pre- step of heat, mechanical or chemical lysis can be performed before the RPA reaction. RPA has also been used to detect many pathogens, including *Leishmania,* Ebola and SARS-Cov-2.[19,20,21]

Table 3.   Comparison of molecular techniques

Factors	PCR/ rt-PCR	LAMP	RPA
Sample processing prior to amplification	DNA/RNA extraction	Crude samples DNA/RNA extraction	Crude samples DNA/RNA extraction
Amplification temperature	Range of temperatures per amplification	One step temperature (65°C)	One step temperature (37- 42°C)
Amplification time	>60 min	10–60 min	10–40 min
Instrumentation requirement	Thermocycler	Heating block or water bath	Body temperature, heating block or water bath
Post-amplification detection	Electrophoresis; Fluorescence detection	Detection by eye; turbidity measurement; fluorescence detection; electrophoresis	Detection by eye; lateral flow cassette; fluorescence detection; electrophoresis
Applicable to field tested	No	Yes	Yes
False positives	Potential risk minimized by proper set up	High risk due to contamination carry over or primer dimer formation, minimized by robust primer design, use of lock tubes and proper set up.	Potential risk minimized by proper set up

We developed RPA protocols to detect DNA/RNA of pathogens in field settings from pools of insects. The assays were designed to detect any of the six human malaria *Plasmodium* species, *Leishmania donovani, Wuchereria bancrofti* and Dengue 1-4 serotypes. A crude nucleic acid extraction protocol was developed based on a previously published method which involves mechanical and chemical lysis.[22] These protocols, together with methods for data management and calculating costs of their implementation, provide the bases of our novel tool for integrated MX described below.

3.2 TOOL 3: A NOVEL APPROACH USING MX FOR INTEGRATED VECTOR-BORNE DISEASE SURVEILLANCE

The novel MX approach described in this toolkit makes use of both RPA and qPCR, and the workflow is illustrated in Figure 8.
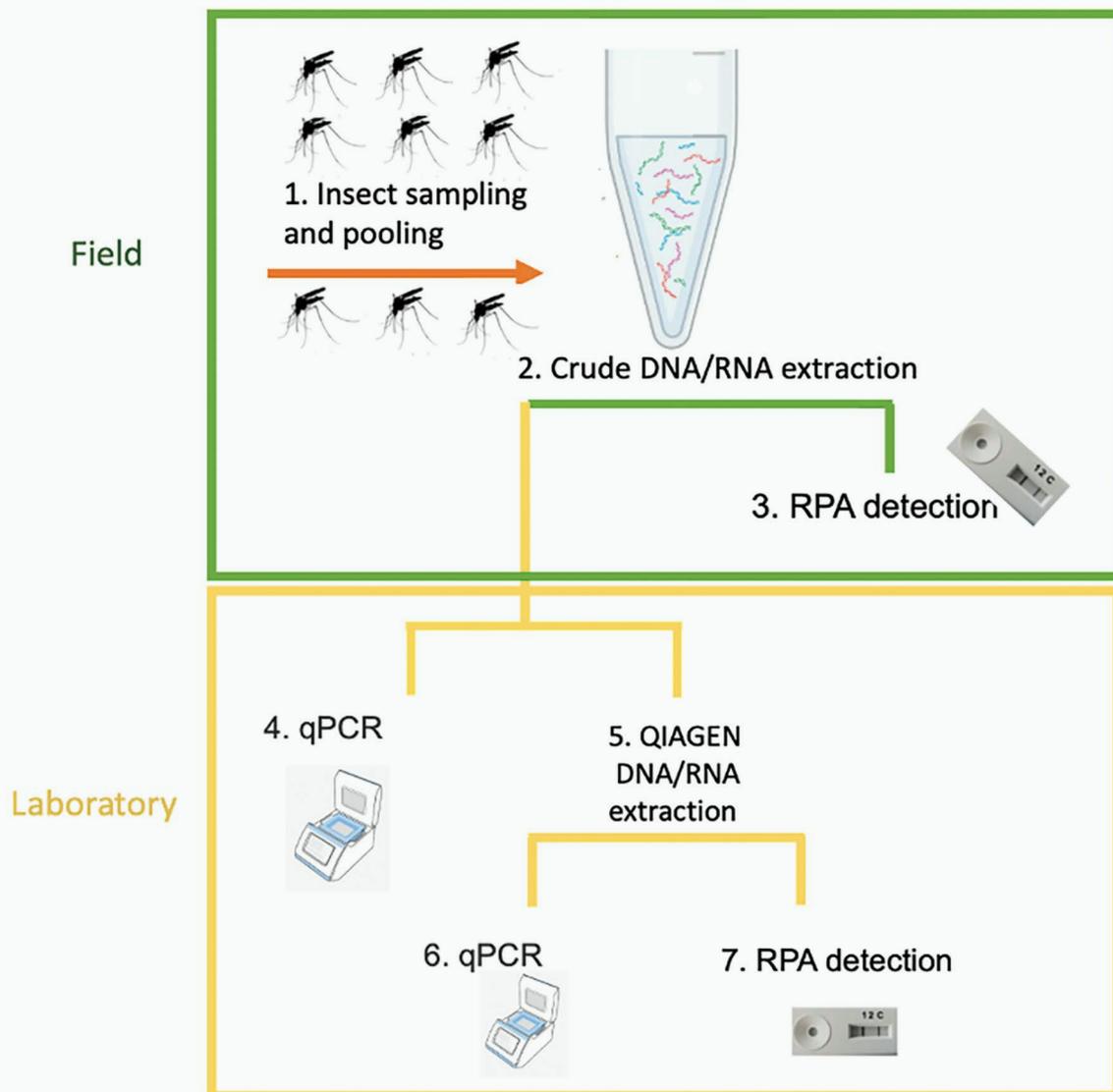
Figure 8.   MX workflow summary used in Bangladesh project.

The specific tools comprise Standard Operating Procedure (SOPs) that were developed for use in a study conducted in Bangladesh and are listed below:

- For Step 1 (Insect Sampling & Pooling), please follow the *Pooling of collected insects SOP* (Appendix 7)

- For Step 2 (Crude Extraction), please follow the *Crude Nucleic Acid Extraction SOP* (Appendix 8).

- For Step 3 (RPA detection on crude extract) please follow the *RPA SOP* (Appendix 9)

- Ensure that transport of samples to laboratory is performed using a cold-chain (see below)

- For Step 5 (Qiagen Extraction)

- For Steps 4 and 6 (qPCR using Crude or Qiagen extracted DNA/RNA), please follow the *DNA Pathogen qPCR SOP* (Appendix 10) or *RNA Pathogen qPCR SOP* (Appendix 11) accordingly.

- For Step 7 (RPA detection), please follow the *RPA SOP* (Appendix 12). Only perform this step for pools which tested positive in Step 6. For each positive pool, 2 repeats should be performed, one using the Crude Extract and one using the Qiagen Extract.

- Data are collected using and corresponding and associated *Vector Collection Record Form* (Appendix 11).

- A tool for estimating the cost for each activity is described in Appendix 13.

If unable to perform **Step 3** or **Step 4, FREEZE** the insect pools for late processing at **-20ºC** (**DNA pathogens**) or **-80ºC** (**RNA detection**).

Cold Chain Storage

From field collection of specimens to DNA/RNA extraction, and a confirmatory qPCR following RPA performed in the field, it is important to have cold chain storage in place as MX involves detection of DNA/RNA. Insulated coolers with ice packs (for transport of specimens after field collection) and -20ºC freezers (for storage of specimens, crude RPA extract and DNA) are easy to source. DNA is relatively stable and -20ºC storage is sufficient. If RNA analysis will be conducted (for detection of arboviruses), a -80ºC freezer would be needed as RNA is much more prone to heat degradation than DNA. Alternatively, samples can be stored in RNAlater solution.

Data management

Since many new data elements are collected at each step in MX, data management can be challenging, especially once the pools are created. Ideally, all MX data should be collected and stored in a central, electronic repository for easy cross-referencing (see Appendix 12). It is recommended that data be collected electronically, whenever possible. A standardized data collection form should be used to record sampling data. Handwritten data collection presents various problems including illegible handwriting, accidents from liquid spillage resulting in ink blotting, and forms/books being misplaced or lost. Collecting data electronically for fieldwork would require a data collection application for mobiles or tablets, or a laptop. If paper forms are used data should be entered into the database each day. For laboratory work it is presumed that the computer being used for MX would be housed in the laboratory, making data collection during sorting and molecular analysis simple.

Estimating cost for implementation

As detailed above, MX integrates fieldwork and molecular analysis of collected vector specimens, both of which involve significant allocation of human, equipment, laboratory and other resources. An SOP developed for estimating resource needs and costs of MX of *P. argentipes* for detecting *L. donovani* in Bihar, India is presented in Appendix 13.

This tool can also be used to:

- estimate costs associated with individual MX activities

- estimate the time burden of MX activities

- characterize factors that influence the cost of the different activities.

For the purposes of this work, MX was divided into five main activities: trap installation, specimen collection, specimen sorting, specimen pooling, nucleic acid extraction, and molecular analysis using PCR.

Several factors may influence the amount of resources and time needed for each stage (i.e. number of specimens collected, travel distance to field site, number of pools created, type of assay). Therefore, it is important to observe each stage and record the total resources and time needed, with collection of this information across multiple observations to allow for variations that may occur at each stage. The more observations per activity, the better the estimates for time and resource burden. It is the role of the observer to include all physical and human resources used during the observation, and collect information on the amount of time it takes to complete activities. Ideally, the observer should be someone not directly involved in MX activities to reduce potential bias in reporting of observations.

3.3 REPURPOSING THE MX TOOLS FOR OTHER CONTEXTS

Epidemiological data on reported cases (VL and PKDL, malaria, LF, dengue) may be used for deciding where to implement MX and which type of traps to use. The number of collections (within a collection day and over time), the objective of collections, and collection site locations (indoor and/or outdoor) will need to be considered early in MX planning. Depending on the objective of the study collection sites might need to be randomized and dispersed across the selected site

as best as possible; alternatively, collections might be focused around disease transmission hotspots where many human cases occur. The sampling scheme for MX should be robust, standardized, and replicable across the different sites.

Since MX requires systematic and replicate collection events until the appropriate sample size is achieved, it involves coordination with the local population to avoid disruption to daily routines that may result in refusal at any point during MX. Trust and commitment from the local population is critical to ensure sustained support for MX over its course. Consent from village householders will be needed if the sampling strategy includes any indoor or peri-domestic sampling.

Collection of adult sand flies, mosquitoes and other hematophagous insects can provide information on their distribution, bionomics, is suitable for pathogen detection, blood meal analysis, detection of insecticide resistance as well as evaluation of control interventions.[23,24,25]

Each collection event must have a unique identification code/ID to accurately interpret downstream data and connect field samples with the results of molecular analysis. The ID for collection event should contain the minimum information needed to determine:

· Location of collection at the site-level and within-site-level

· Date of collection

Appropriate labelling can be complex if MX is being performed across many selection sites. Additionally, the ID may need to be short enough to be written on small tubes. Selecting an appropriate ID system is critical and should be thoughtfully considered early in the planning process. Use of barcodes (e.g. Appendix 2) or QR codes can be used.

High magnification (at least 40x) microscopes are recommended for species identification of sand flies and mosquitoes. USB microscopes are also available that provide good magnification and are relatively inexpensive compared to bench-top stereomicroscopes. However, when the focus is not specifically on vectors because all hematophagous insects are being collected, detailed species identification might not be necessary.

MX staff should be trained in medical entomology to recognize unique morphological characteristics of sand flies (especially *Phlebotomus argentipes* in comparison to other sand fly species and insects), and to distinguish between *Anopheles, Culex, Aedes* and other culicine mosquitoes, as well as other insects that feed on blood

such as biting midges. These insect groups can be separated quite easily by trained personnel.

Specimens should be sorted based on insect genus, sex, and physiological stage. The physiological status of females (unfed, fed, or gravid) is important as pathogen detection will only be possible in insects that have previously fed on blood.

The ID for sorted specimens should contain the minimum information needed to determine:

· Location of collection at the site-level and within-site-level

· Date of collection

· Physiological status (sex, fed status)

A high number of female insects (especially vectors) may be needed to detect low infection rates during peri- and post-elimination stages.[26] Pooling is an appropriate strategy when the likelihood of positive detection of a DNA/RNA target is low.[27] This approach is cost-effective and timesaving, allowing quick analysis of pooled specimens.

The strategy of organizing specimens into pools should be agreed beforehand and records on pool composition will be required. Misidentification of a positive pool could result in misdirection of resources to area where no action is needed and lack of follow-up and
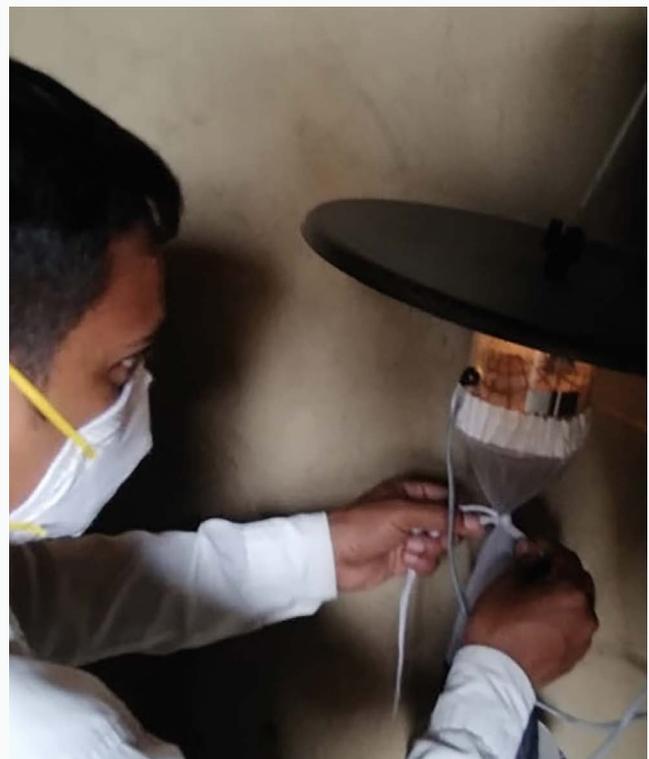
CDC-LT set up inside household of village (field collector wearing PPE during covid-19 pandemic).

intervention in an area of concern. It is recommended that pools are created from specimens within a selected site rather than across multiple sites, so it is easy to determine which village specifically had insects with pathogens.

Use of Excel spreadsheets for data storage can be problematic when there are many data fields, making the system prone to data entry errors. Epi Info 7, both for mobile use and for Windows operating systems, is a good data collection tool. It is open-source and allows data to be entered as presented on a form that can be printed if needed. Forms can also be easily edited to add and remove data fields. Open Data Kit (ODK) is a free, open-source application and a great alternative to Epi Info 7 for collection and management. Alternatively, Google Forms and Google Drive can be used for data entry and storage (as shown in Appendix 12).

Analysis of MX data may range from simple counts to more complex statistical analysis (i.e. regression analysis) depending on the purpose. Epi Info 7 is not only a good system for data storage but also contains features for descriptive and statistical analysis; additionally, data can be line-listed as a Microsoft Excel spreadsheet for analysis within this program. For more advanced statistical analysis, applications like R Studio or STATA may be required.

Ideally, MX data would be integrated with other VBD data systems. This would require data to be uploaded to the system by a person with approved access and training on how to input data. As detection of pathogens (within insect pools) may trigger downstream enhanced surveillance and events, an additional notification system should be considered, especially if a positive pool is detected where presence of a certain pathogen is not expected.

Field team isolating sand flies from a CDC light trap collection

4

Tools for short- and long-term prediction of block-level VL diagnoses

4 Tools for short- and long-term prediction of block-level VL diagnoses

4.1 BACKGROUND TO DEVELOPMENT OF PREDICTION TOOLS

The WHO target of elimination of VL as a public health problem was the motivation for developing this tool. The aims were to explore the feasibility of projecting recent trends in incidence forward at (i) short and (ii) long-time horizons in order to better monitor the progress of each block towards this target and to forecast trends and prevent any resurgence during post-elimination era.

Routine surveillance monitors incidence rates per block in order to identify high-, moderate- or low-endemicity and hence determine intervention plans. Block-level incidence was also used to monitor regional elimination status with respect to the number of blocks above or below the target. While the short-term prediction modelling approach (i) does not take into account that risk is shared across administrative boundaries and, as such, a block defined as low- or non-endemic does not imply that it is at low risk of transmission or reintroduction in the future, the long-term prediction modelling approach (ii) takes account of the risk shared by both administrative boundaries and time as well as risk that could vary due to environment, climatic and demographic features across block.

Both models (i) and (ii) use the monthly block-level summarized VL case data as reported in the Kala Azar Management Information System (KA-MIS).

4.2 TOOL 4: SHORT-TERM PREDICTION MODEL WITHOUT COVARIATES

This prediction tool employs an existing statistical framework[28] to model the dependence of monthly, block-level incidence rates on the recent past, both within the same block and across its neighbours.

The tool serves to explain the analysis pipeline written to obtain short-term predictions of visceral leishmaniasis diagnoses at the block level in Bihar, India, employing a model published by SPEAK India.[29] The purpose of this work was to estimate potential incidence over the next 3-4 months based on historical incidence patterns over time and between neighbouring blocks. These may be used to monitor progress towards elimination goals and potentially to identify blocks for closer attention, in which patterns diverge from the regional trends.

All code has been written and tested in *R version 3.6.3 (2020-02-29) and requires the following packages:* tidyverse, lubridate, reshape2, surveillance, hhh4addon, rgdal, spdep, here, sf, tictoc. The code can be accessed through a public repository at https://github.com/esnightingale/vl-short-term-prediction.

Data

Raw linelist data are currently downloaded manually from the KA-MIS web portal (https://ka-mis.org/) via a secure login provided by CARE India.

The dataset of interest is the state-level resident diagnosis table, consisting of individual diagnosis records for both VL and PKDL with date and block of residence. To ensure all relevant records are included, data are downloaded without filter and the time span restricted within a later data cleaning step.

This data table currently may only be downloaded by state, so to include multiple states in the analysis requires downloading the separate files and appending before running the data aggregation step.

The raw data are read in, cleaned, split by case type (VL or PKDL) and aggregated by block and month of diagnosis (Figure 9).
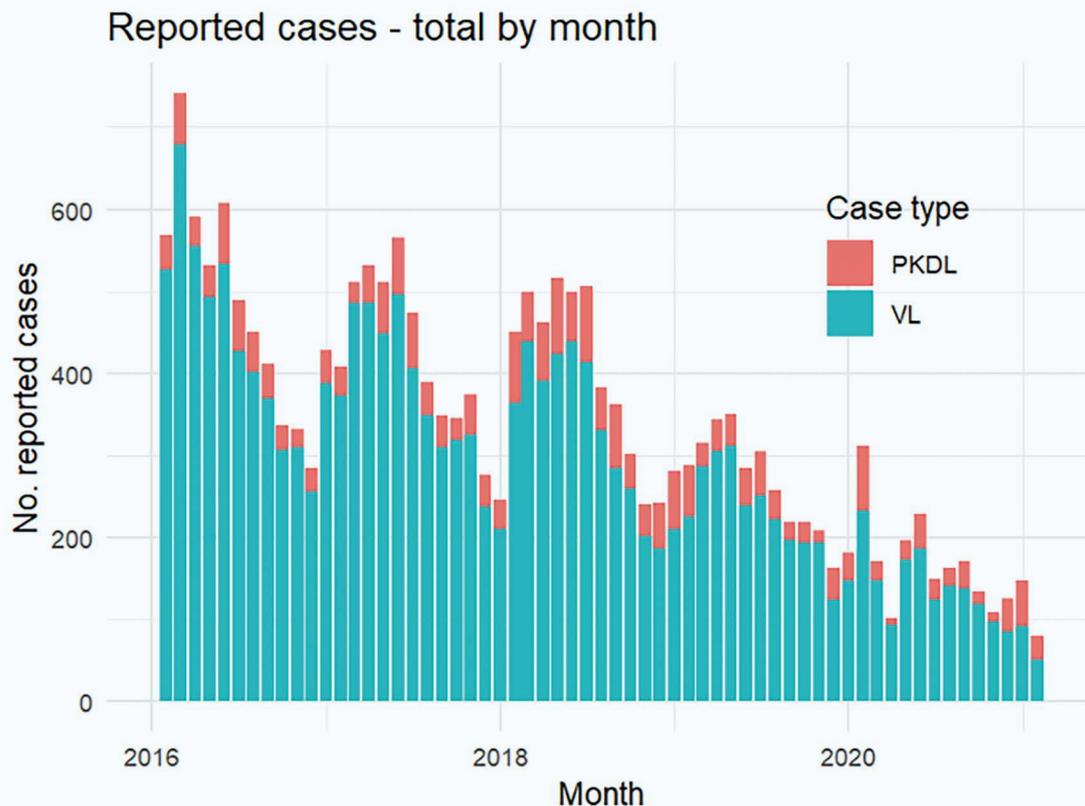
Figure 9.   Raw data of VL and PKDL cases by block and month of diagnosis.

Pipeline

The pipeline consists of the following steps:

1. **Set up populations –** Read block population estimates from the most recent census (2011) and project forwards per month according to estimated decadal growth rates.

2. **Clean and aggregate linelist –** Read in raw data, clean and filter to specified period, then aggregate by block and month for analysis.

3. **Set up data formats for HHH4 model –** The HHH4 model framework requires a certain structure of input data (defined as a "spatial time series" object or stsObj), consisting of matrices of case and population counts, a shapefile and neighbourhood matrix for the spatial units, start date and frequency of observations.

4. **Fit the model and predict ahead –** Predictions are obtained by drawing simulations of the trajectory n months beyond the fitted data, on a rolling basis. *A test period is also specified (currently set to the most recent six months) with which to check predictive accuracy.*

5. **Summarise and plot the predictions –** Calculate quantiles over simulated trajectories, by block, state and month, and plot these prediction intervals.

These steps are implemented in sequence via the script run_all.R. Parameters which can be adjusted are:

· Start and end dates between which to aggregate data and fit model

· Number of months to include as a test period for prediction

· Number of months to forecast ahead of observed data

· Number of simulations to draw

If the specified end date is beyond the observed range of diagnosis dates in the raw data, the end date is redefined from the last observed diagnosis date rounded down to the start of the last month (in order to exclude diagnoses from the latest, incompletely reported month).

Outputs

The following are example outputs based on a model fit to diagnoses from 2016-01-01 to 2021-02-01, with rolling 3-month-ahead predictions (Figures 10-13).
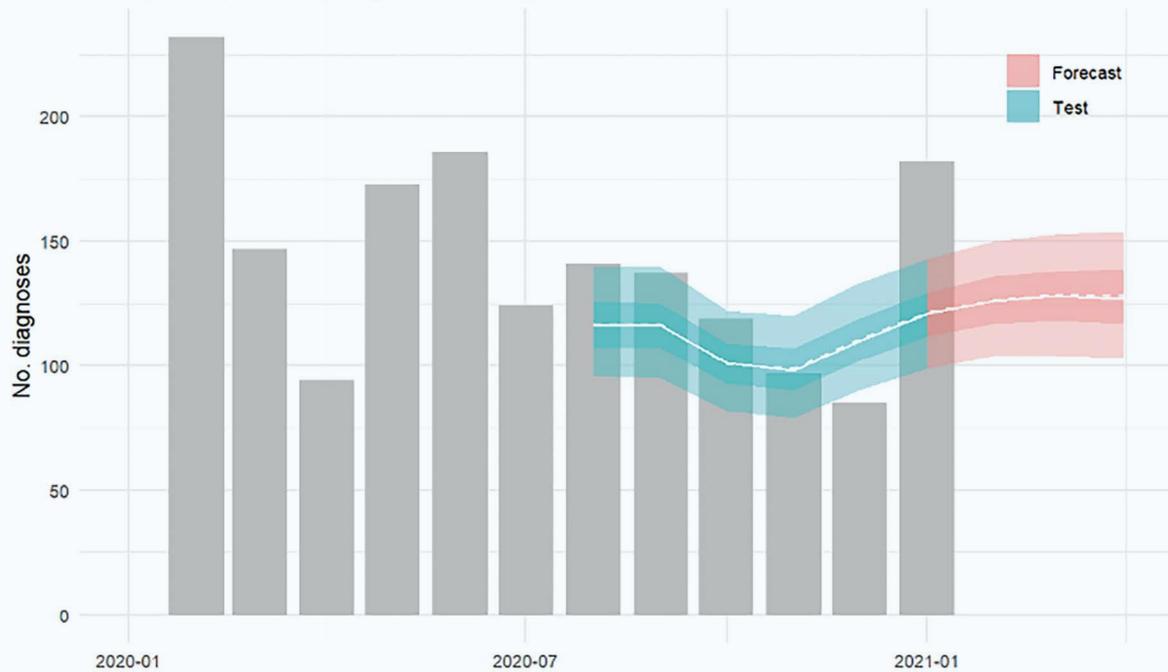
Figure 10.   State total counts with three-month-ahead predictions based on 1000 simulations from the model, for a test period of six months and a forecast period of three months. For each predicted month, the model is refit to include the latest observed data.
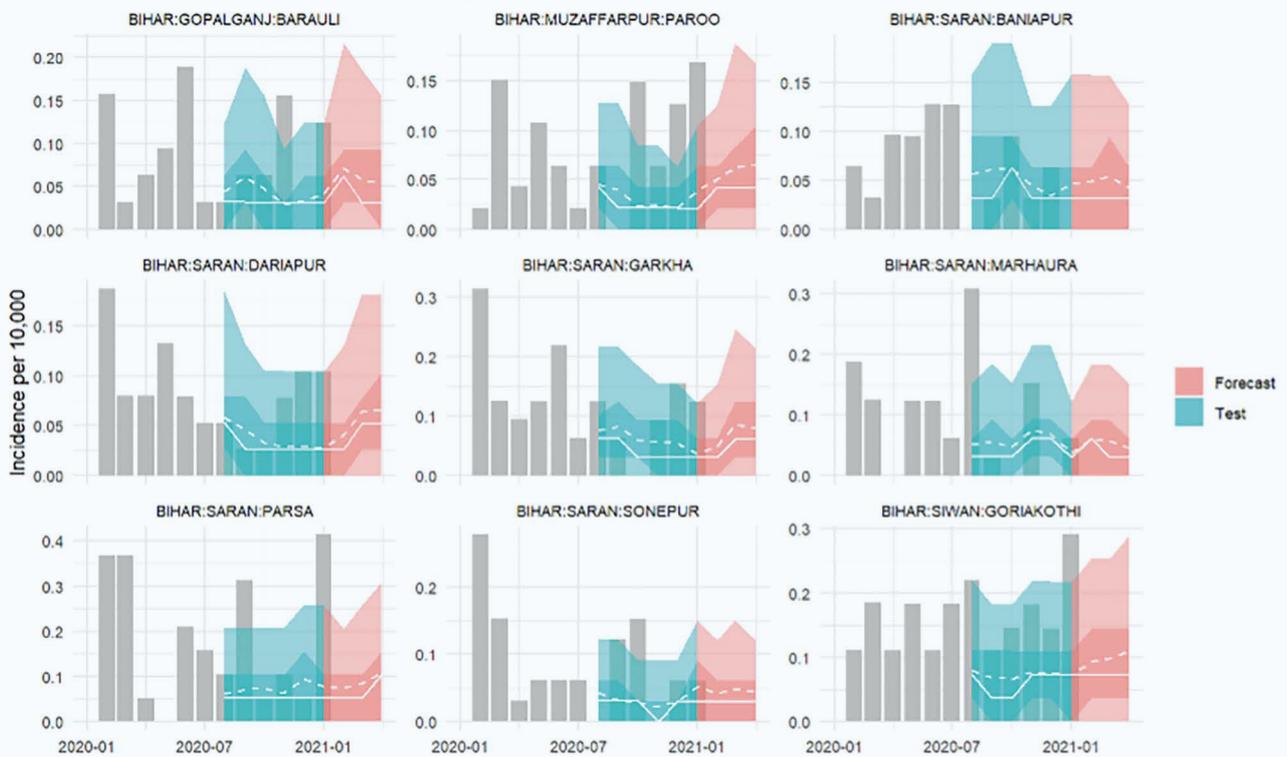
Figure 11.   Equivalent predictions at the block level, for blocks with highest total incidence since 2019.
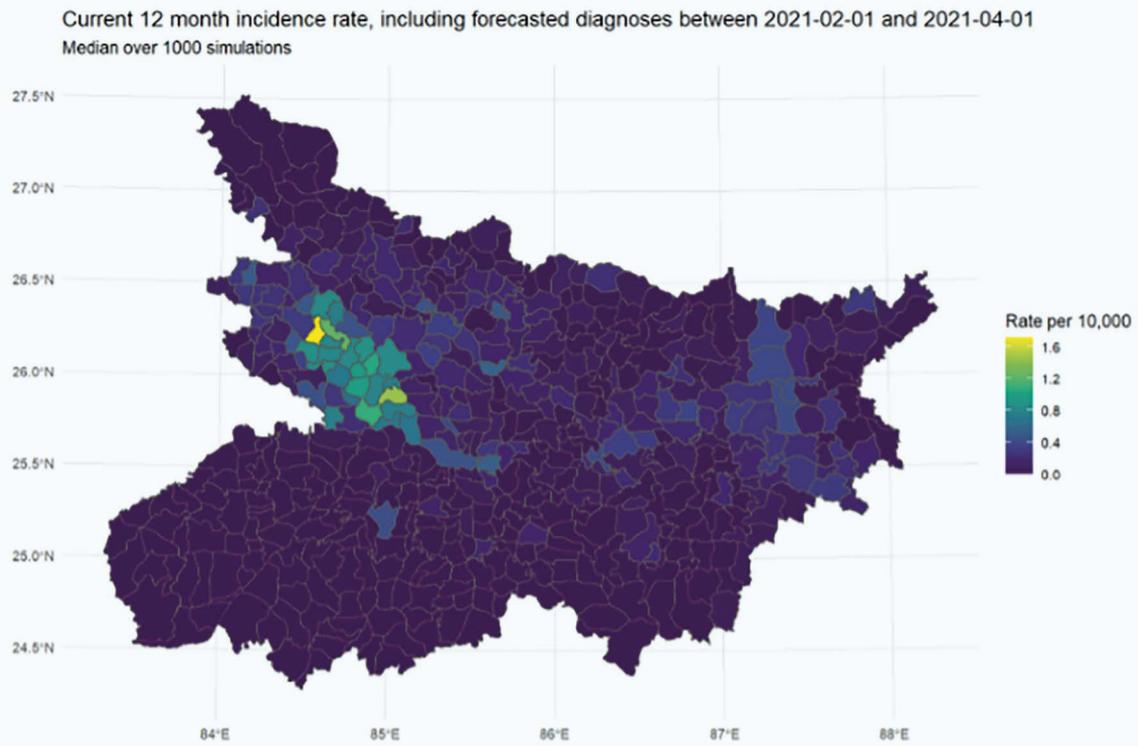
Figure 12.   Estimated block-level incidence rates for the most recent twelve months, including the three month forecasts just obtained.
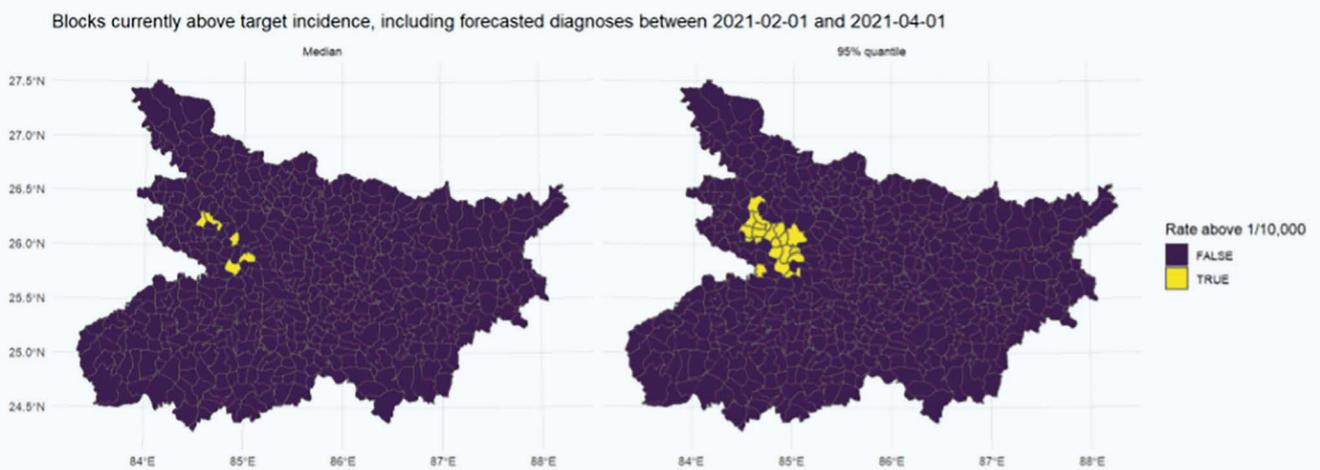
Figure 13.   Blocks above the 1 per 10,000 target incidence rate according to estimated 12 month incidence, based on median and upper 95% quantile of simulations.

4.3 TOOL 5: LONG-TERM PREDICTION MODEL WITH COVARIATES

This prediction tool is based on the existing Bayesian spatiotemporal modelling framework that has been employed for mapping and predicting risk of diseases such as malaria[30], lymphatic filariasis[31], dengue[32]. All the inferential procedures were carried out using the Integrated Nested Laplace Approximation (INLA) approach[33] by means of the R-INLA package 22.12.12-1 built in 2022-12-11 (www.r-inla.org). All code was written and tested in R version 4.2.2 (2022-10-31 ucrt) and requires the following packages: INLA, spdep, rgdal, tmap, sf, ggplot2, dplyr, rgeos, maptools, ggthemes, raster, viridis, ggalt.

A spatiotemporal model incorporating potential risk factors of environment, climatic and demographic features that could account for spatial heterogeneity in VL incidence was developed and validated using block level monthly VL incidence data from endemic and non-endemic blocks of both Bihar and Jharkhand. The model can be used to predict and forecast annual incidence at block level that can guide the programme to monitor progress towards the elimination goal and to prevent or reduce risk of resurgence during post elimination settings.

Data

The data needs to be prepared as per the following steps:

1. **Population data:** as described in step 1, Section 4.2, Tool 4.

2. **VL case data:** VL case data can be downloaded and summarized by block for each month as described in step 2, Section 4.2 Tool 4.

3. **Covariates data:** Block level monthly data for the following variables can be obtained as described below:

    a. LST (Land Surface temperature) and EVI (enhanced vegetation index) from MODIS (MODerate-resolution Imaging Spectroradiometer) data base using 'MODISTools' package in R.[34]

    b. Soil moisture, minimum and maximum temperature and land surface temperature can be extracted from TerraClim web portal.

    c. The 3 bioclimatic variables (Mean temperature [BIO 1], isothermality [BIO 3], and precipitation [BIO 12], from the WorldClim database (ver. 2.1,

released in Jan 2020, https://www.worldclim.org/) using 'Biovar' function in R.

4. **Set up data formats for INLA model:** The INLA model framework requires a certain structure of input data (defined as a "data" object), consisting of matrices of block wise case, population counts, data for each covariate per month and for each year, a shapefile and neighbourhood matrix for the spatial units. A variable to split the data for model training and validation.

5. **Fit the model and predict ahead:** Predictions are obtained by drawing 1000 simulations of the trajectory for the required duration of months beyond the fitted data.

6. **Summarise and plot the predictions:** Calculate quantiles over simulated trajectories, by block, state and month, and plot these prediction intervals.

Outputs

The following are example outputs based on a model fit to the training data (Jan 2013 to Dec 2018). Figure 14 shows the estimated spatial (block) pattern of risk of VL (A) and the probability that the risk is > 1 (B).
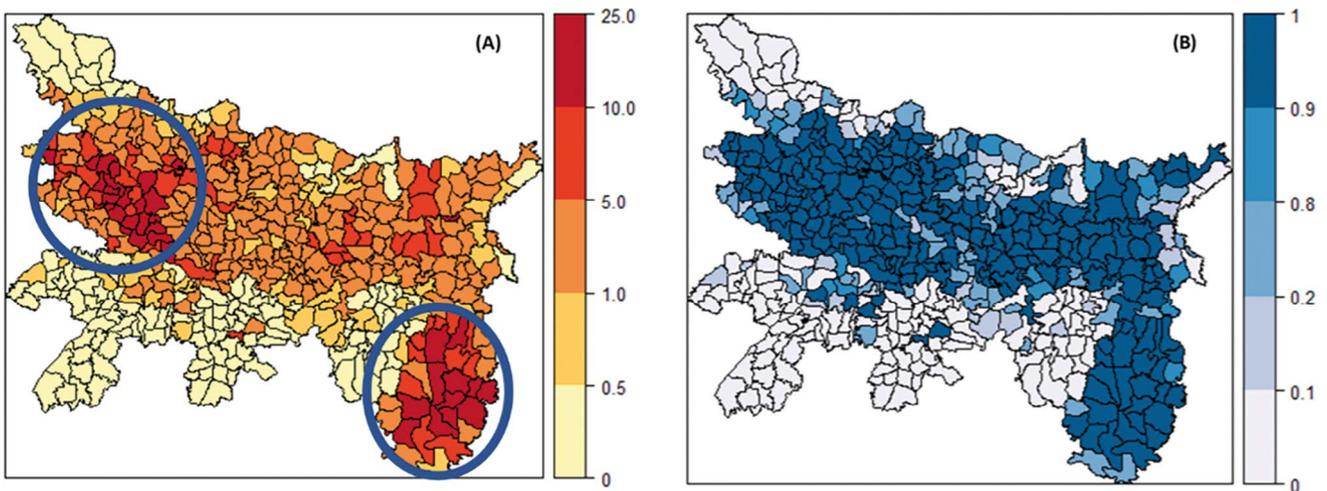
Figure 14.   Posterior means of the block-specific (A) relative risks and (B) posterior probabilities that the relative risks are greater than 1. Relative risk is >5.0 for blocks lie within circles (A). Probability that relative exceeds 1.0 is >90% for most of the blocks and is across the centre of Bihar and almost all the blocks in Jharkhand (B).

Figure 15 compares the observed and predicted annual VL incidence for the year 2019 (training) and 2021-2022 (test periods). Model predicted incidence indicates that in3 blocks (Kathikund in Dumka district, Amrapara and Hiranpur in Pakaur district are above the elimination threshold of 1 per 10,000 population during 2021 and 2022. Considering the upper 95% credible interval for the incidence, a few more blocks in Bihar (n=26) and Jharkhand (n=21) may exceed the elimination threshold in 2022. The values in parentheses indicate the observed and model predicted number of blocks exceeding elimination threshold.
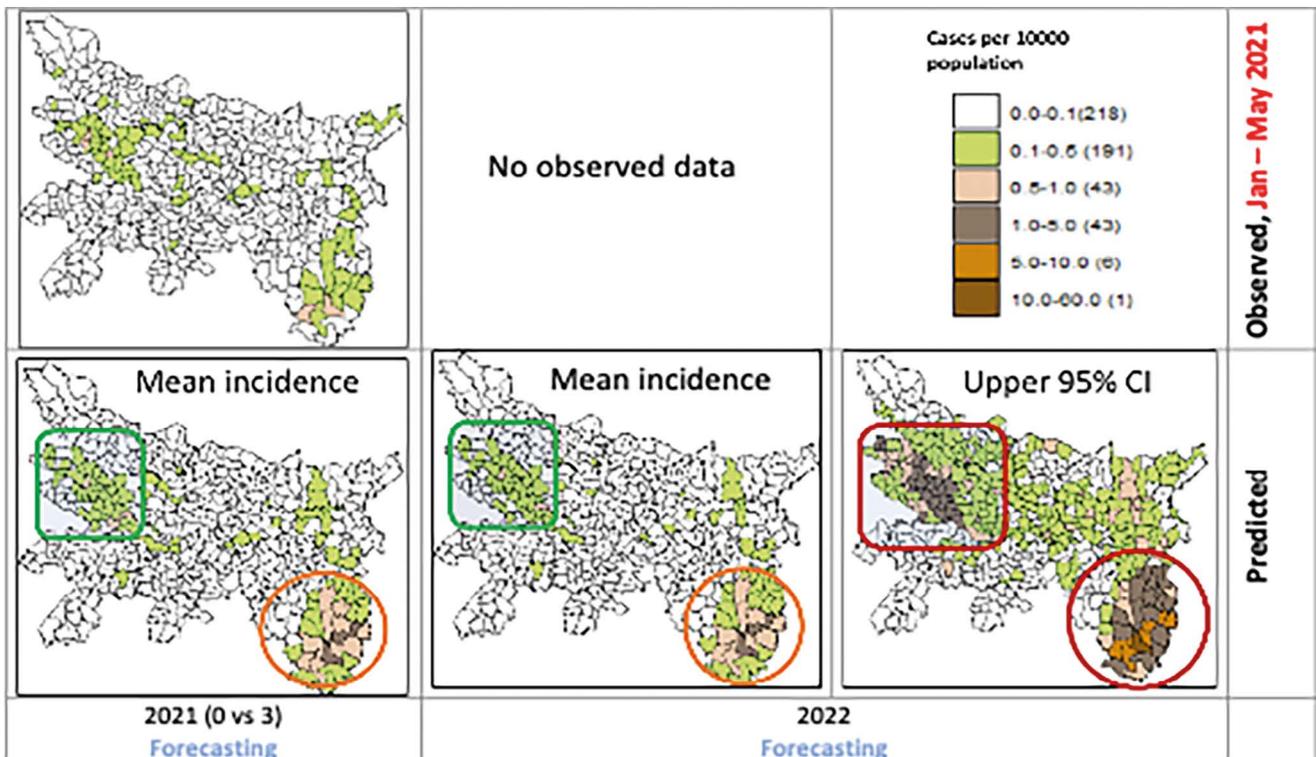
Figure 15.   Spatial pattern in observed and predicted annual incidence per 10,000 population during 2021-2022 (forecasting period) in the states of Bihar and Jharkhand.

Figure 16 shows the spatial temporal variation in selected blocks of Bihar and Jharkhand where the annual incidence in 2013 was > 6 (Bihar) and > 12 (Jharkhand) per 10000 population. Although both observed and predicted trends decline and tend to go below or above the elimination threshold over time, the 95% credible intervals suggests that in all the selected blocks the incidence is likely to exceed the elimination threshold in 2022.
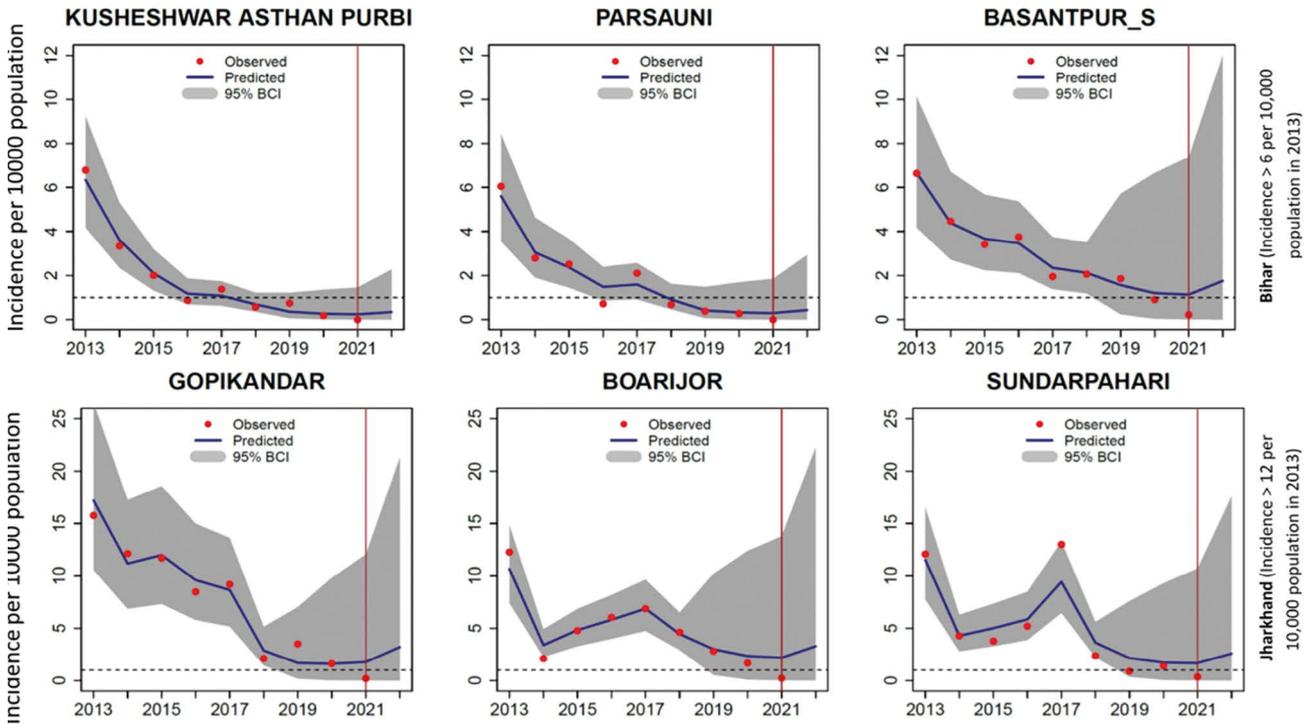
Figure 16.   Spatiotemporal variation in 3-blocks each with annual incidence > 6 or > 12 per 10,000 population in 2013 respectively in Bihar (top panel) and Jharkhand (bottom panel). The predicted trends closely mimicked the declining observed trends in all the 3 blocks for both Bihar and Jharkhand. The upper 95% credible intervals (CI, grey band) for 2022 indicates the likelihood of exceeding the elimination threshold (1/10,000 population) in these selected blocks. The red vertical line indicates the period up to which VL case data are available (up to May 2021).

4.4 REPURPOSING THE PREDICTION TOOLS FOR OTHER CONTEXTS

Provided that relevant administrative boundary and population data were available alongside observed case counts, the tools may be adapted to other settings (with respect to geographic region and/or disease). However, two key limitations of the described approach are, firstly, that predictions at this administrative level will have less practical use as case counts fall to very low levels, and secondly, that the predictions only reflect patterns of *reported* cases and not of underlying incidence or transmission. These limitations should be taken into account when considering applying the tools in new contexts.

5 The way forward

Effective surveillance and control of vector-borne diseases, as recommended by the WHO Global Vector Control Response 2017-2030[35], has been compromised by inadequate capacity for evidence-based decision-making and implementation tools which have been identified as priority needs.[36]

Yet, effective surveillance is crucial to prevent resurgence of diseases in the endgame of disease elimination[6] and, indeed, is a strategic priority in the 'Regional Strategic Framework for accelerating and sustaining elimination of kala-azar in the South-East Asia Region, 2022–2026'.[13]

In this toolkit we have described tools that were developed specifically to strengthen surveillance of VL in post-elimination settings in the Indian Subcontinent, but they also have flexibility to be used for other diseases and in other settings with low endemicity. The prediction modelling tools may be used to determine where resources and efforts are best targeted, including surveillance, diagnostics, treatments and vector control. Incorporating the prediction tools onto the dashboard of a disease reporting database will further improve model predictions. The molecular xenomonitoring tools can be used in sentinel sites, the selection of which may be informed by the prediction tools, to determine whether a pathogen is circulating in an area to serve in an early warning system or to assess any increased risk of transmission. The human surveillance tools provide opportunities to strengthen case-based and population-based surveillance. The tools that investigate disease transmission in the field use hand-held devices for data entry and for immediate reporting to the end-user to enable a rapid response. The tools also lend themselves to integrated disease surveillance, which is also an important priority for accelerating and sustaining elimination of Neglected Tropical Diseases.[13] The MX tools and serology sampling tools are multiplex, requiring single samples from vector and/or human populations respectively, and may play important roles in post-validation surveillance.

Further operational research may be required to ensure that the tools are implemented for maximum impact in different contexts and an integrated approach should be considered. In India, integration of three eliminable disease control programmes, malaria, lymphatic filariasis and visceral leishmaniasis, is recognized as a desirable goal since the distributions of the diseases often overlap and control is administered by the same health care workers at different levels.[37] Indeed, the National Centre for Vector Borne Diseases Control has produced a manual on integrated vector management in India.[38] We believe that implementation of the multiplex molecular tools and data transfer in real time onto digital platforms, as described herein, will help contribute to an integrated vector-borne disease surveillance system.

Appendices

LIST OF APPENDICES 33

Appendix 1

Standard Operating Procedure
'Improving VL surveillance at PHC level'

INTRODUCTION

This SOP describes the procedures for new kala azar patients in the eight Block PHCs involved in the study described in the 'Improving VL surveillance at PHC level' protocol of SPEAK. For this purpose two Block PHCs in each of four States, Bihar, Jharkhand, UP and West Bengal have been selected. Incident VL patients as well as patients registered 1, 3 and 5 years earlier will be enrolled.

New patients will be asked for a blood sample that will be tested with qPCR at BHU. Also they will be interviewed to document healthcare seeking behaviour and costs incurred. They will be referred for HIV testing to the nearest ICTC and they will be visited in their homes for their contacts to be screened for kala azar. All persons screened will also be screened for signs of leprosy and PKDL.

Kala azar patients registered 1, 3 and 5 years earlier will also be visited in their homes to be screened for PKDL. At the same time their relatives will be screened for kala azar. All persons screened will also be screened for signs of leprosy and PKDL.

Below we will describe in more detail the procedures involved.

PROCEDURES:

Part A: New kala azar patients

1. Enrolling new kala azar patients:

Any new kala azar patient will be explained about the study and provided information sheet 1 in annex 1 of the protocol. If they agree they will sign the informed consent form (ICF), form 1 in annex 5. Please make sure to ask them whether they agree to a home visit, if they refuse please check the box 'I do not wish to be visited at home'.

At the same time take a copy of form 2 (annex 6), which is the informed consent form to be used for screening of household members. On this form serial number 'FSN' is preprinted at the top. Please copy this number to the empty boxes behind 'FSN' in the header of form 1 (annex 5). Make sure to have three identical barcode stickers, one of which is stuck in the top right corner of form 1 (annex 5). A second identical barcode sticker is stuck on the PHC register in which the patient is recorded on the same line. The third will be used to label a blood sample. Now fill out the header of form 2 (annex 6, from which you copied the FSN) and enter name, age and gender of the patient on the first line. If the patient agrees to a home visit, ask him/her to sign for consent behind his/her name, if (s)he does not agree you may cross out the rest of the form but do keep it together with form 1.

2. Interviewing new kala azar patients:

The next step is to interview the patient. For this purpose please use the app 'SPEAK_interview' on an android tablet or smartphone. The first thing to enter is the form serial number (FSN) which van be copied from the header of form 1 (the ICF). The next question is on whether or not the patient is a newly diagnosed patient, the answer will be 'yes'. You will then be asked for State, District, Block and Village name, followed by the date of the interview. Then there will be a question on the symptoms that were present, date of onset and date of first healthcare seeking action. This is followed by questions on providers visited, on place and date of diagnosis, on history of kala azar, whether or not the patient sleeps under a bed net and whether or not (s)he sleeps outside for part of the year. Then there are some questions about money spent on diagnosis and treatment and finally a question on whether or not the patient would agree to be contacted for a more in-depth interview at a later stage. You will then be asked to save the form and exit the app. As soon as you have internet connection again you may send it to the server.

3. Collecting a blood sample:

Collect a 2ml venous blood sample, stick a barcode sticker on it identical to the one on the ICF (and the register). Put the sample in the refrigerator and inform the study coordinator who will arrange for it to be forwarded to BHU.

4. Refer for HIV testing:

Explain the patient about the need for HIV testing and refer him/her to the nearest ICTC. Make sure to follow up until you have received the result from the ICTC. As soon as you have the result use the app 'SPEAK_Test_Result' to enter it and send it to the server.

5. Home visit for VL/PKDL screening:

All patients who have agreed to a home visit are to be visited in their homes for their household members to be screened for kala azar. Before going there, first get the copy of 'Form 2' (annex 6) in which the header and the name, age and gender of the index case have already been filled out.

When you arrive at the household, explain about the study and provide a copy of information sheet 2 (Annex 2). Then fill out name age and gender of each remaining permanent household member (whether present or not) on form 2 (annex 6) and ask all household members present to sign for informed consent. Check the box 'Present' for those that are present, leave it blank for those that are not present.

Ask whether anyone has been suffering from fever recently and if so perform an rK39 RDT to check for kala azar or refer the patient to the PHC. Also examine the skin of each person for signs of PKDL and at the same time also look for signs of leprosy. The results are entered in the app 'SPEAK_Screening' on an Android tablet or smartphone.

The first question asked in the app is the 'FSN' or form serial number. This is the number at the top of the ICF (form 2, annex 6). You will then be asked to identify State, District, Block and village from a list and to record the geographic coordinates. For recording geographic coordinates you have to be outside in front of the patient's home. You then select the date of visit and enter the number of household members. This number should include index case him(her)self. There will be a series of questions on each individual household member, including a question on whether the person ever suffered from kala azar. If you reply 'yes' to that question there will be a next question on whether or not the person is the index case, i.e. the kala azar patient who was the reason for your visit. The key question is on whether or not there were signs of VL (kala azar), PKDL and leprosy.

When you have examined all consenting household members present and entered all data in the app, the next step is to send the data to the server as soon as you have an internet connection.

6. Collection of ICF forms and blood samples:

All ICF forms as well as the blood samples will be collected on a regular basis by a study coordinator. Form 1, which has the name of the patient, barcode and FSN will be entered at the study coordination site in an MS Access database and will allow linking data collected through different Android apps as well as results of HIV testing.

Part B : Kala azar patients registered 1, 3 and 5 years ago

1. Interviewing the (ex)patients:

Kala azar patients registered 1, 3 and 5 years ago (i.e. in 2014, 2016 and 2018 if the study year is 2019) are to be visited in their homes for PKDL screening. Before going to the homes of these (ex)patients please contact the ASHA to obtain verbal consent for a visit. Take along a copy of form 2 (annex 6) as well as a copy of form 3 (annex 7). When you arrive at the household please explain the study procedures and provide information sheet 4 (annex 4). If the (ex)patient agrees to participate, ask him to sign the ICF, form 3 (Annex 7). At the same time take your empty copy of form 2 (annex 6) and fill out the header and the name, age and gender of the index case. This form has a preprinted form serial number (FSN) which you copy to the top of form 3 (annex 7).

You may now start interviewing the (ex)patient using the same Android app (SPEAK_interview) that was used for new patients. Again the first question is about the form serial number (FSN) which you copy from the ICF, form 2 (annex 6). This time you will reply 'no' to the question on whether or not the index case is a newly diagnosed VL (kala azar) patient and a slightly different set of questions will follow. There will be a question about the year of disease, which is the year in which the patient was diagnosed. This is followed by questions on State, District, block and village, followed by the date of the interview. Next there will be questions on age and gender of the (ex)patient, followed by questions on place and type of treatment. There are questions about treatment outcome, whether or not any additional treatment had been required and whether or not any adverse events were observed. For those treated less than two years ago there are also questions on costs incurred. Finally there is a question on whether the patient agrees to be interviewed more in-depth at some time in the future. Then you may save the form and exit. Whenever you have internet connection again the form can be sent to the server.

2.   Screening for PKDL:

The next step will be to screen the (ex)patients and his/her household members for PKDL or kala azar. At the same time also look for possible signs of leprosy. The procedure is just the same as when screening the household members of a new kala azar patient described in part A5 above. This tile use information sheet 4 (annex 4). The ICF form to be used is form 2 (annex 6) of which you should have brought an empty copy that you used for getting the form serial number (FSN) in part B1 above. Fill out the rest of the header and fill out name, age and gender of each permanent household member, starting with the index case. Check the box 'Present' for those that are present and ask them to sign for informed consent. Then screen them for kala azar, PKDL and leprosy as part A5 above. Make especially sure that you examine the index case for PKDL. Enter all data in the 'SPEAK_screening' app and upload to the server whenever you have internet connection.

Appendix 2

SPEAK surveillance Standard Operating Procedure for new kala azar patients

1. Enrolment in the study and registration:

Whenever a new kala azar patient is diagnosed in your PHC, please inform the patient about the study and provide him/her with a copy of information sheet 1: 'Improving VL surveillance at PHC level (for incident VL patients)'.

If the patient agrees to participate in the study, fill out form I, *'Informed consent 1 Improving VL surveillance at PHC level (for incident VL patients)'* and ask the patient to sign or provide a thumb print if he/she is illiterate. Ask the patient whether he/she agrees to be visited at home for household members to be screened for kala azar and skin conditions associated to kala azar. If he/she does not agree, check the box next to 'I do not wish to be visited at home'.

Form1　　　　　　　**FSN** ☐☐☐☐☐　　　Barcode: Sticker

Informed consent 1 'Improving VL surveillance at PHC level' (for incident VL patient)

I been invited to participate in a study on Kala Azar. I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this study and understand that I have the right to withdraw from the study at any time.

I have been informed that a member of the study team will visit me in my home for a short interview. In case I do not want to be visited at home I will ask for the box below to be checked.

☐　　I do not wish to be visited at home

I have been informed that the blood sample taken will be used for other tests for Kala Azar.

Block name: ......*Kanti*...

Village name: ......*Awari*...............................

Name of participant: ...*Paritosh Singh*...............................

Gender of participant: F ☐　　M ✓

Age of participant: ...*42*...

Date: ...*20/05/2018*.......... (Day/month/year)

Name of parent/custodian (if applicable): ....

Signature/thumb print of patient or parent/ custodian: ....

Signature/thumb print of patient for assent (if applicable): ....

Blood sample collected ☐

Name of a staff member: ...*CP Malaviya*................

Date: ...*20/05/2018*.. (Day/month/year)

Signature staff member: ....

If the patient agrees to a home visit, take a copy of form 2 *'Informed consent 2 Improving VL surveillance at PHC level (for VL and PKDL screening)'* and fill out the header, i.e. block, village and date, and then use the first row of the form to enter the name, age and gender of the patient. In the 'remarks' column indicate 'index case'. Leave the date blank for now.

FORM 2
Informed consent 2 'Improving VL surveillance at PHC level' (for VL and PKDL screening)

Block __Kanti__   Village __Awari__   FSN __0235__   Date_____

Name	Age	Sex	Present	Consent	Assent	Remarks
Paritosh Singh	42	M	Yes			Index case

Now copy the form serial number 'FSN' from form 2 to the header of form 1. This is very important because it allows us to link the forms belonging to the same patient.

Form1   → FSN [0][0][2][3][5]   Barcode: Sticker

Informed consent 1 'Improving VL surveillance at PHC level' (for incident VL patient)

I been invited to participate in a study on Kala Azar. I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this study and understand that I have the right to withdraw from the study at any time.

I have been informed that a member of the study team will visit me in my home for a short

Next take three identical barcode stickers and stick one on form 1, *'Informed consent 1 Improving VL surveillance at PHC level (for incident VL patients)'* (shown right), a second one on a tube for collecting a venous blood sample and a third on the line of the entry for this patient in your PHC register.

Form1   FSN [0][0][2][3][5]   Barcode: Sticker |||||||| 05705

Informed consent 1 'Improving VL surveillance at PHC level' (for incident VL patient)

I been invited to participate in a study on Kala Azar. I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this study and understand that I have the right to withdraw from the study at any time.

2.   Interview with new kala azar patient:

The next step is to interview the patient, results are to be recorded on the tablet in the app 'SPEAK_Interview'. Unless mentioned otherwise, the questions are all about the current episode of kala azar.

In your apps, find the icon of ODK Collect 📋 and tap on it to open it. Then tap on 'Fill Blank Form' and scroll

to 'SPEAK_Interview'. Tap on it to open a form. The first question is to enter the FSN (form serial number), this is copied from form 2, 'Informed consent 2 Improving VL surveillance at PHC level (for VL and PKDL screening)', of which you have just filled out the header. In this example it is 235, as shown above.

Fill in '235' as 'FSN' and press on the arrow pointing to the right to proceed. In the next question you are asked whether the patient is a newly diagnosed VL patient, here the answer is 'Yes'.

Click on the arrow to proceed.
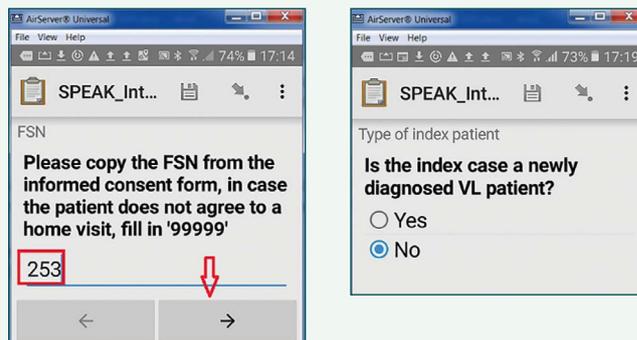
Next you will be asked to fill in 'State', 'District', 'Block' and 'Village'. This is about the village in which the patient lives. If you select the right state, district and block, all the villages from that block should be listed. If you really cannot find the village, do not fill in anything and proceed. You will then be asked whether you found the village in the list, to which you should reply 'No' and proceed. The next question will be the name of the missing village, enter the name and proceed. Please note that this is exceptional, normally all names of villages should be in the list.
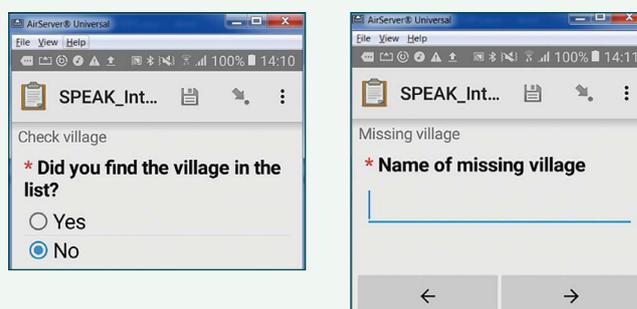
The next field is the date of interview, by default it is set to today. If you tap on the 'Select date' button, you will see three dates listed of which the date of today is in the center. Just tap 'OK' and proceed.

The next screen you get is about the symptoms the patient experienced, fever, malaise, loss of appetite, weight loss etc. By default all are set to 'No'. Change to 'Yes' where appropriate, you may select more than one. If you select the option 'Other' there will be an additional question allowing you to list these other symptoms.

Next there are questions about date of onset of symptoms and date of first seeking medical care. The latter date can never be earlier than the former.

The next question is about where you first sought medical care for your current episode of kala azar. Only one option can be selected. If the actual first provider is not in the list, use the 'Other' option and you will be presented with an additional question to explain which other provider it was. The next question is whether apart from the first provider you just listed you visited any other providers. Here multiple providers can be selected and again there is an 'other' option in case one of the providers consulted would not be in the list.

Then there is a question on the provider where you were diagnosed with Kala Azar, here only one answer is allowed. Again there is an 'other' option in case your provider is not in the list. You will then be asked when you first reported to that provider and when you were diagnosed. The date of diagnosis cannot be earlier than the date of first reporting and the latter cannot be earlier than the data of first healthcare seeking action recorded earlier.

Next there are questions about whether the patient ever suffered from Kala Azar before, whether (s)he normally sleeps under a bed net and whether (s)he sleeps outside for at least one month per year.

The following set of questions is on costs incurred on transport, diagnostic tests, drugs, fees and any other costs as a result of the current Kala Azar episode. All amounts are in Indian Rupees. Tt's probably best to take a piece of scrap paper to write down the expenditures before entering them in the app. In the next question the patient is asked about the total costs incurred, please make sure that the two questions tally as much as possible.

There is one more question about whether or not the patient agrees to be contacted at a later stage for an in-depth interview. Finally you will get to the screen shown below. Tap on 'Save Form and Exit'.
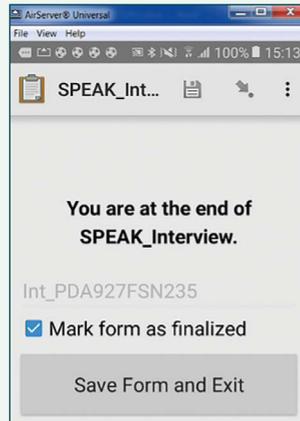
As soon as you have access to internet, please upload your form to the cloud. The ☰ button on top of the screen allows you to sort the forms, by date, newest first, so your latest form will be on top. Select it and click on 'send selected', as shown below. If you see the 'Success' message on the right you may close your smartphone.
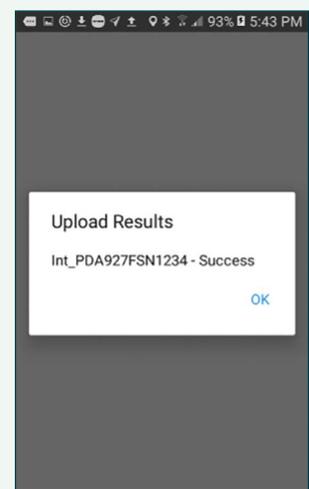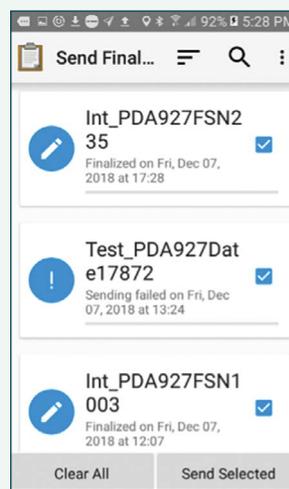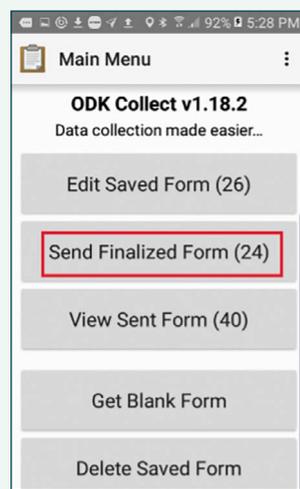
3. Other procedures:

Make sure to store Form 1 and Form 2 in a place where you can easily find them back. Eventually they will be collected by the study coordinator.

Take the blood collection tube on which you had stuck a barcode identical to the one on Form 1. Take 2 cc venous blood and store the tube in the fridge. Inform the study coordinator who will come and collect it.

Refer the patient to the nearest ICTC for HIV counselling and testing. When the result of HIV screening comes back from the ICTC, it is to be entered in another app. The app is called 'SPEAK_TestResult'.

Open the app in the usual way and first enter State, District and Block. The Block is the Block of your PHC. You will then be asked for the date of the result, if available this should be the date provided by the ICTC. If such date is not available just enter the date on which you received the result.
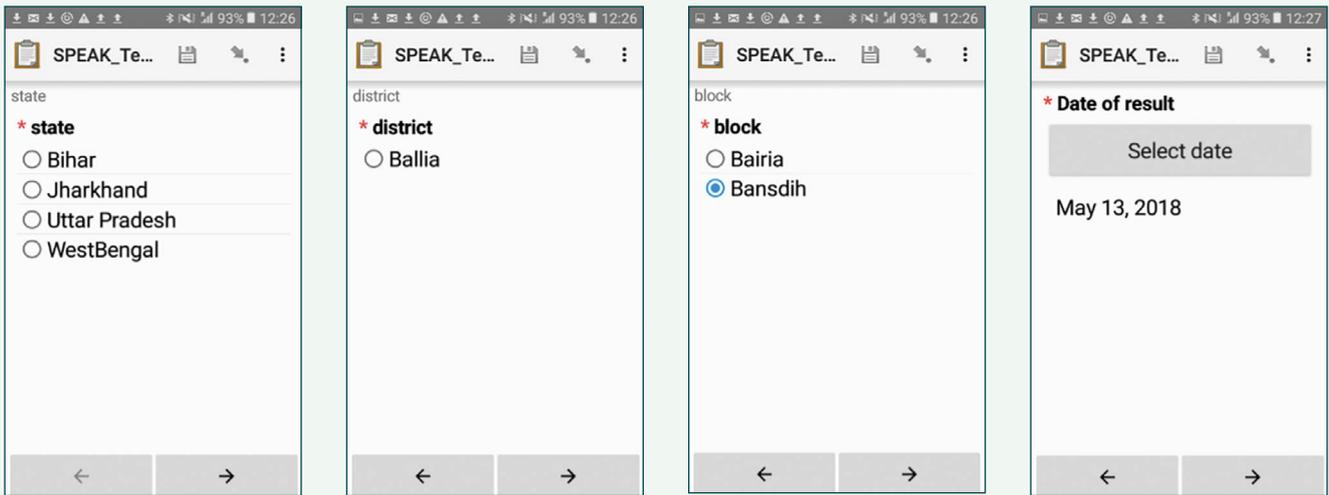
Next you will be asked to scan the barcode and enter the test result. This barcode can be scanned from the sticker on form 1 (if it has not yet been collected by the study coordinator) or the sticker you put in the register, they have to be identical. Next you will be asked for the result of the HIV test, the relevant screens are shown on the right.
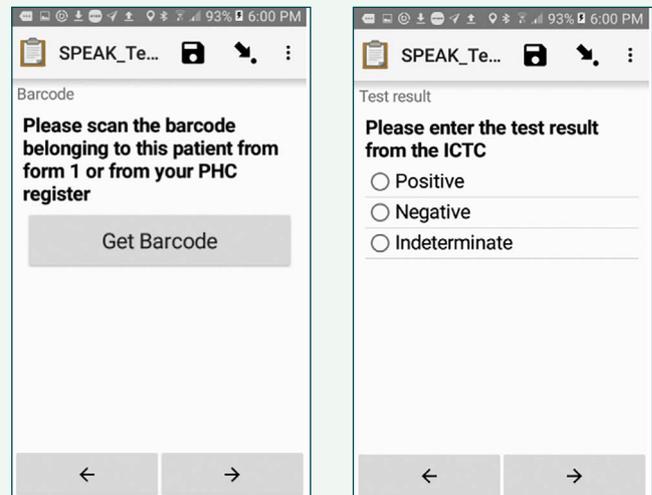
You will then reach the screen 'Save form and exit'. This information too is to be uploaded to the cloud as soon as you have access to the internet. The procedure is the same as for the 'SPEAK_Interview' app.

If the patient has agreed to a home visit, you may already ask for a convenient time and for directions to his village and write them on the back of form 2 *'Informed consent 2 Improving VL surveillance at PHC level (for VL and PKDL screening)'.*

Appendix 3

SPEAK surveillance Standard Operating Procedure for home visits to new kala azar patients

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1. Registering and examining the family members:

This SOP describes the procedures during a home visit to a newly registered kala azar patient. We assume that the interview with the patient has already taken place at the PHC and that the patient has given permission for a home visit. The purpose of this home visit is to screen all household members for kala azar and PKDL. This requires examining for skin lesions. If you find any skin lesions suspect for PKDL this should be reported in the form that you will fill in via the app on your tablet. You should look not only for PKDL but also for signs of leprosy or other skin conditions that require referral for further examination and management.

Before you leave the PHC, make sure that you take with you the copy of form 2 *'Informed consent 2 Improving VL surveillance at PHC level (for VL and PKDL screening)'* on which you had already filled out the name, age and gender of the index patient. The form has a pre-printed form serial number 'FSN' that you should have copied already on the informed consent form (form I, *'Informed consent 1 Improving VL surveillance at PHC level (for incident VL patients)'*) of the patient.

The header of form 2 is below. Now first explain the purpose of your visit and if the household members agree, record for each individual name, age and gender, and ask to sign or put a thumb print for consent.

If the household member is a child, ask the parent/guardian to sign (or put a thumb print). If the child is older than 12 years, ask the child to also sign (or put a thumb print) in the 'Assent' column. Also record the date of the visit in the form header.

FORM 2
Informed consent 2 'Improving VL surveillance at PHC level'
(for VL and PKDL screening)

Block __Kanti__   Village __Awari__   FSN __0235__   Date_____

Name	Age	Sex	Present	Consent	Assent	Remarks
Paritosh Singh	42	M	Yes			Index case

Make sure to register all permanent household members, also those who are not present. Once you have done that you can ask for signs and symptoms of kala azar and inspect the skin for signs of PKDL, leprosy or other skin conditions. If you find anything you can make a note in the 'remarks' column.

An example of form 2 (without signatures or thumb prints) is shown opposite:

FORM 2
Informed consent 2 'Improving VL surveillance at PHC level' (for VL and PKDL screening)

Block __Kanti__   Village__Awari__   FSN __0235__   Date_31-05-2018__

Name	Age	Sex	Present	Consent	Assent	Remarks
Paritosh Singh	42	M	Yes			Index case
Sangeeta Singh	41	F	Yes			
Kamlesh Singh	12	M	Yes			Fever 3 wks Swollen abdomen
Puja Singh	16	F	Yes			
Jaya Singh	34	F	No			

2. *Recording the data on the tablet:*

The next step is to enter the data though the SPEAK Screening app. In your apps, find the icon of ODK Collect and tap on it to open it. Then tap on 'Fill Blank Form' and scroll to 'SPEAK_Interview'. Tap on it to open a form. Please locate it on your tablet as shown below. The first thing you will be asked to enter is the FSN, which you should copy from Form 2, in this case it is 235.
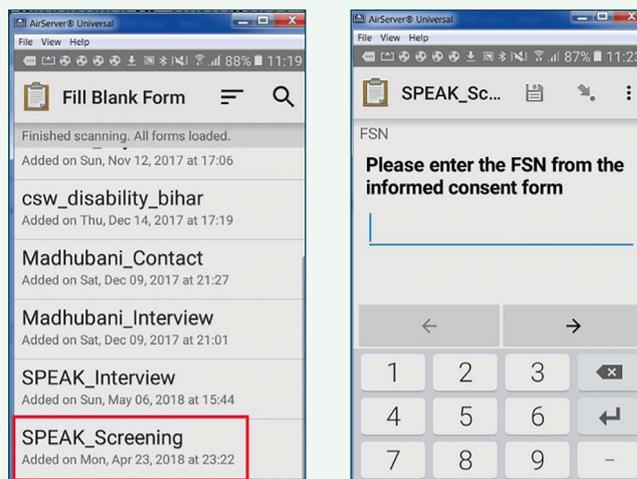
You will then be asked whether the index case is a newly diagnosed VL patient, to which the answer is 'Yes'. (Depending on your answer different questions will pop up. The 'No' answer is for visits to cases diagnosed in earlier years, where the purpose of the visit is mainly to screen for PKDL and interview the patient).

Next you will be asked to enter State, District, Block and Village. As with the 'SPEAK Interview' app, there is the option not to select any village and manually type the village name in case it would be missing from the list. This should be exceptional though, normally the village has to be in the list. Please check carefully, perhaps the spelling is just slightly different from what you are used to.

The next thing to do is to record the geographic coordinates of the household. To do this you should be outside, with a clear view of the sky.

When you tap on the button 'Start GeoPoint' your screen will change as shown below. Either wait till you see the coordinates as on the screenshot below on the right side tap on 'Save GeoPoint' as soon as the accuracy is within 10m.

The next question is on the date of visit, select the date (set by default to today's date), tap on 'OK' and move on to the next question about the number of people in the household. Since you have already filled out form 2, you know exactly how may there are. Please fill out accordingly, you will then get a series of questions on each individual household member as shown in the screenshots below. Start with the first person on the form, which is the index case. Fill in surname, name, gender and age, followed by the questions on whether he/she ever suffered from kala azar or PKDL.

If you reply 'Yes' to the question on kala azar (VL), the next question that will pop up is whether this person is the index case. The answer here is 'Yes', there will be an additional question on the year when the person suffered from VL. In this case this will be the current year. You will then proceed to the question on whether or not the person is present. The answer here is 'Yes'.
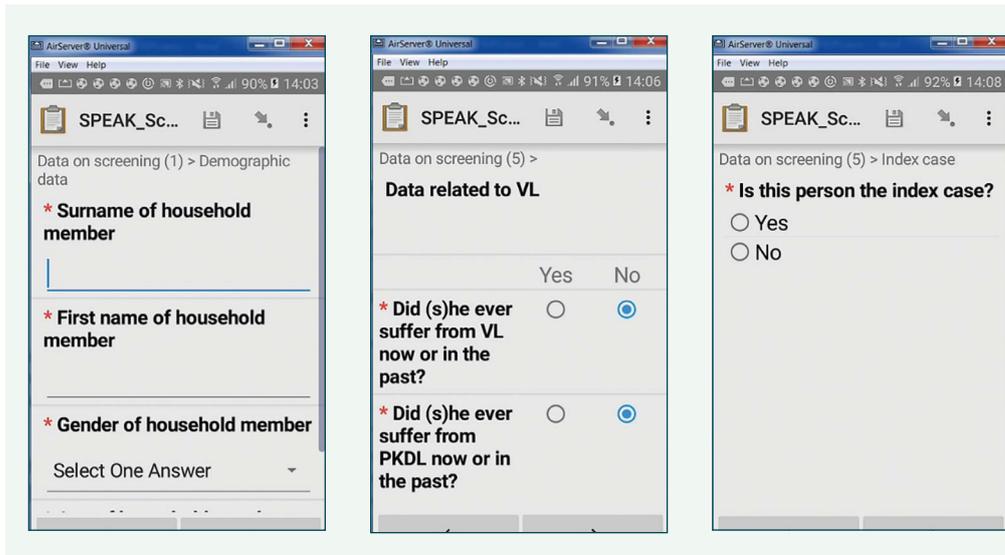
To the next question 'has (s)he been examined'. If you answer 'No' you'll be taken to the next household member. However you may still examine the person for leprosy in which case the answer is 'Yes'.

You will then be asked whether there were signs of VL, PKDL or leprosy. In this case the answer on VL may be 'Yes' bit it's less relevant because anyhow this person is a current VL case.
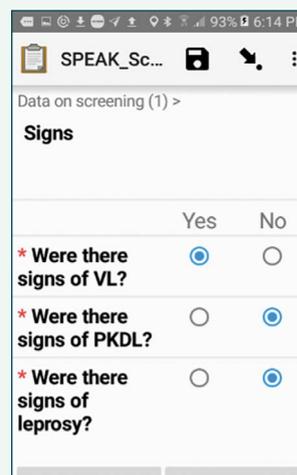
You will then proceed with the next household member. Fill in name, age, gender and whether or not the persons suffered from VL or PKDL now or in the past. 'Now' refers to a patient already diagnosed at the time of your visit, if the person is diagnosed during your visit please fill in 'No'.

You will then be asked whether the person is present and if 'Yes', whether (s)he was examined.
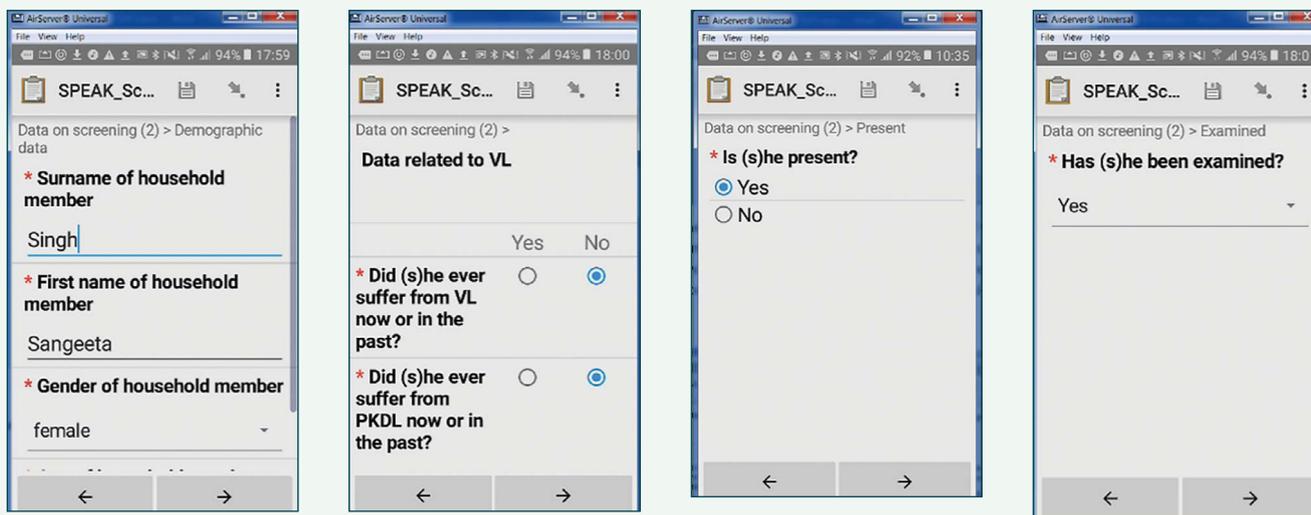
If the persons was examined you are asked about whether there were signs of kala azar (VL), PKDL, or leprosy. Please consider the signs and symptoms listed below and reply 'Yes' if such symptoms are present. You may reply 'Yes' for more than one disease if appropriate.

Kala azar:

- ever of more than 2 weeks duration, not responding to anti-malaria treatment
- enlarged spleen/ abdominal swelling
- weight loss/ malaise in combination with fever and/ or enlarged spleen
- dark coloration of the skin in combination with fever and/or enlarged spleen
- a positive rK39 RDT in combination with fever and or enlarged spleen

PKDL:

- Patches or nodules anywhere on the skin, without loss of sensation with or without a history of kala azar

Leprosy:

- Patch with loss of sensation
- Loss of sensation of hands and/or feet
- Deformities of hands and/or feet (clawing of toes and/or fingers, wrist drop, foot drop, loss of fingers and/or toes)
- Eye complications in combination with any of the above signs and symptoms

Importantly, any suspect case of kala azar, PKDL or leprosy needs to be followed up. If you can do an rK39 on the spot for a VL suspect, do it and refer the patient for treatment if positive. Note down the contacts details of the patient or suspect case (phone number, name of ASHA, phone number of ASHA) and ensure that the patient/suspect case is further evaluated or treated.

Having completed the questions for all household members you will get to the end of the questionnaire, please tap on 'Save Form and Exit'. As soon as you have access to internet, please upload your form to the server just like you did earlier for the 'SPEAK_interview' app.

Appendix 4

SPEAK surveillance Standard Operating Procedure for previous kala azar patients

1. Enrollment in the study and registration:

For this part of the study you will receive a line listing of all kala azar patients registered in your Block in 2014, 2016 and 2018. These ex-patients are to be visited in their homes and interviewed. Also they and their household members should be screened for signs of PKDL, kala azar and leprosy.

We will use form 3 for informed consent for the interview and form 2, also used for new VL cases, as informed consent for screening of household members. To avoid possible stigmatization first ask the ASHA to contact the ex-patient and get his/her verbal consent for your visits. When you arrive at the house first explain to the ex-patient why you came and ask him/her if he/she agrees to cooperate. If so, ask to sign informed consent form 3 and interview the ex-patient using the SPEAK_Interview app as explained below. Already you may also prepare a copy of form 2 on which you will list all permanent household members, starting with the index case. Copy the form serial number (FSN) from form 2 to the informed consent for the patient, form 3, as you did for new patients.

2. Interview with previous kala azar patient:

As for new patients, we will use the app 'SPEAK_Interview'. The questions are all about the past episode of kala azar, if there were multiple episodes just focus on the most recent one.

In your apps, find the icon of ODK Collect 🗒 and tap on it to open it. Then tap on 'Fill Blank Form' and scroll to 'SPEAK_Interview'. Tap on it to open a form. The first question is to enter the FSN (form serial number), this is copied from form 2, 'Informed consent 2 Improving VL surveillance at PHC level (for VL and PKDL screening)', on which you have just entered name age and gender of each of the household members. In this example it is 653.

Fill in '653' as 'FSN' and press on the arrow pointing to the right to proceed.
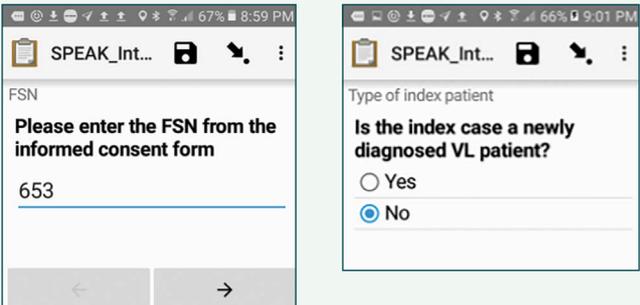
In the next question you are asked whether the patient is a newly diagnosed VL patient, here the answer is 'No'. Click on the arrow to proceed.

You will then be asked in which year the patients suffered from VL, let's assume it was in 2018.

Next you will be asked to fill in 'State', 'District', 'Block' and 'Village'. This is about the village in which the patient lives. If you select the right state, district and block, all the villages from that block should be listed. If you really cannot find the village, do not fill in anything and proceed. You will then be asked whether you found the village in the list, to which you should reply 'No' and proceed.

The next question will be the name of the missing village, enter the name and proceed. Please note that this is exceptional, normally all names of villages should be in the list.
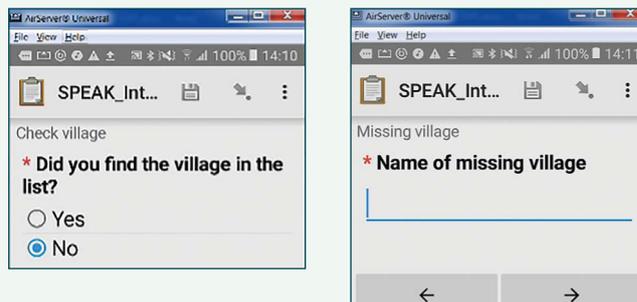
The next field is the date of visit, by default it is set to today. If you tap on the 'Select date' button, you will see three dates listed of which the date of today is in the center. Just tap 'OK' and proceed.

The next screen you get is about age and gender of the index case. Then you will get a screen about where the (ex)patient was treated. This refers to the first treatment taken after having been diagnosed with kala azar, only one option can be selected. In case the patient still took another treatment later for the same disease episode, just list here the place where he/she took his first specific kala azar treatment. There will still be a question later on whether or not he/she took further treatment(s).

The next question is on the type of treatment, tablets, injections or infusion(s), followed by a question on whether or not he/she knows the name of the treatment. If the answer to the latter question is 'Yes', you should fill in the name in the next field. Then there will be questions on the duration of treatment (how many days) and on whether or not the patient took all treatment prescribed. Next there is a question on the patient's state of health after the treatment, followed by a question on whether or not he/she experienced any side effects. Then there is a question on whether or not he/she took any further treatment for kala azar, so this refers to specific kala azar treatments after the first treatment for the kala azar episode. If the answer to that question is 'Yes' there will be a follow-up question on where that treatment took place, which type of provider.

The next questions are about treatment expenditures but these will only appear for patients treated in 2018 or later. As with the interview of new patients, it's probably best to take a piece of scrap paper to write down the expenditures before entering them in the app. You will be asked for the total expenditures in a separate question.

Finally there is the question on whether or not the ex-patient agrees to be contacted for a more detailed interview. Finally you will get to the screen shown opposite. Tap on 'Save Form and Exit'.
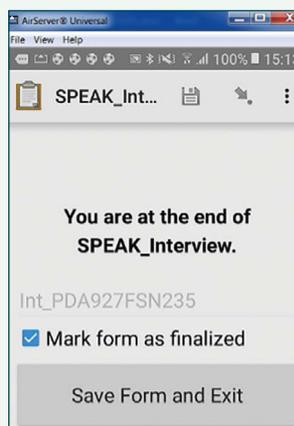
3. Screening for PKDL, kala azar, leprosy, malaria and tuberculosis:

Now explain to the household members present that you would like to screen them for screen the index case and all household members for kala azar and skin conditions related to kala azar and provide them with a copy of the information sheet (information sheet 4). Ask them to sign for informed consent on form 2, just as with the screening of household members of new cases. If they agree, ask for signs of kala azar and check for signs of PKDL or leprosy. If signs of any of these conditions are found, indicate so in the 'remarks' column of form 2.

Kala azar:

- fever of more than 2 weeks duration, not responding to anti-malaria treatment
- enlarged spleen/ abdominal swelling
- weight loss/ malaise in combination with fever and/ or enlarged spleen

- dark coloration of the skin in combination with fever and/or enlarged spleen
- a positive rK39 RDT in combination with fever and or enlarged spleen

PKDL:

- Patches or nodules anywhere on the skin, without loss of sensation with or without a history of kala azar

Leprosy:

- Patch with loss of sensation
- Loss of sensation of hands and/or feet
- Deformities of hands and/or feet (clawing of toes and/or fingers, wrist drop, foot drop, loss of fingers and/or toes)
- Eye complications in combination with any of the above signs and symptoms

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4. Data entry of screening results

The next step is to enter the data though the 'SPEAK_ Screening' app, just as you did for new kala azar patients. Please locate it on your tablet as shown below. The first thing you will be asked to enter is the FSN, which you should copy from Form 2.
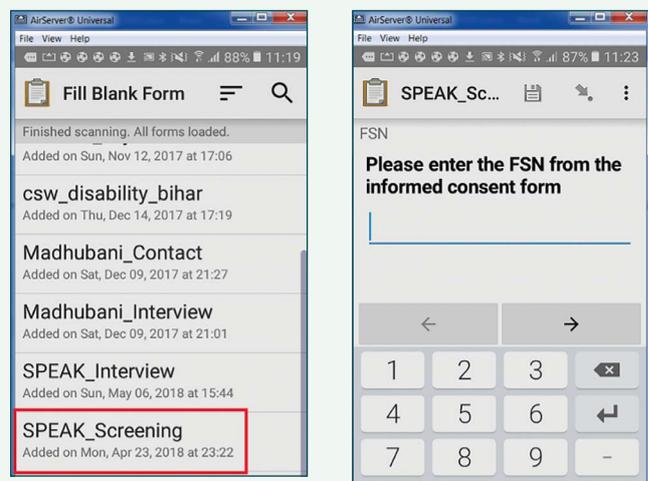
You will then be asked whether the index case is a newly diagnosed VL patient, to which the answer is 'No'.

Next you will be asked to enter State, District, Block and Village. As with the 'SPEAK Interview' app, there is the option not to select any village and manually type the village name in case it would be missing from the list. This should be exceptional though, normally the village has to be in the list. Please check carefully, perhaps the spelling is just slightly different from what you are used to.

The next thing to do is to record the geographic coordinates of the household. To do this you should be outside, with a clear view of the sky. When you tap on the button 'Start GeoPoint' your screen will change as shown below.

Either wait till you see the coordinates as on the screenshot below on the right side tap on 'Save GeoPoint' as soon as the accuracy is within 10m.

The next question is on the date of visit, select the date (set by default to today's date), tap on 'OK' and move on to the next question about the number of people in the household. Since you have already filled out form 2, you know exactly how may there are. Please fill out accordingly, you will then get a series of questions on each individual household member as shown in the screenshots below.

Start with the first person on the form, which is the index case. Fill in surname, name, gender and age, followed by the questions on whether he/she ever suffered from kala azar or PKDL. If you reply 'Yes' to the question on kala azar (VL), the next question that will pop up is whether this person is the index case. The answer here is 'Yes'. This is followed by a question on the year when the person suffered from VL.

The next question is on whether or not the person is present. The answer here is 'Yes'. The screens are shown in the figure opposite.
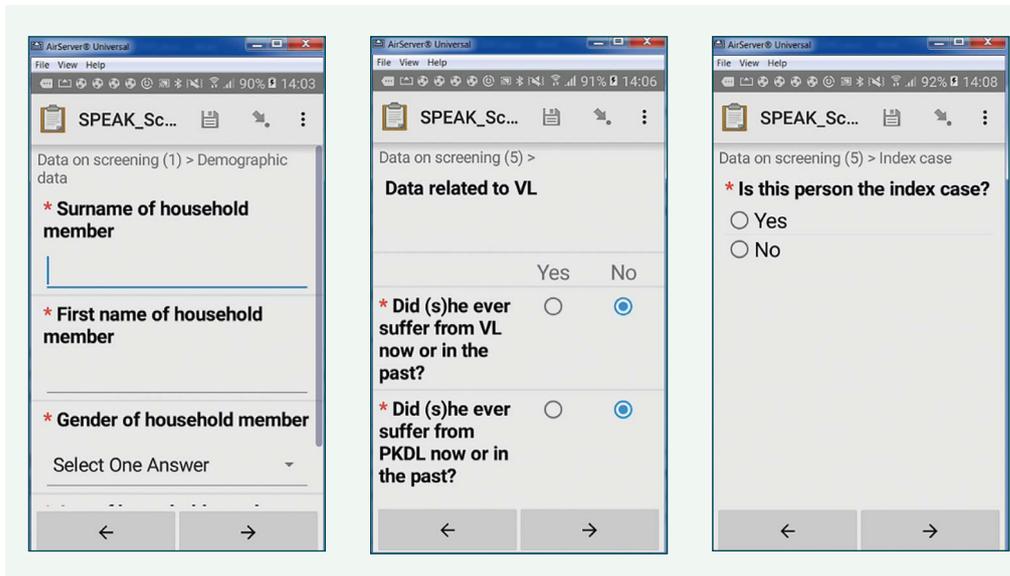
To the next question 'has (s)he been examined' you may answer 'Yes' if appropriate and then fill in the results of the examination for VL, PKDL and leprosy.

By default these are set to 'No', change as appropriate.
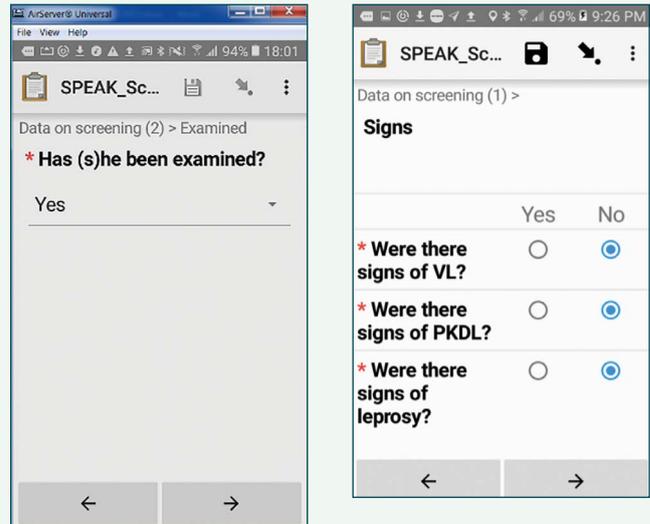
Tap on the arrow to the right and you'll be taken to the second household member. Usually this person has not suffered from VL in the past but in case he/she did have VL in the past, i.e. was a household contact of the index case, answer 'No' to the question on whether he/she was the index case. Per household there can be only one index case. Continue until all household members have been entered. Now you may save your form and exit.

Importantly, any suspect case of kala azar, PKDL or leprosy needs to be followed up. If you can do an rK39

on the spot for a VL suspect, do it and refer the patient for treatment if positive. Note down the contacts details of the patient or suspect case (phone number, name of ASHA, phone number of ASHA) and ensure that the patient/suspect case is further evaluated or treated.

When you have internet connection again, go to 'Send Finalized Form' and locate your two forms, 'Screen.....' and 'Int.....', select them and send them to the server. If you see the 'Success' message you may close your smartphone (image below).
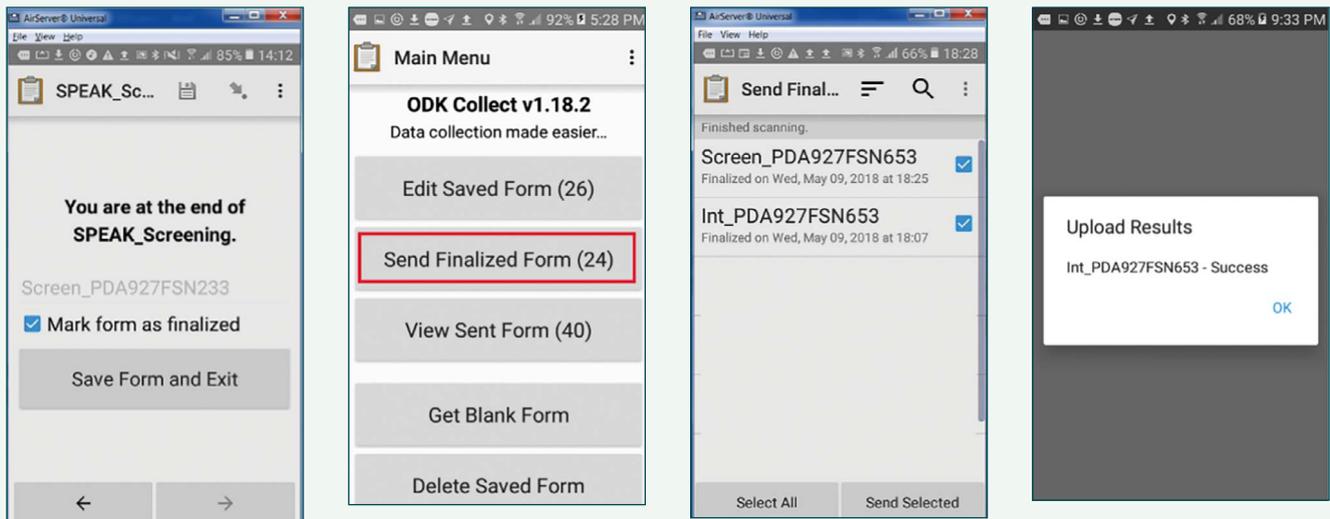
Appendix 5

SPEAK surveillance Standard Operating Procedures for the sero-survey

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1.   Enrollment in the study and registration:

This SOP describes the procedures of the SPEAK sero-survey. For this survey door-to-door screening is performed and all consenting permanent household members of 2 years of age and above are enrolled. For each subject a finger prick blood sample is collected on filter paper and data is entered through an Android app. Each filter paper has a unique barcode that needs to be scanned in the corresponding record in the app.

When you arrive at the household, first inform the inhabitants of the survey using the information sheet. If they agree, start filling out a copy of Form 1, shown in figure 1 below. First fill in the name of the Block and the village and the date. Write name, age and gender of the head of the household on row number 1. Then write down the names of all other permanent household members with age and gender on the rows below and ask all to sign for informed consent (except if they are minors) in the 'Consent' column (or put a thumb print if illiterate). For minors ask parents or guardians to sign, if the child is 12 years or older the child itself should also sign in the 'Assent' column. Record all permanent household members, also those who are temporarily absent. Use the 'Present' column to indicate whether or not they are present at the time of your visit. Ask whether any ever had kala azar in the past. If so, ask in which year the VL episode happened and ask for documentation, such as prescriptions. Make a note in the 'Remarks' column, e.g. 'KA 2015, no docs', as a reminder for when you enter the data in the app. In case a household has more than 18 permanent members you may use additional copies of form 1.

Form 1 : Informed consent 'Monitoring transmission of L.donovani'

(Sero survey)

Block: _____   Village:_____   FSN: __10017__   Date:____/_____/_____

	Name	Age	Sex	Present	Consent	Assent	Remarks
1							
2							
3							
4							
5							

Figure 1.
The ICF form (form 1).

Prepare a filter paper for each person present (ages ≥ 2 years) on which you write his/her name. Next collect a finger prick blood sample on the corresponding filter paper for each household member and allow it to dry. While the samples are drying, open the SPEAK_ SeroSurvey app to record the data.

2. Recording the data in the SPEAK_SeroSurvey app

In your apps, find the icon of ODK Collect and tap on it to open it. Tap on it to open a form, then choose 'Fill Blank Form' and select 'SPEAK_SeroSurvey' as shown in the images to the right.

Figure 2.   Opening the app.
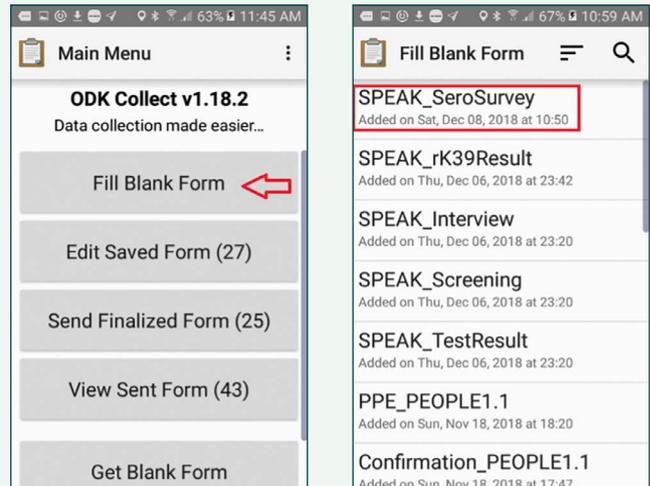
This will open the 'SPEAK_SeroSurvey' app. The first field will ask you to identify the cluster, let's assume in this case it was 'Fanda'. Select 'Fanda' as shown in figure 3 below and click on the arrow pointing to the right at the bottom of the screen to proceed.

Figure 3.   Selecting the cluster.
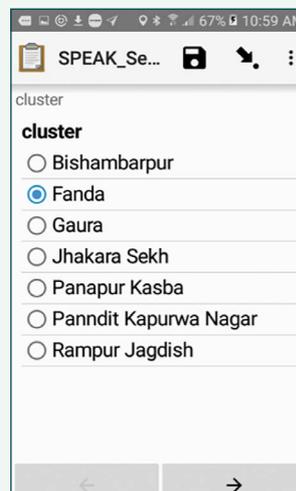
The next entry is form serial number (FSN), this is very important, it has to be correct. If not we will not be able to link the result of the screening test to the person. You will find it at the top of form 1 (see figure 1). The FSN in use are between 10000 and 20000, all other values will be refused.

Figure 4.   Entering the FSN.
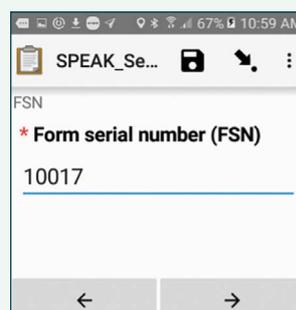
The next field is the geographic coordinates of your location. Use the 'Start GeoPoint' tab shown below to get them. This will only work when you are outside, with a clear view of the sky. If you tab the button, the device will start searching for the satellite, which may take a while. You will see the screen in the middle below. Once accuracy is within 5 meter it will automatically save the point and you will see a screen like the screen on the right. If it takes too long, you may press on 'Save GeoPoint' shown in the image in the middle as soon as the accuracy is within the 10 meter range.

Figure 5.
Recording the geographic coordinates.
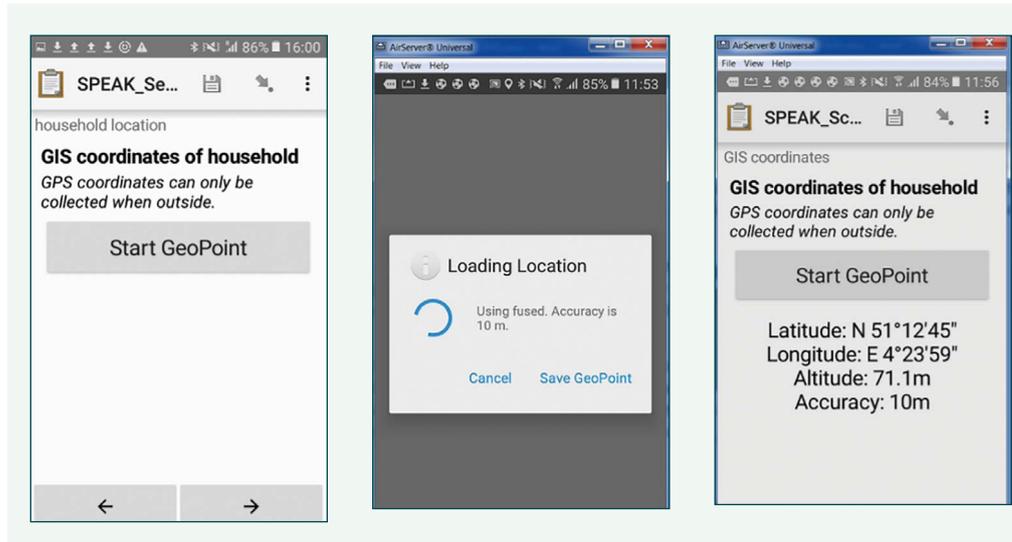
The next field is the date of visit, which should be the same as the date indicated at the top of form 1 (figure 1). This is followed by the number of household members. Include only permanent household members, even those that are not present the day of your visit. The total number has to be the same as the number indicated on form 1. If the household has more than 18 permanent members you will have used two (or more) copies of form 1. Just complete the registration in the app for the first form and then proceed wit the next.

Figure 6.   Date and number of household members.
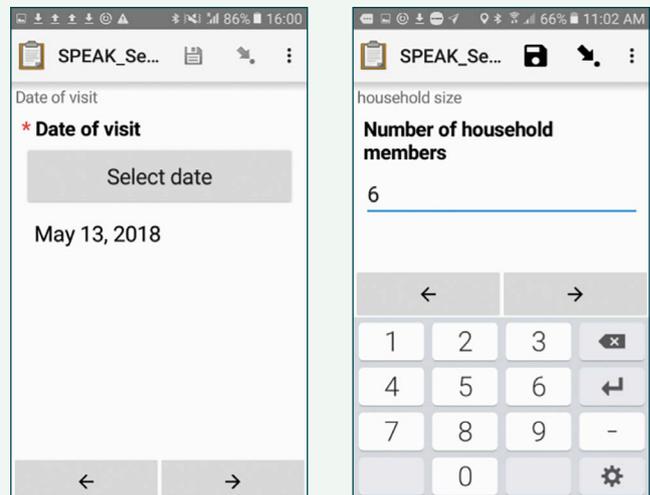
You will then be asked to enter for each individual the age of the subject and the gender.

In the next screen you will be asked whether or not the person is present, whether he/she ever suffered from kala azar (VL) and whether a filter paper has been collected.

In case you reply 'Yes' to the question on having suffered from kala azar, there will be additional questions about the year of disease and whether or not any documentation is available. The screens are shown in figure 7 below.

Figure 7.
Presence, history of VL and filter paper collected.
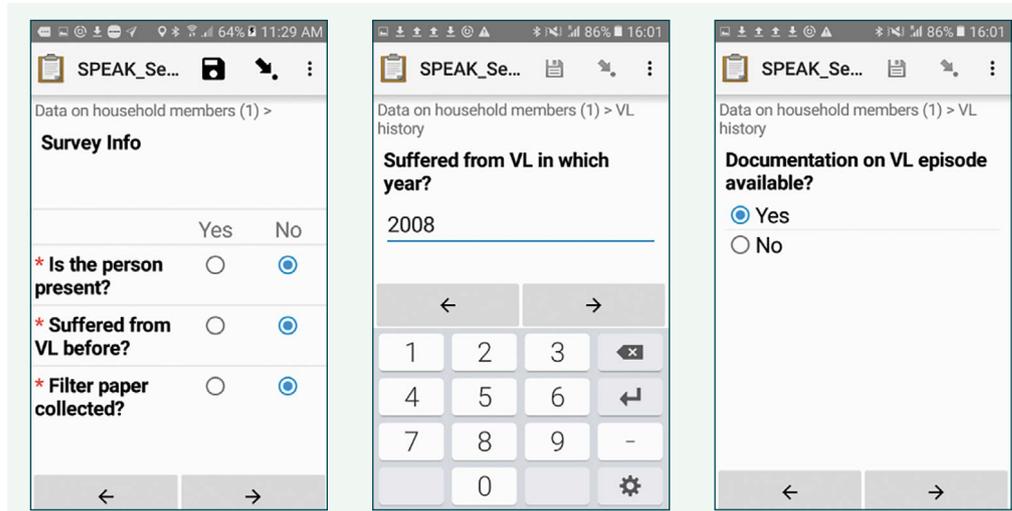
If you replied 'Yes' to the question on where or not a (blood sample on) filter paper was taken, you will be asked to scan the barcode. Make sure you scan the correct barcode by carefully comparing the name written on the filter paper to the one on form 1, in this case for the first household member. You can see which household member you are entering at the top of your screen. In this case it shows: 'Data on household members (1)>', so we are dealing with the first household member listed on Form 1.

Hold the lens of the phone above the filter paper and tap on the 'Get Barcode' button (figure 8).

Your screen will change and you will see a line that should be placed above the barcode as in the middle image below. Once it has captured the barcode the screen will change again into the image shown on the right. You can then proceed to the next household member.
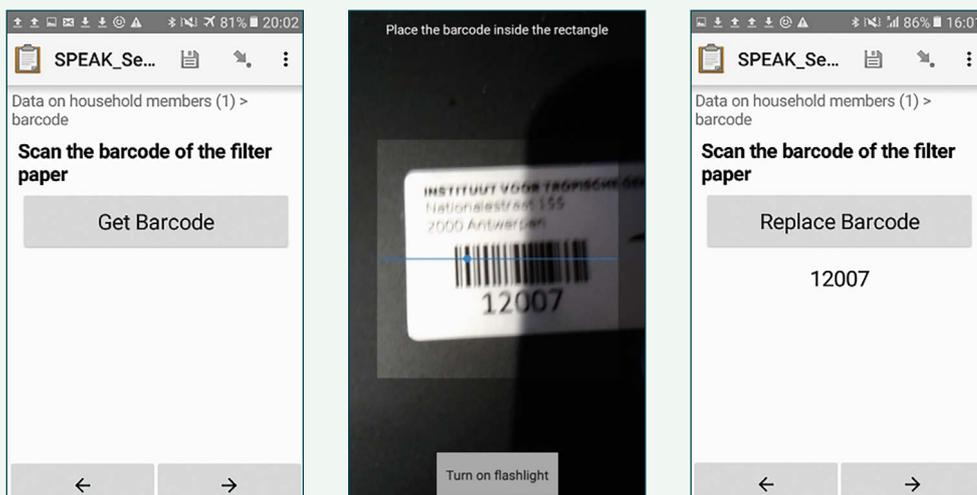
Figure 8.
Scanning the barcode.

For the next household member the questions will be just the same, this time at the top of your screen you will see: 'Data on household members (2)>', indicating that you are supposed to be entering the second household member on form 1. When you have entered all, you will see the screen in figure 9 below on the left. Just tap on 'Save Form and Exit'. This will take you back to the main screen of ODK (figure 9, screen on the right side).
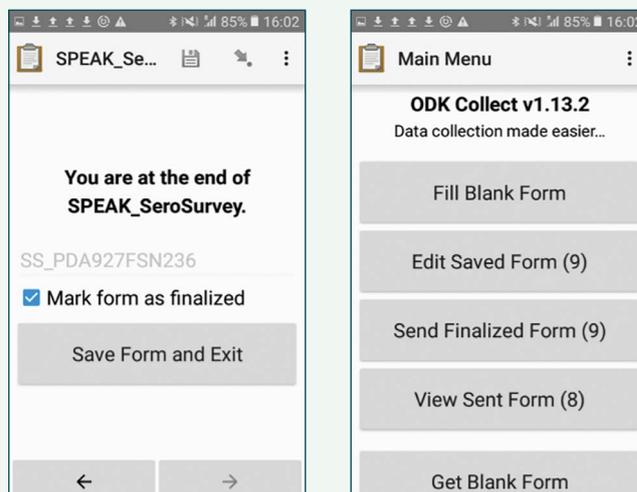
Figure 9.   Saving your form.

3.   *Sending the data to the cloud:*

When you have internet connection again, open ODK and from the main screen select the option 'Send Finalized Form'. If you tap on it you will get a list of froms to be sent. If you have no other ODK apps on your phone you will only see the survey forms, 'SS_PDA***FSN***'. You may select them all and then tap on 'Send Selected'. If this works well you should see a message that the xx forms have been sent successfully and the forms should no longer be visible on your phone. In case you have difficulty locating your forms, use the 　 icon at the top of your screen to sort your forms by: 'Date, newest first'.
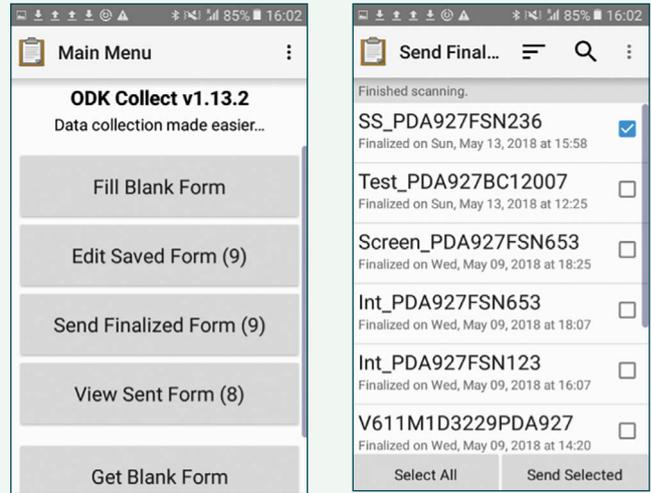
4. Further procedures:

All filterpapers are taken to the lab for testing with rK39 ELISA and DAT. The ICF sheets (form1) are entered in an MS Access database (Village, FSN and row number (1-18), name, age and gender of each subject). This will allow linking with the data collected through the app, provided FSNs are correct and individuals have indeed been entered in exactly the same order as the one in which they are written on form 1. The age and gender info is entered in the app as well as in the Access database which will allow to check whether data entry is correct and will allow to locate a record within an ICF (form 1), provided the FSN is correct.

The filter paper samples are all barcoded and all barcodes have been scanned in the app, allowing us to link the laboratory results to the data entered in the app. In the app we also have FSN and geographic coordinates, so we can plot a map of all households (one FSN is one household), allowing us to easily find back a household.

Anyone testing positive either on rK39 ELISA or on DAT has to be revisited to collect a venous blood sample for qPCR. For this purpose a list with name, age, gender, FSN and barcode needs to be prepared per cluster of all those who need to be revisited. Matching barcode stickers are to be prepared to label the new samples collected. Results are added to the MS Access database, with the corresponding barcodes.

Appendix 6

Standard Operating Procedures for collecting haematophagous insects

6A CDC MINIATURE LIGHT TRAPS

CDC Miniature Light Traps (CDC-LTs) were developed by the US Centers for Disease Control and Prevention (CDC) for surveillance of mosquitoes (especially *Culex* spp. and *Anopheles* spp.) but they are also very effective for collecting adult *P. argentipes*.[39,40,41] These lightweight, battery-operated traps (less than 1kg) are hung overnight at a distance above the ground suitable for catching appropriate insect species. For *P. argentipes,* CDC-LTs are commonly hung close to the ground due to the low flight height of sand flies. They use light to attract insects and once near enough, a fan draws them into a mesh sleeve and down into a collection pot. After collection, the sleeve is tied to prevent trapped insects from escaping.

Assembly of CDC-LTs is simple and quick. As produced by the manufacturer (John W. Hock), a 6 volt, 12amp DC battery is required for operation. It is important that fine mesh collection cups are used otherwise sand flies may escape through the wide mesh collection cups more commonly used for trapping mosquitoes.

For indoor collections, CDC-LTs should be installed inside of houses in a room where at least one person sleeps at night, under a bed net. Traps should be mounted 2 cm away from the wall and 2 cm above the ground, whenever possible, if the main goal is to collect sand flies. For mosquito collection, the CDC-LTs should be installed about 1 m from the ground.

To understand exophilic behaviour of different biting insects in relation to human infection, CDC-LTs should be installed on the veranda/in front of the house where household members sleep at night during warmer months (March – November). Traps should be installed near where household members sleep and at the same distance from the wall and height from the ground as with indoor traps.

CDC-LTs can also be installed in vegetation, cattle enclosures, or near other areas where sand flies likely rest and breed. Traps should not be set in locations where there is competing light, including moonlight.

SOP CDC Light Traps

Materials
☐ CDC miniature light trap (CDC-LT) (Supplier: John W. Hock, https://www.johnwhock.com/products/mosquito-sandfly-traps/cdc-miniature-light-trap/)
☐ 6 V 12 amp rechargeable battery (Supplier: John W. Hock – but such batteries can also be bought elsewhere)
☐ Battery charger (Supplier: John W. Hock)
☐ Spare light trap bulb
☐ Spare charged battery
☐ String
☐ Data collection form/device
☐ Pens/pencils
☐ Optional: CO_2 source (yeast, dry ice, etc.)

INSTRUCTIONS

Prior to collection: explain the study to the household occupants, describe the information on the informed consent form and obtain consent.

If members of the household will be required to help by switching on the trap at a certain time, make all the necessary arrangements and if possible, follow up with a phone call/message to check the trap is on and working properly.

1. Gather all trap components.

2. Make sure that each CDC LT is functioning before leaving for the field. This includes checking that each battery is charged and each trap is functional (the bulb works, the fan work, etc.).

3. Connect the collection cup to the trap bag. Place the end of the trap bag (without the elastic drawstring, from thicker material) over the open top of the cup. Push the cup ring over the sleeve down to the cup. Screw the ring firmly on top of the cup, attaching the sleeve to the cup.
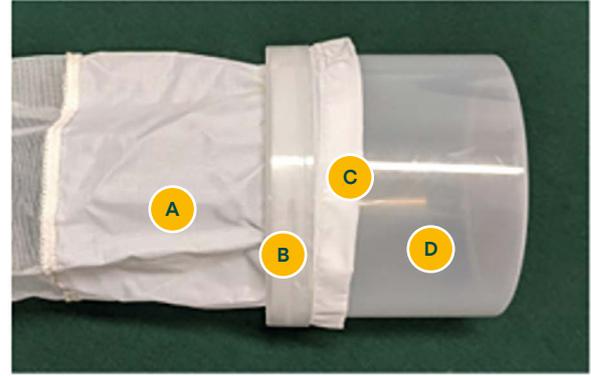
A. Sleeve
B. Cup ring
C. Collection cup
D. Thick end of collection bag should overhang all around the cup.

4. Assemble the trap body by connecting the metal brackets to the trap body with screws. The bracket does not need to be removed after each trapping session.

5. Connect the trap body to the plastic lid by using the black trap screw – attach from the top side of the lid (the lid should curve downwards). The string for hanging the trap should be attached to the screw.
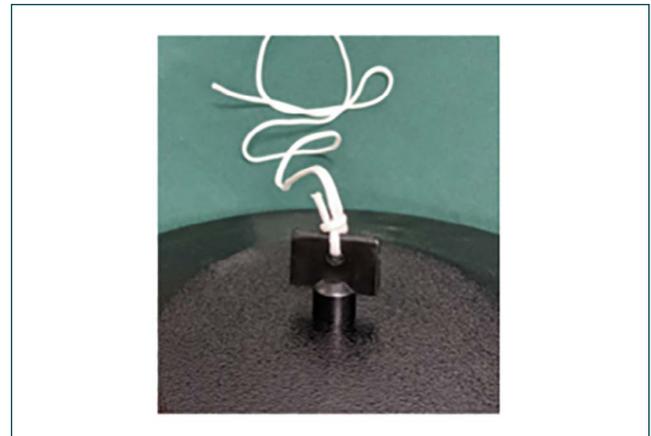
6. Connect the battery to the trap body to ensure that it works. The red cable end should be connected to the battery's red terminal and the black cable end should be connected to the battery's black terminal.

    ·   Check that the light bulb is working.

    ·   Check that the fan is working and that there is nothing obstructing it (there should be no loud buzzing noise).

7. Connect the trap bag to the trap body. Fit the elastic drawstring over the rubber ring at on the trap body and pull the drawstring tight so that the trap is secure.
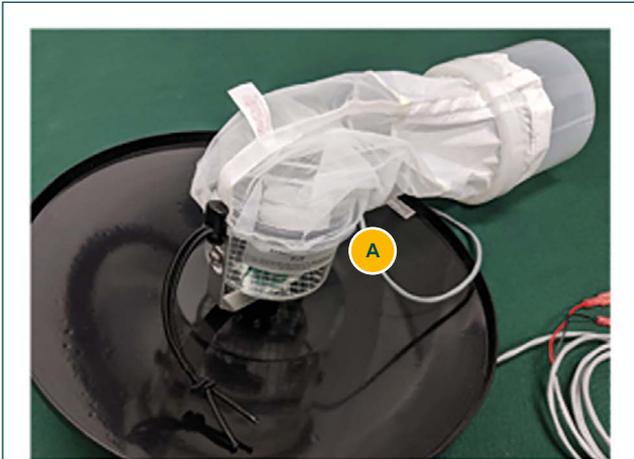
A. Elastic drawstring fitting over the black rubber ring.

8. At the time of collection, it is important to keep the CDC-LT running and the mesh trap bag attached before removing it from the trap body to prevent insects from rapidly escaping. Tap the trap bag to force any insects within it down into the collection pot, to reduce the potential loss of specimens above the tie-off area of the trap bag.

These steps are summarised in the figure below, and a checklist of materials to take to the field is also provided.

For further details see [42, 43].

APPENDIX A. ASSEMBLY AND USE OF CDC MINIATURE LIGHT TRAPS

ASSEMBLY OF CDC LIGHT TRAPS

Unscrew nut with attached string from light trap apparatus.

Screw the protective lid onto light trap, then screw string back onto light trap.

Fit collection sleeve onto the base of the light trap.

RUNNING CDC LIGHT TRAPS

Match alligator clips to the appropriate color on battery
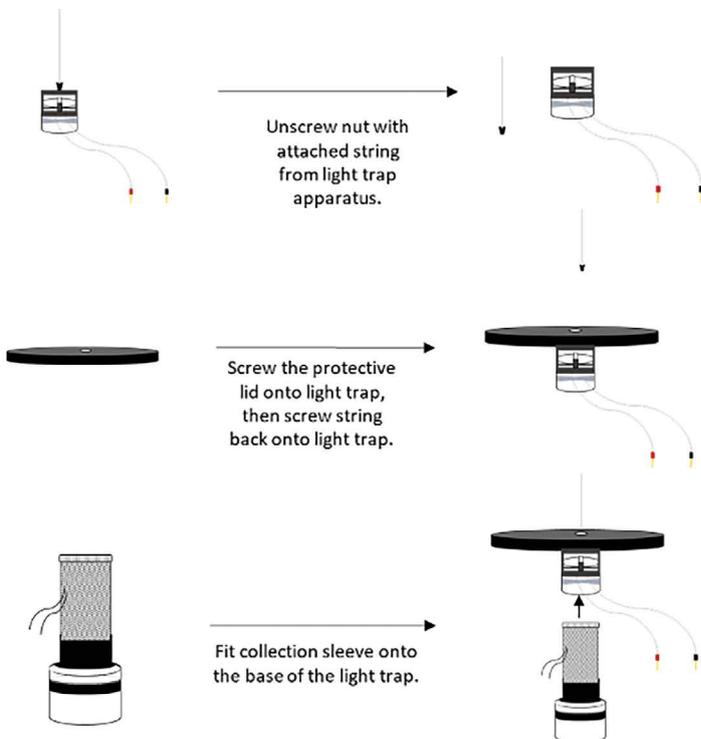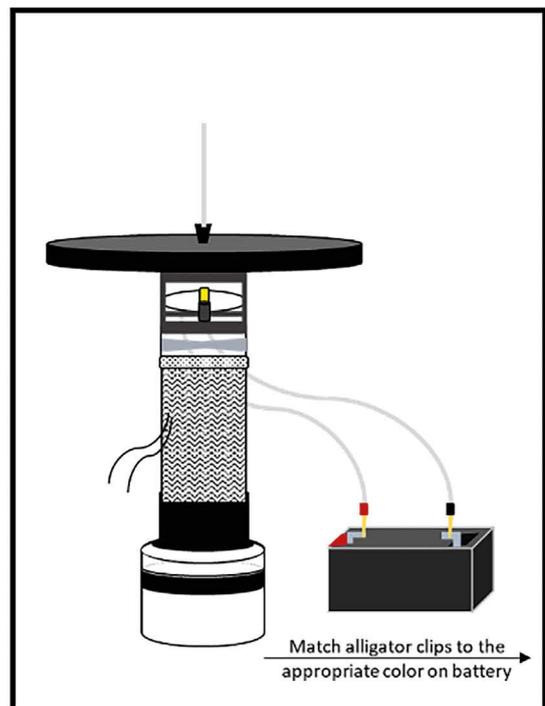
Example of Checklist for CDC Light Trap Sampling   (can be adapted according to context)

BEFORE INSTALLATION DAY

☐ Clean collection pots with warm soapy warm and leave them to dry facing downward.

☐ Wash mesh collection sleeves (if possible) and hang to dry at least overnight. If sleeves cannot be washed, invert and shake them rapidly to remove large debris. Manually remove smaller debris trapped in mesh.

☐ Brush the fan are of the CDC Light Trap to remove large debris. Wipe area clean with a damp cloth.

☐ Charge batteries (including a few extras) that will be used for field collection

☐ Arrange vehicles that will be needed for both days of field work.

☐ Put ice packs in the -20°C freezer.

☐ Assemble CDC Light Trap collection pots and sleeves.

☐ Charge and clear previous readings from data loggers.

☐ Inform ASHA and/or villagers of planned visit.

INSTALLATION DAY

☐ Check batteries to make sure all are fully charged.

☐ Check each light trap to make sure that all bulbs are working.

☐ Turn on data logger upon trap installation at household.

☐ Ensure all equipment needed is loaded into field vehicles.

☐ Ensure there is enough freezer space to fit collection pots.

EQUIPMENT NEEDED:
☐CDC Light Traps	☐Batteries	☐Labels/Tape	☐ Markers
☐Data Loggers	☐Storage Containers	☐Notebook and pen	

COLLECTION DAY

☐ Leave for field before 8AM.

☐ Ensure that all equipment needed is loaded into field vehicles.

☐ Turn off data logger upon trap uninstallation at household.

☐ Place collection pots in freezer immediately upon returning from the field.

☐ Store all field equipment

EQUIPMENT NEEDED:
☐Insulated Coolers	☐Ice packs	☐Storage Containers	☐Notebook and pen

6B ASPIRATOR COLLECTION

Aspirator collections are used for collection of resting, usually blood-fed and/or gravid sand flies and mosquitoes. Insects are sucked up rapidly and blown into a collection container. There are different types of aspirators, including mouth aspirators, battery-operated mechanical aspirators, backpack aspirators. The Prokopack is a type of battery powered aspirator.

They can be used both indoors and outdoors, for collection of insects resting in both natural and artificial shelters. This method allows collection of resting adults of both sexes, all physiological states, and will collect many species. Collections should take place during period of minimum flight activity. For *Aedes* mosquitoes, preferably from mid-morning to early afternoon. For

Anopheles mosquitoes, preferably in the early morning. For *P. argentipes* sand flies, preferably in the morning around dawn or dusk corresponding to their diurnal resting behaviour.

Aspirator collections should be standardized before collections start in terms of how long the collection should take per house. That can be more difficult outside, due to differences between resting sites. It should also be standardized in terms of where aspiration begins within the household and the path of aspiration along the walls. Aspirator collection should be standardized based on complete passes along walls. Aspiration can occur either with progressive down-and-up or side-to-side motions across the height and length of walls, covering each area once. Walls should be aspirated to a specified height, and the direction of aspiration should be consistent. Speed should also be consistent (e.g. 1m/second).
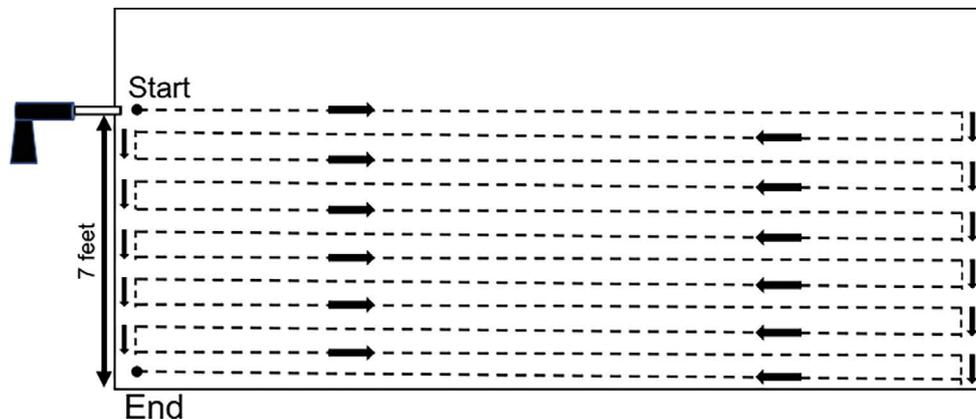
A possible collection scheme is to start a collection on the wall immediately to the right of the doorway of each household and continue in a clockwise direction around the room. Start at an agreed height (this will depend on the type of house) moving from the left side of the wall to the right side, gradually shifting downwards in a hairpin fashion at an aspiration speed of approximately 30 sec/m^2.

Aspirators can be noisy and cumbersome to operate. Especially use of larger aspirators such as backpack aspirators or Prokopack can be labour intensive and requires trained staff. It can be difficult to find resting insects. A torch or a headlamp should be used to illuminate dark areas where the insects may be resting. At times, two people are needed: one to perform the aspiration and another to remove objects in the aspiration path.

SOP Aspirator collection of indoor resting insects

Materials
☐ Aspirator (mechanical/backpack/Prokopack) (Prokopack supplier: John W. Hock, https://www.johnwhock.com/products/aspirators/improved-prokopack-aspirator/)
☐ Mouth aspirator (Supplier: John W. Hock, https://www.johnwhock.com/products/aspirators/mouth-aspirators/)
☐ Torch or headlamp
☐ Collection cups
☐ Rubber bands
☐ Cotton wool
☐ Netting/mesh for cups (e.g. supplier and product: Point North (International) Ltd; Insect Midge Net Fine, MM34-white)
☐ Falcon tubes (e.g. supplier and product: Fisher Scientific Ltd; Cat. No. 10788561)
☐ Batteries for torches
☐ Pencil/pen/marker
☐ Scissors
☐ Masking tape
☐ Timer
☐ Box or bag for transporting samples
☐ Data collection form/device

INSTRUCTIONS

Prior to collection: explain the study to the household occupants, describe the information on the informed consent form and obtain consent.

Start the collection at the appropriate time – depending on the type of insects targeted for collection (e.g. between 06:00 to 08:00 am if collecting *Anopheles* mosquitoes).

1. Gather all the components and organize equipment, including the collection cups/tubes.

2. Collect resting insects from each sampling point. With the aid of the torch, look for mosquitoes/sand flies on walls, ceiling, the roof, behind and under furniture. Insects seen flying around should also be collected, if possible. Mosquitoes could also be resting on wall hangings and curtains, and inside large pots and jars.

3. Search and collect systematically starting from the door and move clockwise around the inside of the house or room.

4. Aspiration can occur either with progressive down-and-up or side-to-side motions across the height and length of walls, covering each area once. Aspirator should be moved back and forth at approximately 1 meter per second.

5. Collection should be carried out for a fixed period in each house per collector.

6. After collection is completed, make sure that the collection cup is closed before the aspirator is switched off (Prokopack) or carefully transfer all the collected insects from the aspirator tube holding insects into the falcon tube, then cover it with netting. If insects need to be transferred into another cup, use the mouth aspirator. Label all the collection cups/tubes until processing and record the details in the form/using a collection device.

7. Repeat the sampling in the same selected households over 6 days.

   For further details see [44, 45]

6C CDC GRAVID TRAPS

CDC Gravid Traps (John W. Hock) are particularly good for collecting virus-positive mosquitoes as they were designed specifically to collect gravid *Culex* mosquitoes, which would have blood fed at least once, making the capture of mosquitoes harbouring arboviruses more likely. However, other insects will get trapped as well – including sand flies.[46]

The trap consists of an oviposition medium contained in a plastic bowl/pan below the trap. It operates by creating an upward current of air from within the confines of the pan, so that the mosquitoes are blown into the collection bag while they try to examine the oviposition medium before oviposition.

These traps are often used with an oviposition attractant – the hay infusion (made by adding hay to tap water and allowing the infusion to incubate for 5 days). The trap is placed at the desired collection site at least 1 hr before sunset and the bowl is filled with 4 L of infusion solution. Captured mosquitoes should be removed early the following morning to ensure maximum survival of the insects and any virus that may be present. New medium should be used each night.

The CDC Gravid Trap require a 6 volt, 12 amp DC battery.

These traps often catch other types of insects as well.

SOP CDC Gravid Trap

<div style="background:green;color:white;">

Materials

</div>

- ☐ CDC Gravid Trap (Supplier: John W. Hock, https://www.johnwhock.com/products/mosquito-sandfly-traps/cdc-gravid-trap/)
- ☐ Power cable (Supplier: John W. Hock)
- ☐ Double-ring collection bag (Supplier: John W. Hock)
- ☐ Oviposition pan (Supplier: John W. Hock)
- ☐ 6 V, 12 amp DC battery (Supplier: John W. Hock – but such batteries can be bought elsewhere)
- ☐ Battery charger (Supplier: John W. Hock)
- ☐ Pencil/pen/marker
- ☐ Box or bag for transporting samples
- ☐ Data collection form/device

INSTRUCTIONS

Make the hay infusion by adding 0.5 kg of hay to 114 L of tap water (adjust the quantities in this same ratio according to needs). Allow infusion to incubate for 5 days.

1. Gather all the components and organize equipment. Make sure the battery is charged before going to the field.

2. Set up the trap in the chosen location at least 1 hour before sunset:

    - Place the unit consisting of the aluminum supports and an ABS foam-core tube containing the suction motor on the rim of the oviposition pan. Make sure that the shorter tube without the large holes in the sides is protruding downward.

    - Slip the double-ring collection bag over the upright tube. Slide the sleeve downwards towards the aluminum supports until the "bottom" of the bag is resting on the top end of the trap.

    - Add 4 L of the oviposition medium (hay infusion) to the pan.

    - Connect the trap to a 6 volt DC battery. The red (copper) lead should be connected to the (+) and the black (tinned) lead should be connected to the (-) terminal on the battery.

If wrongly connected, the fan will move in the wrong, reverse direction and no insects will be collected.

A. Double-ring collection bag
B. ABS foam-core tube with suction motor
C. Aluminum supports
D. Oviposition pan
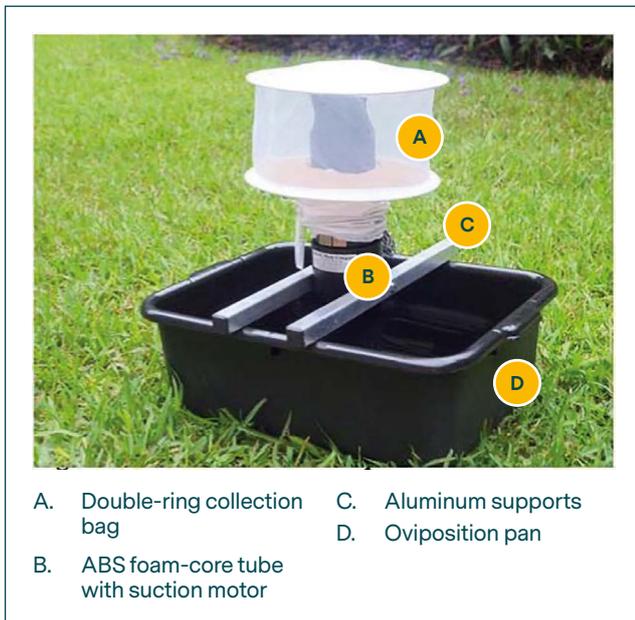
3. Remove captured insects early the following morning – especially if checking for the presence of arboviruses as this will ensure maximum survival of trapped insects and viruses that may be present.

The traps can be used outdoors, next to houses or even indoors. If used indoors, hay infusion should be replaced with tap water.

For further details see [23, 46-48].

6D BG-SENTINEL TRAPS

The BG-Sentinel trap from Biogents is a mechanical trap that employs visual cues to attract mosquitoes, which can be combined with olfactory lures (e.g. CO_2, BG-Lure®). When used with BG-Lure, which mimics the odour of human skin, it is supposed to be especially attractive to *Aedes aegypti* and *Ae. albopictus*. It is a good general mosquito trap when used with CO_2, which makes it attractive to *Culex* and *Anopheles* mosquito species. It captures mosquitoes of both sexes and all physiological stages.

The BG-Sentinel is a lightweight, collapsible fabric container with a white lid with holes covering a black entrance funnel in the middle. Air is sucked into the trap by a small electrical fan, drawing approaching mosquitoes into a catch bag.

This trap needs a 12V battery or mains power to operate. It can be deployed up to a week if running on mains but captured mosquitoes will degrade after a couple of days.

SOP BG Sentinel Trap

Materials

☐ BG-Sentinel trap (Supplier: Biogents, https://www.bg-sentinel.com/)
☐ Black entrance (intake) funnel (Supplier: Biogents)
☐ Entrance funnel net (Supplier: Biogents)
☐ Trap body (poles can be separate or a sort of frame already built in, depending on the version of the trap) (Supplier: Biogents)
☐ Catch bag (Supplier: Biogents)
☐ White gauze trap cover (Supplier: Biogents)
☐ Inner cylinder suction tube with a fan attached (Supplier: Biogents)
☐ Catch bag storage container (Supplier: Biogents)
☐ 12 V battery (Supplier: Biogents)
☐ Battery cable (Supplier: Biogents)
☐ Battery charger (Supplier: Biogents)
☐ BG-Lure cartridge (optional) (Supplier: Biogents)
☐ Pencil/pen/marker
☐ Labels
☐ Data collection form/device

INSTRUCTIONS

Before going to the field make sure the battery is charged.

Check with the members of the household that they are happy with the trap location.

1. Remove the trap components from the bag.

2. Unfold the trap and assemble according to the instructions that came with the trap as different versions of BG-Sentinel traps differ slightly in their construction.

❶	Black Catch Pipe
❷	Funnel Net
❸	Catch Bag (x2)
❹	White Gauze Cover with Drawstrings
❺	White plastic trap
❻	Mounting Poles
❼	Mesh Pouch for Lures
❽	Inner Suction Tube
❾	Fan
❿	Fan Cables
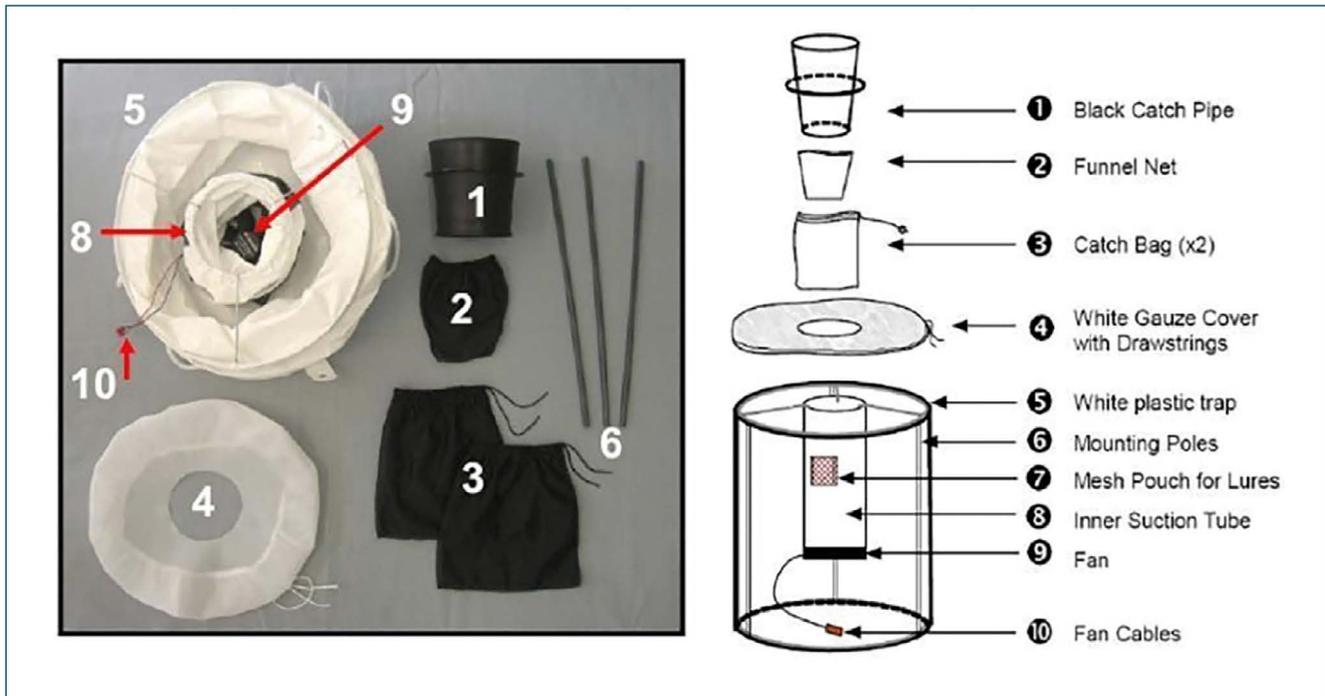
The current BG-Sentinel Manual can be downloaded from the BioGents website at https://www.bg-sentinel.com/downloads/BG-Sentinel_Manual.pdf or https://www.bg-sentinel.com/downloads/BG_Sentinel_2_Manual_EN_web.pdf and an alternative set of instructions can be found at PacMOSSI Consortium's website at https://pacmossi.org/carbon/assets/2022/06/PacMOSSI-SOP_BG-Sentinel-Assembly-and-Deployment.pdf

3. Make sure that the intake funnel, funnel net and catch bag are correctly assembled and inserted into the trap body.

4. Make sure the battery cable is correctly connected to the fan cable. The battery can be placed inside the body of the trap (especially on windy days so the trap does not get blown over).

5. Test the trap – when powered, the fan can be heard, and the lid should tilt open.

6. Location of the trap will greatly affect its capture rate. Things to consider when choosing the location:

   · Place the trap on the ground.

   · Location should be sheltered from wind, rainfall and direct sunlight.

   · Place the trap close to preferred mosquito resting areas, such as bushes or foliage.

   · The trap works on the basis of visual clues, so should not be hidden in the bushes or tall vegetation and should not be surrounded by many objects.

   · Trap should be placed near the areas where productive larval habitats are likely to be present.

   · Trap should be near areas used by people (e.g. sitting areas where people gather).

   · The trap can also be used indoors.

7. Before switching off the trap remove and close the catch bag currently in use. This will ensure that caught mosquitoes cannot escape.

For further details see [49, 50].

Appendix 7

SOP Sorting and Pooling of Collected Insects Protocol

Materials and Supplies
☐ Petri dishes, glass or plastic (e.g. supplier and product: Fisher Scientific Ltd.; Cat. No. 11339283)
☐ Forceps (e.g. supplier: Watkins & Doncaster, entomological forceps, https://www.watdon.co.uk/acatalog/Forceps2.html)
☐ Brushes (e.g. supplier: Watkins & Doncaster, paint brush size 0, https://www.watdon.co.uk/acatalog/T1260-paint-brush-size-0.html#SID=232)
☐ Frozen ice packs
☐ Plastic or enamel trays/shallow boxes of different sizes so they can be nested
☐ Eppendorf/1.5 ml tubes (e.g. supplier and product: Scientific Laboratory Supplies; Cat. No. E0030108051)
☐ Cryobox(es) for tube storage (e.g. supplier and product: VWR International; Cat. No. AXYGTR8300-A)
☐ A4 paper
☐ Insulated cooler
☐ Ethanol resistant marker
☐ Dissecting microscope/USB microscope
☐ Adjustable desk lamp
☐ Power strip with sockets
☐ Nitrile gloves
☐ Lab coat

Traps used for insect collections

Indoors

- CDC light traps – 6pm to 6am indoors
- Prokopack/mechanical aspirator – 1 hour per household

Outdoors

- CDC gravid traps – daily placed in the morning when the CDC light trap is collected and removed the following morning (24 hrs)

- BG Sentinel traps – daily placed in the morning when the CDC light trap is collected and remove collected insects the following morning (24 hrs collection)

INSTRUCTIONS

1. Place ice packs in a larger plastic tray, then place the smaller A4 tray with paper on top. While DNA is a very stable molecule, it is best to keep specimens cool through cold chain storage until molecular analysis.

2. Empty the traps as per standard protocol.

3. Kill collected insects using chloroform or freezer.

   · If sorting and RPA are to be done in the field, kill insects in the field just before sorting.

   · If sorting and RPA are to be done in the local laboratory, bring live insects to the laboratory and kill the insects just before the sorting.

4. If freezer is used make sure insects are left in it for long enough so they do not start waking up during sorting – or work in batches. Remove one trap collection pot at the time from the -20°C freezer and place contents in A4 tray by inverting the pot firmly. This will keep specimens contained within the tray area.

5. Sort insects by trap type. Discard males but record how many were collected. Keep female mosquitoes and sand flies and females of other blood feeding insects (such as midges).

6. Group the females by insect genus: *Anopheles, Culex, Aedes* mosquitoes, 'other culicines' (=group any other culicine genera together), *Phlebotomus argentipes* sand flies and 'other sand flies' (group *Sergentomyia* spp. together), and 'other blood feeding insects' (=group any other blood feeding insects together).

7. Sort each of the insect groups by physiological stage:

   · Blood fed (freshly fed) and half fed

   · Gravid

   · Unfed

8. Create a pool of 10 insects belonging to the same genus and the same physiological stage in a 1.5 ml Eppendorf Tube. If any remain and are <10, create a smaller pool.

9. Label the tube pool using an agreed coding system that may use bar codes or QR codes.

10. Proceed with **SOP Crude Nucleic Acid Extraction.**

Figure 2x. Sorting of Phlebotomus sand flies by sex.

Enter collection-related data in a spreadsheet or database (Appendix 7).

Appendix 8

Standard Operating Procedure Crude Nucleic Acid Extraction

Reagents and equipment

- ☐ Potassium Hydroxide (Merck, Product Number: 757551)
- ☐ Trizma Base (Merck, Product Number: T1503)
- ☐ Glacial Acetic Acid (Merck, Product Number: A6283)
- ☐ Poly(ethylene) glycol (Merck, Product Number: P2139)
- ☐ Distilled water
- ☐ PCR clean, Eppendorf tubes (1.5ml or 2ml)
- ☐ Plastic pestles (sterile) (e.g. supplier and product: Fisher Scientific Ltd: Cat. No. 13236679)
- ☐ Battery-operated pestle-holder grinder (e.g. supplier and product: Merck Life Science Limited: Cat. No. Z359971-1EA, manufactured by Kimble)
- ☐ RLT buffer (Qiagen, Product Number: 79216)

Reagent Preparation

Create 2M stock of Potassium Hydroxide (40 ml)

- ☐ Dissolve 4.49 grams of Potassium Hydroxide in 40 ml of distilled water

Create 2M stock of Trizma Base (40 ml)

- ☐ Dissolve 9.69 grams in 40 ml of distilled water

Create 2M stock of Acetic Acid (40 ml)

- ☐ Add 4.6 ml of Glacial Acetic Acid (17.4 M) to 35.4 ml of distilled water

Crude Lysis Buffer (60 ml)

- ☐ 3 ml of 2M Potassium Hydroxide
- ☐ 15 grams of Poly(ethylene) glycol
- ☐ Top up to 60 ml with distilled water

Crude Neutralisation Buffer (20 ml)

- ☐ 10 ml of 2M Trizma Base
- ☐ 7.5 ml of 2M Acetic Acid
- ☐ 2.5 ml of distilled water

PROTOCOL

1. Create a pool of 10 insects in a 1.5 ml Eppendorf Tube. (**See Pooling of Collected Insects SOP**).

2. Add **200µl of Crude Lysis Buffer** to the Eppendorf Tube, ensuring all insects are fully submerged. Push insects to the bottom of Eppendorf tube using the plastic pestle.

3. Manually homogenise the insects using the pestle – or use the battery-operated grinder which works with the pestles. On completion seal the Eppendorf tube and mix the solution through inverting the Eppendorf tube. Subsequently leave the lysis reaction for **30 minutes.**

> ! *Ensure all insects are homogenised.*

> ! *Following homogenisation, wash the pestle in 100% ethanol followed by water.*

4. Add **67 µl of Crude Neutralisation Buffer** to the 1.5 ml Eppendorf tube to stop the lysis reaction. Subsequently seal the Eppendorf tube and mix via inversion.

5. Split the final solution into two aliquots (aliquot A with 200 µl, Aliquot B with 60 µl). Use aliquot A for RPA reaction and Qiagen extraction and freeze Aliquot B for later use.

Detection of RNA viruses Only

For detection of Dengue virus or other RNA viruses add 600 µl of **RLT buffer** (from the RNAeasy Miniprep Kit) to aliquot A just after the RPA reaction. This aliquot will subsequently be used for Qiagen-based RNA Extraction. *This is to prevent RNA degradation during transport and storage.*

Footnotes

Poly(ethylene) glycol enhanced alkaline lysis has been previously validated and combined with PCR.[22]

- Whilst we have validated the use of 200 µl of Crude Lysis Buffer for pools of 10 mosquitos, this volume could potentially be reduced to minimise the dilution factor introduced, however the subsequent impact of assay sensitivity remains to be tested.

- To stop the lysis reaction, the crude neutralisation buffer should be added to the crude lysis buffer at a ratio of 1:3 (E.g. 50 µl : 150 µl).

- Whilst we have validated incubating the lysis reaction for 30 minutes at room temperature (22°C), theoretically this can be shortened but the impact on assay sensitivity needs to be gauged.

Appendix 9

Standard Operating Procedure Recombinase Polymerase Amplification

Decontamination
☐ Prepare master mix without primers/ probes in advance (as table below 2)
☐ Prepare all master mixes in a negative pressure fume hood.
☐ Always use a new aliquot molecular grade water when preparing master mixes.
☐ Decontaminate workstation and pipettes using DNA / RNA eliminator and / or UV light.
☐ Change gloves regularly.

Reagents
☐ Primer and Probes (Integrated DNA technology)
☐ TwistAmp® Basic (TwistDx, Product Code: TABAS03KIT)
☐ Endonuclease IV (New England Biolabs, Product Code: M0304L)
☐ MMLV reverse transcriptase (New England Biolabs, Product Code: M0253L)
☐ Molecular grade water
☐ PCR clean, Eppendorf tubes (1.5ml or 2ml)
☐ PCR Tubes & Caps, Rnase-free, 0.2 mL (8-strip format): **Catalog number:** AM12230
☐ PCRD Lateral Flow Cassette (Abingdon Health, Product Code: FG-FD51673)
☐ PCRD Buffer (Abingdon Health, Product Code: FG-FD51673)

Pathogen species	Forward Primer sequence	Reverse Primer sequence	Probe sequence
Plasmodium species (Detects any human malaria parasite)	GAGTCGATCAGGAAGGTTT CATCCTTAAAT	/5Biosg/GTCTCTATGCCTTGA ATGGAGCACTGGATTGG	/56-FAM/GTTAAGGTGCTCAG GGTCTTACCGTCGGGCCGTA T/idSp/ATTCCACATA/3SpC3/
Leishmania donovani	ACCTATGCATGTTTGATTAC CAGAA	/5Biosg/CCTGTATGTATAACA GATCAATTTG	/5DigN/TGAATTGAGTGTATT GTTAGCAAG/idSp/GTGGTA TTAA/3SpC3/
Dengue 1-3 serotype	CTCCCTTACAAATCGCAGC AACAA	/5Biosg/GATTCAACAGCACC ATTCCATTTTC	/56-FAM/TCCTGCTGTCTCC TCAGCATCATTCCAG/idSp/ CACAGAACGCC/3SpC3/
Dengue 4 serotype	GGGGCCCGATGCCAGGAG GAAGCTGTA	/5Biosg/CCTGTTGGATCAAC AACACCAATCCATC	/5DigN/CAGCAGGATCTCTG GTCTTTCCCAGCG/idSp/CA ATATGCTGTT/3SpC3/
Wuchereria bancrofti (Lymphatic filariasis)	TTATTTAGTATTTTGATCATC TGGGAACG	/5Biosg/GCCACTATTCAAGCA CCTTAAATCTGTC	/5Biosg/GTTAGAGTTATCGA TTCAATTTCTGTGCT/idSp/ AATTTTTGTGGA/3SpC3/

Reagent Preparation

Important: When preparing reagents, it is essential to use fresh molecular grade water which is DNA/RNA free.

a.   Primer-Probe Combinations to select for each RPA reaction.

Pathogen	Standard primer	Biotinylated primer	Probe	Tag	Cassette band
Plasmodium spp.	Pan_RPA_FP	Pan_RPA_Bio_RP	Labelled_Pan_ FAM PR	FAM	2
Leishmania donovani	Ld kinetoplast _FP	Ld kinetoplast _Bio_RP	Ld_kinetoplast _DIG_PR	DIG	1
Dengue 1-3 serotype	Dengue1:3_FP	Dengue 1:3_Bio RP	Dengue1:3_FAM_ PR	FAM	2
Dengue 4 serotype	D4_FP	D4_Bio_RP	D4_DIG_PR	DIG	1
Wuchereria bancrofti	Wuch_FP	Wuch_Bio_RP	Wuch_DIG_PR	DIG	1

b.   Primer-Probe 10x Master Mix (4 μM Primer and 2 μM Probe).

Each primer and probe stock provided is pre-diluted to a 100 μM concentration using molecular grade water.

To prepare a 10x Primer-Probe Master Mix for a single pathogen:

Primer – probe (10x master mix)	
Standard primer (100 μm)	4 μl
Biotinylated primer (100 μm)	4 μl
Probe (100 μm)	2 μl
Molecular grade water	90 μl

Note. This mix can be prepared in advance and stored at -20ºC when in the lab. When transporting to field site maintain cool-chain and use within 24hrs.

c)   RPA Enzyme Master Mix

This master mix can be prepared in advance and stored at -20ºC when in the lab. When transporting to field site maintain cool-chain and use within 24hrs.

·   **DNA Pathogen** (*Plasmodium, Leishmania* or *Wuchereria*). Prepare the RPA reaction master mix according to the number of reactions necessary. One pathogen per reaction (single plex)

DNA detection: RPA Enzyme Master Mix	1 reaction	10 reactions
Rehydration Buffer	29.5 μl	295
Endonuclease IV	3 μl	30
Molecular grade water	8 μl	80

Freeze until necessary to use.

·   **RNA Pathogen Detection** (Dengue1-3 or Dengue4). Prepare the RPA reaction master mix according to the number of reactions necessary. One pathogen per reaction (single plex)

RNA detection: RPA Enzyme Master Mix	1 reaction	10 reactions
Rehydration Buffer	29.5 µl	295
Endonuclease IV	3 µl	30
MMLV reverse transcriptase	2.5	25
Molecular grade water	5.5 µl	55

Freeze until necessary to use.

PROTOCOL

1. Add **40.5 µl of RPA Enzyme master mix** to each **RPA lyophilised pellet tube**. Upon addition, seal the reaction tubes and invert manually to ensure the pellet is fully dissolved.

2. Add **5 µl** of **Primer-Probe 10x Master Mix.**

3. Open tube and add **2 µl of the appropriate sample.** (Crude Nucleic Extract or Qiagen-cleaned DNA).

4. Add **2.5 µl of Magnesium acetate** and mix via pipetting.

5. Incubate the reaction at **39°C for 45 minutes.** For field-based RPA utilise the heating block in the portal lab, or using human body temperature. (Make sure to record what heating method was used).

6. Add **80 µl of PCRD Buffer to a clean 1.5 ml Eppendorf Tube.**

7. Add **10 µl of RPA reaction to the Eppendorf** tube and mix by pipetting up and down.

8. Subsequently **add 75 µl of the mixed solution into the PCRD Lateral Flow cassette.** Wait **15 minutes** and record the presence of any bands.

Footnotes

On the PCRD Lateral Flow Cassette. Line 1 = DIG Probe and Line 2 = FAM Probe.

When in the field keep the RPA Lyophilised Pellets, Endonuclease IV and MMLV cold.

Positive and negative control.

Appendix 10

Standard Operating Procedure Multiplex real time qPCR reactions for DNA Pathogen

DNA Pathogen (Plasmodium, Leishmania donovani or Wuchereria bancrofti).

Reagents

- ☐ TaqPath ProAmp Master Mix (Thermo Fisher, A30865)
- ☐ PrimeTime Standard (Primer-Probe Combinations 1-tube) (Integrated DNA technology)
- ☐ Molecular grade water
- ☐ 1.5 PCR clean, eppendorff tubes
- ☐ qPCR plates and optic covers (dependent on qPCR instruments used)

Pathogen species	Forward Primer sequence	Reverse Primer sequence	Probe sequence*
Pf **Plasmodium falciparum**	CCGACTAGGTGTTGGATGAAAGTGTTAA	AACCCAAAGACTTTGATTTCTCATAA	Cy5-AGCAATCTAAAAGTCACCTCGAAAGATGACT-BHQ2
Pv **Plasmodium vivax**	GCTTTGTAATTGGAATGATGGGAAT-	ATGCGCACAAAGTCGATACGAAG-3	56-FAM/AGCAACGCTTCTAGCTTA/3IABkFQ
Ld **Leishmania donovani**	GCGACGTCCGTGGAAAGAA	GGCGGGTACACATTAGCAGAA	56-FAM/CAACGCGTA/ZEN/TTCCC/3IABkFQ/
Wb **Wuchereria bancrofti**	ATTTTGATCATCTGGGAACGTTAATA	CGACTGTCTAATCCATTCAGAGTGA	Cy5-/ATCTGCCCATAGAAATAACTACGGTGGATCTCTG/BHQ2

* There is a wide selection of possible dyes that can be used, please check which excitation and detection wavelength are compatible with the instrument light source and detection system in place.

Dilute PrimeTime Standard stocks by adding 500ul of water (20Xdilution).

PROTOCOL

1. **Testing only 1 pathogen.** Prepare the qPCR reaction master mix according to the number of reactions necessary.

Reagents	1 reaction	50 reactions
Taqpath Proamp master mix	10 µl	500
Primer probe assay	0.9 µl	45
Molecular grade water	7.1 µl	355

2. **Testing 2 pathogens.** Prepare the qPCR reaction master mix according to the number of reactions necessary.

Reagents	1 reaction	50 reactions
TaqPath Proamp master mix	10 µl	500
Primer probe assay FAM*	0.9 µl	45
Primer probe assay CY5*	0.9 µl	45
Molecular grade water	6.2 µl	310

* Use dyes that have excitation and detection wavelength compatible with the instrument light source and detection system in place. Combine dyes which have different excitation and detection wavelength.

· Add 18 ul of the master mix to each qPCR tube.

· Add 2 ul of sample and mix by pipetting up and down.

· Prepare a negative control by adding 2 ul of water instead of sample.

· <u>Always do the positive control last to avoid contaminations.</u>

· Spin down.

Run the real – time PCR instrument with the following programme:

Step	Temp	Time	Cycles
Enzyme Activation/ denaturation	95˚C	10min	1
		For 40 cycles:	
Denature	95˚C	15 seconds	For 40 cycles
Anneal/ extend (capture data)	60˚C	45 seconds	

Analyse results. Confirm if negative control is negative (Ct = not detected or 0) and positive control has a Ct results. Manually inspect the curves for positive CT values. Save plate layout, raw fluorescent results, and Ct Values.

Appendix 11

Standard Operating Procedure Multiplex real time qPCR reactions for RNA Pathogen

RNA Pathogen (Dengue 1-4)

Reagents

- ☐ Quantinova Probe RT-PCR kit (Qiagen, 208352)
- ☐ PrimeTime Standard (Primer-Probe Combinations 1-tube) (Integrated DNA technology)
- ☐ RNAase free water
- ☐ 1.5 PCR clean, eppendorff tubes
- ☐ qPCR plates and optic covers (dependent on qPCR Thermocycler used)

Pathogen species	Forward Primer sequence	Reverse Primer sequence	Probe sequence*
Dengue All serotypes	AGGACYAGAGGTTAGAGGA GA	CGYTCTGTGCCTGGAWTGAT	56-FAM ACA GCA TAT TGA CGC TGG GAR AGA CC 3IABkFQ/
Dengue 1-3	GARAGACCAGAGATCCTG CTGTCT	ACCATTCCATTTTCTGGCGTT	Cy5-/ AGCATCATTCCAGGCAC/ BHQ2

* There is a wide selection of possible dyes that can be used, please check which excitation and detection wavelength are compatible with the instrument light source and detection system in place.

Dilute PrimeTime Standard stocks by adding 500ul of water (20Xdilution).

Decontamination

- ☐ Prepare master mixes in advance (Prior to sample processing).
- ☐ Prepare all master mixes in a negative pressure fume hood.
- ☐ Use **fresh** molecular grade water when preparing master mixes. Aliquot and store molecular grade water at -20oC once opened to prevent microbial growth.
- ☐ Decontaminate workstation and pipettes using DNA / RNA eliminator and / or UV light.
- ☐ Change gloves regularly.

PROTOCOL

1. **Testing 2 pathogens.** Prepare the qPCR reaction master mix according to the number of reactions necessary.

Reagents	1 reaction	50 reactions
2x Probe RT-PCR mix	10 µl	500 µl
Qn Probe RT- Mix	0.2 µl	10 µl
qn rox reference dye	0.1 µl	5 µl
Primer probe assay FAM*	0.9 µl	45 µl
Primer probe assay CY5*	0.9 µl	45 µl
RNAase free water	5.9 µl	295 µl

* Use dyes that have excitation and detection wavelength compatible with the instrument light source and detection system in place. Combine dyes which have different excitation and detection wavelength.

- Add 18 ul of the master mix to each qPCR tube.
- Add 2 ul of sample and mix by pipetting up and down.
- Prepare a negative control by adding 2 ul of water.
- Always do the positive control last to avoid contaminations.
- Spin down.

Run the real – time PCR instrument with the following programme:

Step	Temp	Time	Cycles
RT step	45˚C	12 min	1
Enzyme activation/ denaturation	95˚C	5 min	1
Denature	95˚C	5 seconds	For 40 cycles
Anneal/ extend (capture data)	60˚C	40 seconds	

Analyse results. Confirm if negative control is negative (Ct = not detected or 0) and positive control has a Ct results. Manually inspect the curves for positive CT values. Save plate layout, raw fluorescent results, and Ct Values.

Dengue probe will detect any dengue serotypes. **Dengue 1-3** only detects serotypes 1-3.

Appendix 12

Standard Operating Procedure Creating a Google Form to Collect Field Data

Google Drive provided encrypted and secure access to projects files. It provided an easy and secure method for storing, sharing, and collaborating across team members living in Bangladesh, the United Kingdom, and the United States. Files are accessible to those with access privileges from mobile devices, tablets, or computers. Having files stored in a cloud-based system like Google Drive eliminates the need for local file storage, minimizing the risk of losing important project data. Additionally, all files uploaded to Google are encrypted while in use or inactive. Anyone with a Gmail account can utilize Google Drive.

Within Google Drive is a feature called Google Forms which can be used to create online surveys that can be

Type of Response	Description of response
Short answer	Responses in a few words. Form creator can set rules that respondents must follow (e.g., response must be a number, response must be less than a specific character length).
Paragraph	For longer responses. Form creator can set rules that respondents must follow (e.g., response must be a number, response must be less than a specific character length).
Multiple Choice	Respondents choose one option from a set of options. Form creator can set up form so that only certain questions are seen based on answer.
Checkboxes	Respondents choose many options from a set of options. Form creator can set up form so that only certain questions are seen based on answer. Form creator can set rules that respondents must follow (e.g., response must be a number, response must be less than a specific character length).
Dropdown	Respondents choose one option from a set of options. Form creator can set up form so that only certain questions are seen based on answer.
File upload	Respondents can upload files as a response to a question. Uploaded files will be stored in the Google Drive of the form owner. Specifications for file size and type can be set by the form owner.
Linear scale	Respondents choose option based on a rating scale (i.e., 0 to 10)
Multiple choice grid	Respondents can select one answer per row.
Checkbox grid	Respondents can select multiple answers per row of the grid.
Date	Respondents can input the date (month and day), year, and time.
Time	Respondents can input the time of day or the duration of an event.

sent to others. Google Forms was the preferred method of data management for the SPEAK Bangladesh Point of Need Project due to ease of use and ability to collect and share results in real-time.

Google Forms for Field and Laboratory Data Collection

Google Forms provide a centralized database for collection of project field and laboratory data. Once a form is created, it can be easily shared with others via email, link, or website. Form responses can be analyzed in real-time, allowing for rapid feedback of data and data quality checks. Form links can be shortened to reduce the number of URL characters needed.

The table on the previous page shows the types of form response options available in Google Forms. More information on the type of form responses can be found here.

1. Review the Data Dictionary for the Field Data Collection Form (Appendix 7a).

2. Review the paper field data collection form (Appendix 7b) for completeness and take a photo of the form.

3. Access the project data collection form at the link provided by the form owner and complete with information from the paper form.

4. For the "Photo of paper form" question, click on "Add file" to upload the photo of the paper form.

5. Review form for completeness. Click "Submit" to record form.

6. If additional forms need to be completed, click "Submit another response."

Collecting Data from Forms

The form owner and collaborators who have access privileges can view and edit individual forms, allowing for quick and easy data quality checks. The Owner version of the form (vs. the Respondent version) provides options reviewing the data: "Summary" provides descriptive information about responses. The example below shows a summary pie chart of the percentage of responses for each of the different methods of collection used in the study.
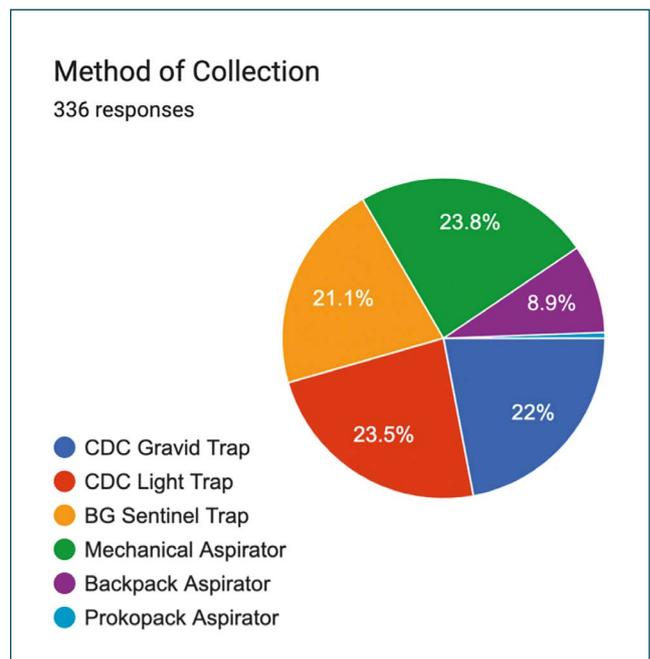
	C	D	E	F	G	H	I
1	Index Case Type	Method of Collection	District Name	Upazila Name	Union Name	Mauza Name	Village Name
2	VL/PKDL	CDC Light Trap	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
3	VL/PKDL	BG Sentinel Trap	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
4	VL/PKDL	Mechanical Aspirator	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
5	VL/PKDL	Mechanical Aspirator	Mymensingh	Trishal	Rampur	Kakchor	Kakchor
6	VL/PKDL	CDC Light Trap	Mymensingh	Trishal	Rampur	Kakchor	Kakchor
7	VL/PKDL	BG Sentinel Trap	Mymensingh	Trishal	Rampur	Kakchor	Kakchor
8	VL/PKDL	CDC Gravid Trap	Mymensingh	Trishal	Rampur	Kakchor	Kakchor
9	VL/PKDL	Mechanical Aspirator	Mymensingh	Trishal	Rampur	Kakchor	Kakchor
10	VL/PKDL	BG Sentinel Trap	Mymensingh	Trishal	Rampur	Kakchor	Kakchor
11	VL/PKDL	CDC Gravid Trap	Mymensingh	Trishal	Rampur	Kakchor	Kakchor
12	VL/PKDL	CDC Light Trap	Mymensingh	Trishal	Rampur	Kakchor	Kakchor
13	VL/PKDL	CDC Gravid Trap	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
14	VL/PKDL	CDC Gravid Trap	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
15	VL/PKDL	CDC Light Trap	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
16	VL/PKDL	Mechanical Aspirator	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
17	VL/PKDL	BG Sentinel Trap	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
18	VL/PKDL	CDC Light Trap	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
19	VL/PKDL	CDC Gravid Trap	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
20	VL/PKDL	BG Sentinel Trap	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
21	VL/PKDL	Mechanical Aspirator	Mymensingh	Trishal	Balipara	Kazigram	Kazigram
22	Malaria	Mechanical Aspirator	Bandarban	Ali Kadam	Ali Kadam Sodar	Toiynfa	Langori para

Photos of the paper forms are stored in Google Drive of the project owner. Within the Google Sheet is a column that contains a link to where the photo is stored. Clicking on a link gives you the option to view the photo, copy the link to the photo, or edit the link to the photo.

Creating a Pivot Table to Summarize Data

Pivot tables are useful way to summarize data. It is a table of grouped values that aggregate individual responses from a selected area of the Google Sheets. Summaries can be sums averages, or other desired statistics.

1. Click on the spreadsheet (if there are multiple spreadsheets within one file) for which you want to create a pivot table. In the figure below, data are being pulled from cells within the "Form Response 1" sheet. Go to "Insert, the select "Pivot Table."

2. The data range contains all rows and columns, and data from the "Form Responses 1" sheet will be inserted to a new sheet within the file.

The figure below shows sample data on the total (sum) of each physiological stage of insects collected by District and Method of Collection. The district totals are highlighted in yellow for easy display. The sheet of the Pivot Table was renamed "Insect_Counts_Method."
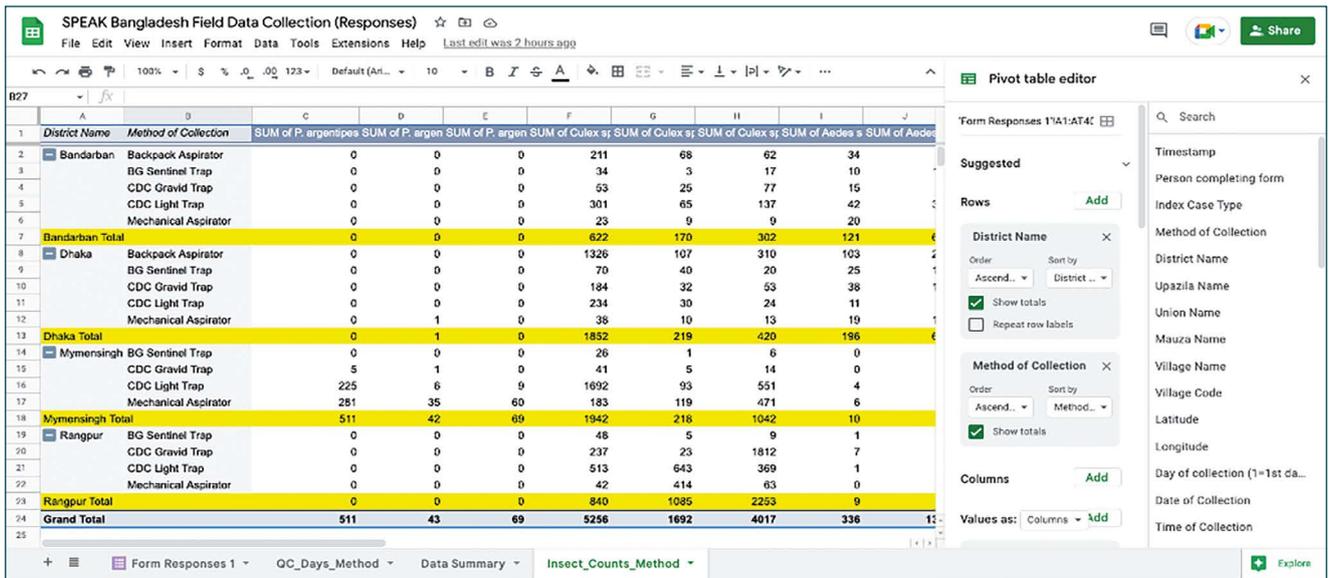
Once a Pivot Table is created, functions can be used to do quick summary analyses. The summary data below were generated using the SUM function and by adding, subtracting, or dividing data. The negative value for *Cx. quinquefasciatus* indicates that 2709 more were collected than what was needed to reach collection goal of 3000 gravid and bloodfed female mosquitoes.

Insect of Interest	Gravid and Bloodfed	Left to Collect	% collected (bloodfed and gravid)	% collected (all stages)
P. argentipes	112	1388	7.47%	41.53%
Cx. quinquefasciatus	5709	-2709	190.30%	365.50%
Anopheles	2962	38	97.47%	162.13%
Aedes	165	335	33.00%	100.20%

The following table shows examples how to generate the summaries using functions with the Pivot table data above.

Summary data of Interest	Description	Function	Result
# of gravid and bloodfed *P. argentipes* left to reach sample size goal ("Left to Collect")	The sample size goal was 1500 gravid and bloodfed *P. argentipes.* Sum data from Columns D and E	=1500– sum(D24:E24)	1388 gravid and bloodfed *P. argentipes* still need to be collected to reach sample size goal
# of gravid and bloodfed *P. argentipes* collected	The sum of Columns D and E	=sum(D24:E24)	112 gravid and bloodfed *P. argentipes* have been collected over the study

Exporting Form Data for Analysis

Form data can be downloaded for subsequent analysis with statistical software packages. Go to File, then "Download," and select the file type to save the data.
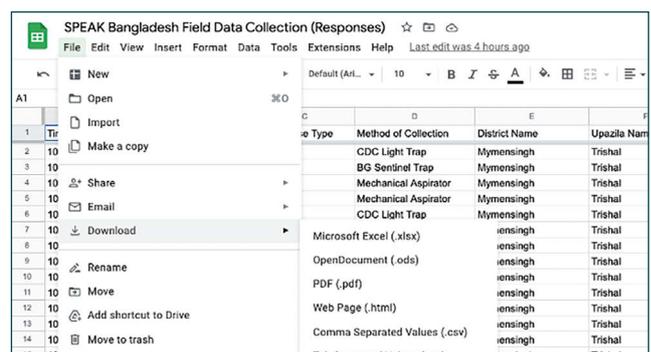
12A   DATA DICTIONARY FOR PAPER AND GOOGLE FIELD DATA COLLECTION FORM

Field Label	Description	Options
Person completing form	Name of person who completed form	Short answer – text
Index Type	Type of case	1, VL/PKDL,	2, LF/TAS+,	3, Malaria,	4, Dengue
Method of Collection	Indicates which of the four methods was used for vector collection	1, CDC Gravid Trap	2, CDC Light Trap	3, BG Sentinel	4, Mechanical aspirator	5, Backpack aspirator |6, Prokopack aspirator
Number of collection method used	Indicates how many traps/aspirators were used	Integer
District Name	District where sampling occurred based on index case's home	Short answer
Upazilla Name	Upazilla where sampling occurred based on index case's home	Short answer
Union Name	Union where sampling occurred based on index case's home	Short answer
Mauza Name	Mauza where sampling occurred based on index case's home	Short answer
Village Name	Village where sampling occurred based on index case's home	Short answer
Village Code	Specifies the village if many villages within a mauza are being sampled	Short answer – Integer
Index case number	Specifies the case within the village used to select households for sampling	Short answer – Integer
Latitude	Latitude coordinates where insect collection occurred	Short answer – Number
Longitude	Longitude coordinates where insect collection occurred	Number
Day of collection	1=1st day, 2=2nd day, 3=3rd day,4=4th day, 5=5th day, 6=6th day, etc.	Short answer – Integer
Date of Installation	Date CDC gravid, CDC light, and BG sentinel traps were installed; Date aspiration occurred	Short answer – Integer
Time of Installation	Time CDC gravid, CDC light, and BG sentinel traps were installed; Time aspiration ended	Short answer – Integer
Total P. argentipes females collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total unfed P. argentipes collected	Total collected by the collection method on the date of installation	Short answer – Integer

Field Label	Description	Options
Total fed P. argentipes collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total gravid P. argentipes collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total female sand flies collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total unfed sand flies collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total fed sand flies collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total gravid sand flies collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total female Anopheles spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total unfed Anopheles spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total fed Anopheles spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total gravid Anopheles spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total female Culex spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total unfed Culex spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total fed Culex spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total gravid Culex spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total female Aedes spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total unfed Aedes spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total fed Aedes spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total gravid Aedes spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total female culicine spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total unfed culicine spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total fed culicine spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer

Field Label	Description	Options
Total gravid culicine spp. collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total female blood feeding insects collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total unfed blood feeding insects collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total fed blood feeding insects collected	Total collected by the collection method on the date of installation	Short answer – Integer
Total gravid blood feeding insects collected	Total collected by the collection method on the date of installation	Short answer – Integer
Photo of paper form	Upload image of paper form used in the field to collect data	Upload

12B   PAPER FIELD DATA COLLECTION FORM

Title of the Research Project: Pilot test of a multiplex molecular point-of-need vector surveillance tool in Bangladesh

Vector Collection Record Form

Initials of Person Completing Form:

Index Case ID: Index Type:|___| (1=VL/PKDL, 2=LF/TAS+, 3=Malaria, 4=Dengue)

Index HH ID: Method of collection: :|___| (1= CDC Gravid Trap, 2= CDC Light Trap,
3= BG Sentinel, 4 = Mechanical aspirator 5= Backpack aspirator 6= Prokopak aspirator)

District Name: .... Upazila Name: ....

Union Name: .... Mauza Name: ....

Village/Ward Name: .... Village Code:|___||___|

Index No.:|___||___|

Latitude:|__|__|.|__|__|__|__|__|__| Longitude:|__|__|.|__|__|__|__|__|__|

Day of Collection:|___| (1=1st day, 2=2nd day, 3=3rd day,4=4th day, 5=5th day, 6=6th day)

Date of Collection: /_ _/_ _/_ _ _ _/ (dd/mm/yyyy)

Time of Collection:|___||___|:|___||___|

Collection data:

Collected insects	Female			Total
	Unfed =1	Blood Fed = 2	Gravid =3	
P. argentipes				
Other sand flies				
Anopheles spp.				
Culex spp.				
Aedes spp.				
Other culicine mosquitoes				
Other blood feeding insects				
Total				

Appendix 13

Standard Operating Procedure to estimate the costs of MX for VL Surveillance in India

Data Collection – Itemized Lists of Resources and Their Costs

Data Collection for the costing tool is centralized to a Microsoft Excel spreadsheet that contains several individual sheets that will be used for the final costing estimate analyses. The first step is to itemise all resources and their costs.

1. Create an Excel spreadsheet that contains multiple sheets, and name the sheets as follows:

   a. Explanation of Costing Tool – provides information on how to use the costing tool.

   b. I. Resource Use – sheets that are used to track the resources per activity.

   c. II. Unit Costs, III. Summary (combines the average costs of the different activities)

   d. Resource Use – one sheet for each MX Activity.

2. Use the following column headings:

   a. **Item/Human Resource** – the resource to be costed. It is a good idea to group resources by categories:

      i. Human resources

      ii. Laboratory resources

      iii. Field resources

   b. **Cost/Salary** – the unit cost for an item or individual cost for the salary of a project staff member.

   c. **Quantity** – the number of an item/human resource needed for MX.

   d. **Description of Resource** – the purpose of the item/responsibility of the human resource in MX activities.

   e. **Total Cost** – the total (actual or estimated) amount spent on an item/human resource. This can be calculated for a single currency or multiple currencies.

      i. If total costs are being calculated for multiple currencies, utilize functions to obtain the costs based on the currencies of interest (Indian Rupees, US Dollars, and British Pounds). Xe can be used to obtain current or retrospective rates: https://www.xe.com/currencyconverter/. Use Indian Rupees as the primary currency of interest and convert per rupee.

      · Using the conversions below, multiple the total cost in Rupees by 0.012 (with rounding) to obtain the cost in US Dollars and by 0.010 (with rounding) to obtain the cost in British Pounds.

3. Use invoices, proposed budgets, payslips, and other relevant information sources to fill in the spreadsheet.

Item/Human Resource	Cost	Quantity	Description of Resource	Total (Rs)	Total (USD)	Total (GBP)
LABORATORY EQUIPMENT						0
qPCR machine	769231	1	Amplification of DNA	769231	10000.003	8461.541
Freezer (-20C)	35000	1	For storage of collection pots containing field-caught specimens and reagents	35000	455	385
Set of Pipettes	50000	1	A set of pipettes offer the range of volumes needed for molecular work from (0.3ul to 1000ul)	50000	650	550
Multichannel pipette	50000	1	Rather than using a single channel pipette, a mutli-channel pipette can reduce error that may be associated with transfer of liquid to 96 well plates.	50000	650	550
Stereo Microscope	30000	2	One for sandfly identification; one for sandfly dissection	60000	780	660
Laptop Computer	30000	1	A laptop is prefered over a desktop due to portability and cost-savings. One laptop can be used for both laboratory and sorting work.	30000	390	330
Thermo Oven	230000	1	Overnight incubation of samples	230000	2990	2530
Motor pestle						
SUPPLIES (FIELDWORK-RELATED)						
Large Insulated Cooler	5000	2	Field-caught specimens should begin cold storage upon collection to preserve the integrity of specimens and RNA for microscopy and molecular analyses.	10000	130	110
CDC Miniature Light Traps	8400	60	CDC light traps were selected as the best method for collection of *P. argentipes* females and is the preferred method for xenomonitoring of visceral leishmaniasis in India. (Note: for collection of blood fed females, wall aspiration may be run parallel to CDC light traps). The number of CDC Light Traps is based on having two field team installing traps (10-15 traps) in two villages each.	504000	6552	5544
Ice packs	200	20	Field-caught specimens should begin cold storage upon collection to preserve the integrity of specimens and RNA for microscopy and molecular analyses.	4000	52	44
Removable adhesive tape	900	5	Autoclave tape was used during xenomonitoring work to label CDC Light Traps with household and date of installation.	4500	58.5	49.5
Fine tip markers (pack of 8)	500	1	Sharpie markers were used on autoclave tape to label CDC Light Traps by household and date of installation	500	6.5	5.5
Register notebook for field notes	260	10	The Reseach Assistant maintains a notebook containing participating household information includind contact information; also used to take notes in the field. Other members of the team should have a notebook as appropriate.	2600	33.8	28.6

Data Collection – Database Spreadsheets

1. **Explanation**

2. **Resource Use Sheets**

   a. I.VC_TrapInstallation

   b. I.VC_FieldSpecimenCollection

   c. I.VC_SpecimenSorting

   d. I.VC_SpecimenPooling

   e. I.VC_DNA Extraction

   f. I.VC_PCR

3. **Average Cost Sheets**

   a. II.VC_TrapInstallation

   b. II.VC_FieldSpecimenCollection

   c. II.VC_SpecimenSorting

   d. II.VC_SpecimenPooling

   e. II.VC_DNA Extraction

   f. II.VC_PCR

4. **III. VC_MX_Costing**

Data Collection – Individual MX Resource Use Sheets

The "Resource Use" sheets collect observation time and resource allocation data for each MX activity. These sheets are used to collect:

· Activity indicators (examples: number of traps used, number of female *P. argentipes* collected, number of pools screened)

· Time spent on each sub-activity

· Human resources and individual time spent on each sub-activity

· Consumables, equipment, and other resources used for each sub-activity

The first section of this sheet contains the activity indicator of interest for each observation. Below is an example for the Trap Installation activity whereby the indicator of interest is the number of traps installed per observation (five observations).

OBSERVATIONS	1	2	3	4	5
Number of traps installed during observation	20	20	20	30	15

The next section is the Total Duration by observation for each sub-activity within the main MX activity. Continuing with the Trap Installation example, there were four sub-activities: Preparation, Travel to Site(s), Installation of Traps, and Return from Site. The Total Duration column is the sum of the time across the four activities using the SUM function in Excel. To obtain the 495 minutes in cell H9, =SUM(D9:G9) is the function that was used.

SPEAK India Molecular Xenomonitoring Study
Trap installation

OBSERVATIONS	1	2	3	4	5
Number of traps installed during observation	20	20	20	30	15

	DURATION (MINUTES)				
Total length of time (minutes)	Preparation	Travel to Site(s)	Installation of Traps	Return from Site	Total Duration
Observation 1: Chainpur Turki and Panapur Kariyat (11/13/19)	60	226	20	189	495
Observation 2: Saran (9/18/19)	60	112	57	147	376
Observation 3: Muzaffarpur (9/26/19)	30	240	43	134	447
Observation 4: Patna (3/3/2020)	60	188	138	92	478
Observation 5: Madarpur, Nalanda, (3/4/2020)	30	99	57	169	355

The next section breaks down the time needed for human resources to perform sub-activities per observation. In the example below, two field members and a driver were needed for Observation 1. Because the driver was not needed for the preparation sub-activity, no time was entered. The two field members participated in all four sub-activities and have times entered. Data for other human resources who did not participate in the sub-activities remain blank.

		DURATION (MINUTES)				
	Human Resources	Preparation	Travel to Site	Installation of Traps	Return from Site	Total Duration
	Field Member 1	60	226	20	189	495
	Field Member 2	60	226	20	189	495
	Filed Member 3					0
	Field Member 4					0
Observation 1	Driver 1		226	20	189	435
	Driver 2					0
	Community health worker					0
	Village worker					0

The next section breaks down the items and associated quantities needed per observation. In the example 20 CDC Light Traps were needed during the installation sub-activity. One vehicle was used (only inputted in the return from site column) which travelled 150 kilometres during Observation 1.

	Inputs Needed	Preparatio	Travel to Site	Installation of Traps	Return from Site	Total
	CDC Miniature Light Traps (fine mesh collection pots)			20		20
	6V 12 amp Battery			20		20
	Battery Charger 6V 12 amp			20		20
	Vehicle				1	1
	Brady labels	0				0
	Permanent marker	2				2
	Carry crates	4				4
	Universal power strip	8				8
Observation 1	Number of km done by the end of day		75		75	150
	Number of km done by the end of day - Vehicle 2					0
	Lunch field workers					0
	Rubber boots field workers					0
	Bagpack for material		1			1
	Large Insulated Cooler		2			2
	Collection cloth (extra) for CDC Miniature Light Trap					0
	Ice packs					0
	Autoclave tape (6 pack)	1				1
	Register notebook for field notes	1				1
	Replacement bulbs					0
	Pens					0

The duration and quantities sections are repeated for each observation conducted.

Data Collection – Activity and Average Costs Sheets

The Activity and Average Costs Sheets are to track costs per activity and calculate the average cost. Once all observations of an activity are completed and the information is inputted in the Resource Use Sheets, the unit cost sheets can be prepared and completed. In these sheets the average resource use per resource used to implement the activity will be calculated (column B-G). Cost details will need to be completed (column K-R) based on trustworthy information sources (payslips, invoices, pro forma invoices, discussions with experts). This information will allow for calculation of the cost per item or the cost per day for equipment used. Data collection for these sheets rely heavily on Excel functions, so caution must be taken to prevent errors.

Data collected from these sheets include:

· Cost per unit

· Currency type

· Number of items per unit (i.e., number per box, days worked/year)

· Information source

· Item reusability

· Lifespan of reusable item

· Type of cost (staff, equipment, consumable, etc.)

Each Activity and Average Cost sheet has three sections:

1. Average resource use

2. Cost details

3. Average cost in currency of interest

Average Resource Use

In this section of the sheet, data from the Resource Use will be to calculate average using functions. Below shows how the COUNT function is used to get the number of entries in a number field that is in a range or array of numbers. The array of numbers is D5, E5, F5, G5, and H5 from the Trap Installation Resource Use sheet. Because a number was entered in those cells, the function counts each cell, and "5" is resulted in C9 for the total number of observations.

C6 | fx =COUNT(I.VC_TrapInstallation!D5,I.VC_TrapInstallation!E5,I.VC_TrapInstallation!F5,I.VC_TrapInstallation!G5,I.VC_TrapInstallation!H5)

	A	B	C	D	E	F	G
1		SPEAK India Molecular Xenomonitoring Study					
2		TRAP INSTALLATION					
3			Average resource use				
4				Observations			
5		Information regarding observations	Total				
6		Total number of observations	5				
7		Average number of traps installed during observations	21				

To obtain the average number of traps installed during the 5 observations, the IFERROR and AVERAGE functions are used. The average is taken from the numbers inputted in D5, E5, F5, G5, and H5, and the resulting average is 21 traps.

C7 | fx =IFERROR(AVERAGE(I.VC_TrapInstallation!D5:H5),0)

	A	B	C	D	E	F	G
1		SPEAK India Molecular Xenomonitoring Study					
2		TRAP INSTALLATION					
3			Average resource use				
4				Observations			
5		Information regarding observations	Total				
6		Total number of observations	5				
7		Average number of traps installed during observations	21				

The IFERROR and AVERAGE functions are also used to obtain the average number of minutes for activities and sub-activities, and for the average number of items used during the (five) observations. Below, cell D19 shows the average number of minutes (109.5 minutes) spent by Driver 1 to travel to study sites across the five observations.

D19 | fx =IFERROR(AVERAGE(I.VC_TrapInstallation!E20,I.VC_TrapInstallation!E31,I.VC_TrapInstallation!E42,I.VC_TrapInstallation!E53,I.VC_TrapInstallation!E64,),0)

	A	B	C	D	E	F	G	H	I
1		SPEAK India Molecular Xenomonitoring Study							
2		TRAP INSTALLATION							
3				Average resource use					
12									
13					DURATION (MINUTES)				
14		Human Resources	Preparation	Travel to Site	Installation of Traps	Return from Site	Total Duration	Total Duration in days	
15		Field Member 1	40	144.17	52.50	146.20	382.87	1.000	
16		Field Member 2	40	144.17	52.50	146.20	382.87	1.000	
17		Filed Member 3	40	135.00	59.50	124.33	358.83	1.000	
18		Field Member 4	30	100.00	65.00	119.50	314.50	1.000	
19		Driver 1	0	109.25	33.50	168.33	311.08	1.000	
20		Driver 2	0	56.00	28.50	147.00	231.50	1.000	
26									
27					Quantities used				
28		Inputs Needed	Preparation	Travel to Site	Installation of Traps	Return from Site	Total		
29		CDC Miniature Light Traps (fine mesh collection pots)	0	0	21	0	21		
30		6V 12 amp Battery	0	0	21	0	21		
31		Battery Charger 6V 12 amp	0	0	21	0	21		
32		Vehicle	1	0	0	1	2		
33		Brady labels	0	0	0	0	0		
34		Permanent marker	2	0	0	0	2		
35		Carry crates	4	0	0	0	4		
36		Universal power strip	8	0	0	0	8		
37		Number of km done by the end of day - Vehicle 1-2	0	15	0	126.6	141.6		
38		Lunch field workers	0	0	0	2	2		
39		Rubber boots field workers	0	0	0	0	0		
40		Bagpack for material	0	1	0	0	1		

Cost Details

This section provides the specific details about the items to be costed such as the quantity, type of cost, currency used, lifespan (for reusable items), and the information source.

| | K | L | M | N | O | P | Q | R | S |

Cost details

Unit Cost	Currency	Unit	Quantity/unit	Type	Unit	Cost/day	Currency	Information source
195,000	Rs	Annual salary (220 days/Year)	220	Staff				
195,000	Rs	Annual salary (220 days/Year)	220	Staff				
195,000	Rs	Annual salary (220 days/Year)	220	Staff				
195,000	Rs	Annual salary (220 days/Year)	220	Staff				
				Staff				
				Staff				

Unit Cost	Currency	Unit	Quantity/unit	Type	Category	Lifespan goods (Month)	Information source	Questions
150	USD	Piece	1	Specialized equipment	reusable	60		Maybe better to express in #
12	USD	Piece	25	Other - Equipment	Non-reusable	0		
240	Rs	Piece	10	Other - Equipment	reusable	12		
2500	Rs	Cost daily car hire	1	Vehicle running costs	Non-reusable	0		
28.02	GBP	Piece	1	Stationery	Non-reusable	0	amazon.com	
6.16	GBP	Piece	6	Stationery	reusable	1	amazon.com	
1500	Rs	Piece	1	Other - consumables	reusable	12		
5	GBP	Piece	1	Other - consumables	reusable	6		
	Rs	Piece	1	Vehicle running costs	Non-reusable	0	12.20 l/100km - 84.19Rs/l	Fuel cost included in car rental
40	Rs	Piece	1	Other - consumables	Non-reusable	0		
250	Rs		1	Other - consumables	reusable	12		
300	Rs	Piece	1	Other - consumables	reusable	12		

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