BCOMING

Sampling Strategy

Project acronym: BCOMING

Project title: Biodiversity Conservation to Mitigate the risks of emerging infectious diseases

Call: HORIZON-CL6-2021-BIODIV-01

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Executive Summary

Sampling Strategy

BCOMING is a project that brings together a large number of partners and teams who will work in the wild and in the laboratory on samples collected from all terrestrial and aquatic ecosystems, including animal and human compartments, in Cambodia, Cote d'Ivoire, Guinea and Guadeloupe. This deliverable, which concerns the sampling strategy, is therefore very important to create cohesion in the whole project. It is indeed essential that an interoperability between the different samples of the project can be done for an integrative modelling which is at the heart of BCOMING. For this, each partner and team must identify and map in a very precise way with geographical coordinates the location of the samples, the type of samples taken and the number. Additional information on the species collected, the people who took the samples and the date and time of collection must be collected by each BCOMING team. It is therefore essential that all the data collected in BCOMING have a unique identifier at all times that allows the link to a unique sample and that is linked to a set of information about the location of the sample, the type of sample, the status of the sample and the storage of the sample. It is therefore important that all teams involved in BCOMING use this unique identifier.

Data collection will be multiple including genetic sampling, eDNA sampling, animal and human sampling, stable isotope analysis. It is therefore essential to multiply the spatial resolution of the sampling at each site in order to include as much of the variability of the site as possible. Several small samples spread across the site will be more robust in accounting for site heterogeneity, rather than a single large sample at one point in the site. It is also important that the eDNA data, where possible, is complemented by conventional faunal sampling to provide a robust point of comparison.

Sampling will be more extensive in Cambodia than in the other countries in the wet and dry seasons with a wider spatial distribution. For the other countries, sampling will be more punctual and in the rainy season. A set of local actors and logistics of the teams present in the country will facilitate the implementation of sampling. All the data collected will be stored in a centralised BCOMING database where everyone will be able to retrieve the data needed for their analysis.

This sampling plan will be supplemented as the project progresses with more specific information on each site as it is finalised. It is therefore up to each team leader to refer to this sampling strategy and report their data to the BCOMING database as the project progresses.

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Sampling BCOMING

WARNING - It is important that all teams involved in BCOMING use a unique identifier. To do this, the identifier should be alpha numeric and contain information related to the country, site, team and specific sample number. This will give a Two Letter Country - Two Letters Site - Two Letters Team - Unique numerical number. Example CA-ST-CI-1 for Cambodia-Stung Tren-Cirad-first sample. This identification number should be reported on each sample. Also, each sample should be split into two sub-samples, if possible, with one sub-sample being used to do the required analyses and the other sub-sample being stored in a separate freezer or cabinet as a back-up.

Sampling sites

1.1.1. Cambodia

In Cambodia we will sample four different regions that follow a gradient of anthropisation and which are 1) Stung Tren; 2) Siem Reap; 3) Battabang; 4) Bassac both during the dry and the rainy seasons. In each of these regions, sampling of bat caves, aquatic sites and local human populations will be carried out. With regard to aquatic sampling in each region 5 representative aquatic sites will be selected, making a total of 20 sites. These sites are to be defined with our partners in the coming months but will allow taking into account any variability with a region.

1.1.2. Guinea

In Guinea, six sites were selected for sample collection. These sites are Madina Oula, Forécariah, Boffa, Nzérékoré, Yomou and Conakry. These sites will be used to capture and collect samples of aquatic eDNA, bats, rodents, and humans. The samples collected in Conakry can be compared with other sites that have been captured previously. In addition to the animal samples, environmental data such as vegetation phenology, presence of domestic and wild fauna, humidity, temperature, will be collected. It is also planned to record the characteristics of the capture caves during the first missions.

1.1.3. Côte d'Ivoire

In Ivory Coast, three sites in the Cavally region will be sampled. The sites will cover an ecological gradient, spanning from primary forest within Tai National Park, reaching the outskirts of the surrounding villages. The samples will encompass two different ecotypes, pristine forest, and fragmented agriculture. Each of the three sites will be sampled in triplicates, reaching a total of 9 sampling sites. Sampling will happen at the end of the dry season, in April/May 2023 and 2024. At the described sites, bats, rodents and flies will be trapped and sampled. In addition to the animal samples, environmental data such as humidity and temperature will be recorded. Aquatic sampling will be performed in each of the three sites, depending on water availability, aiming to collect at least five samples per site, ending with a total of 15 water sampling sites. These water sampling sites are to be defined with our partners in the coming months but will allow taking into account any variability with a region.

1.1.4. Guadeloupe

In Guadeloupe, a set of 6 sites will be sampled for aquatic sampling according to a gradient of anthropisation (degraded/non-degraded). These sites are Choisy, Merwart, Traversée, Sofaïa,

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Moreau and Tambour. They will be sampled for aquatic eDNA samples. The water points that will be specifically sampled at these sites will be identified later in the project.

Aquatic sampling

1.1.5. Site typology

Although the choice of regions follows a gradient of anthropisation, we will collect a certain number of parameters on each site in order to characterise the typology of the site as well as its geographical coordinates. We will first describe the site, i.e. whether it is a river, or an oxbow, and the degree of vegetation on the site. Then we will take five samples of physico-chemical measurements at the site using a multiparameter probe, which will include water temperature, conductivity, dissolved oxygen, pH as well as the granulometric distribution and transparency of the water.

1.1.6. Snails, Fish, and Invertebrate sampling

Identification and data collection

Invertebrate samples will be collected at five locations, spatially distributed within each site to include a range of microhabitats. Nets will be used to vigorously collect samples from the bottom sediment and the surface of the water column within a meter square area. All collected samples will be placed on a white tray for preliminary sorting in the field and fixed in 70% ethanol for subsequent identification and storage in the laboratory. In addition, an exhaustive sampling of the snails present on the riverbank site will be carried out manually and kept alive in a jar in the first instance. Thanks to an analysis with a blue light, some trematodes will be extracted manually before the final preservation of the snails.

Fish will be collected using five standard baited fish traps and five gill-nets spatially distributed as the dip net locations (see above). The nets and traps will be set overnight before retrieval. The fish will then be gently removed from the nets and placed in large tanks with additional oxygenation (air stones). Three fish of each species will be taken and euthanised with an excess of eugenol-type anaesthetic (400-mg to 1-liter solution of clove oil). The solution will be added gradually, and fish remained submerged for 10 min until death, following the guidelines set out by the American Veterinary Medical Association as advocated by the Institutional Animal Care and Use Committee. Fish will be then preserved in 70% alcohol for sorting and identification back at the laboratory. The other fish will be identified, weight, and gently released into their natural environment as soon as they have fully recovered. Permission to fish will be obtained from the local authorities beforehand.

The length of each invertebrate will be measured, and identification will be conducted predominantly to a family level where possible, using a number of literature sources. Specimens of the same family will be pooled by site and collection date and preserved in 70% alcohol for potential further PCR analysis. Invertebrates containing more than 10 individuals were pooled in sets of 10. Dry weights in milligrams will be calculated using length-to-mass regression equations. Fish will be identified to species level, and for each individual, a standardized length measurement will be taken. Fish weights will also be calculated using length-to-weight regression equations; however, because there is no direct conversion available in the literature for fish to establish dry weights for all species, an average wet-to-dry weight conversion of 20% will be used.

1.1.7. eDNA sampling

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Nature Metrics will provide aquatic eDNA sampling kits to partners undertaking field sampling for eDNA. At each site, up to 10 different aquatic samples for eDNA analysis will be collected. The DNA taken from these filters will be used for the detection of fish and general vertebrates, as well as for the development of single-species assays targeting trematodes within Task 2.4. This will provide us with a list of species whose DNA is present at the site and will allow us to standardise the assessment of the species richness across sites. These data will also be compared to other samples collected (1.2.2.) in order to calibrate the eDNA data. Sampling strategies will vary depending on the type of water body:

Ponds

In a pond, eDNA does not mix well due to absence of flow or wave action, so multiple water samples are key to capture the eDNA present. A pond will be sampled by taking independent samples, each comprised of subsamples (e.g. 2 L comprised of 5 x 400 mL subsamples) taken at multiple locations around the pond perimeter.

When sampling ponds:

- Surface water (sub)samples should be collected from the shoreline at roughly equidistant intervals around the pond perimeter.
- Use the provided sampling bag or ladle, or a clean bottle (Nature Metrics' recommends a small mineral water bottle with the water discarded), to collect water. Deposit the collected (sub)sample in the provided sampling bag. Repeat for each subsample if applicable. Seal the bag and make sure the water is well mixed by shaking for 20-30 seconds. The bag is not self-standing but can be supported by the surveyor or propped against a log, tree stump, or rock to stabilize it for filtration.
- A new bag/ladle/bottle will be used for independent samples and for each pond to avoid cross-contamination.
- Depending on water clarity, it may be possible to pass up to 2 L of pond water through each filter used, but smaller volumes (e.g. 150-250 mL) are more typical for turbid ponds or ponds with dense vegetation.

Lakes

In a lake, eDNA can still be stabilized, so multiple water samples remain key to capture the eDNA present. We will aim to take at least 10 independent samples, each comprised of subsamples (e.g. 2 L comprised of 5 x 400 mL subsamples), which are taken from multiple locations around the lake perimeter (19,20). As a rule of thumb, one independent sample (i.e. using 1 kit) will be taken along approximately 100 m of shoreline, with sampling locations distributed at equidistant intervals around the lake perimeter.

When sampling lakes:

- Surface water samples should be collected from the shoreline at roughly equidistant intervals around the lake perimeter.
- Use the provided sampling bag or ladle, or a clean bottle (Nature Metrics' recommends a 2 L mineral water bottle with the water discarded), to scoop up water every 10 m along a 100 m

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stretch of shoreline. Deposit the collected subsample in the provided sampling bag. Repeat for each subsample, seal the bag, and make sure the water is well mixed by shaking the bag for 20-30 seconds. The bag is not self-standing but can be supported by the surveyor or propped against a log, tree stump, or rock to stabilize it for filtration.

- Depending on water clarity, it may be possible to pass up to 5 L of lake water through each filter used, but smaller volumes (e.g. 2 L) are more typical.
- Sampling when the lake is not thermally stratified is ideal as more mixing of the water will occur. This means there is a higher chance of detecting eDNA from shallow and deep-water aquatic species. However, detection of invertebrates and some other taxonomic groups is generally lower in colder temperatures.

Rivers or streams

In a river or stream, eDNA can be well-mixed depending on local environmental conditions, but flow means that eDNA can be transported hundreds to thousands of metres from its source and be diluted. As such, multiple sampling locations along the length of the stream/river are recommended. In small streams or rivers, at least three sampling locations (i.e. upstream, mid-section, downstream) should be identified. In larger rivers, 20-60 sampling locations should be identified for a comprehensive survey. A minimum of three independent samples (e.g. 1 L) spanning the width of the stream/river section (e.g. left bank, centre, right bank) should be collected from each sampling location. More water samples at each sampling location are recommended for wider rivers and possibly at downstream sampling locations if the river has a large catchment and/or is fast-flowing.

When sampling rivers or streams:

- We will start at the most downstream sampling location and work your way upstream.
- Use of a sampling vessel/device and sampling from the shoreline is recommended. If shoreline sampling is not possible, surveyors should enter the water downstream where they will collect the sample and be careful not to disturb sediment as they move to the collection point. If the water is too deep to enter, a boat or similar should be used for sampling.
- Use the provided sampling bag or ladle or a clean bottle (we recommend a 2 L mineral water bottle with the water discarded) to collect water by holding it with the opening pointing upstream at the water surface. Stand downstream of the sampling bag/ladle/bottle to avoid collecting your own DNA. Deposit the collected sample in the provided sampling bag. Repeat for each sample, seal the bag, and make sure the water is well mixed by shaking for 20-30 seconds. The bag is not self-standing but can be supported by the surveyor or propped against a log, tree stump, or rock to stabilize it for filtration.
- Depending on water clarity, it may be possible to pass up to 5 L of stream/river water through each filter used, but smaller volumes (e.g. 2 L) are more typical.

A field negative control (blank) will also be collected at each site to detect possible contamination during sampling. This is a purified water sample (eg. mineral, deionized, or MilliQ water) which is passed through a filter in the same way as a water sample.

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If individuals collecting the samples enter the water to collect samples, boots or waders should be decontaminated with bleach (containing sodium hypochlorite) in between waterbodies. Bleach will be disposed of responsibly.

Soils

Up to 20 composite soil samples will be collected per site. Each composite soil sample will consist of 9 subsample surface scrapes collected using a NatureMetrics Surface Soil Sampling kit. The soil sample will be collected by marking out a 10m X 10m plot and collecting 9 surface scrapes following a grid pattern into a zip lock bag. To maximise chances of detecting mammals, if visual evidence of mammal activity is present near the plot a surface scrape will be collected in that area. The surface scrapes collected in the zip lock bag will be mixed by shaking and massaging the bag. A portion of the composite soil sample will then be transferred into a sample pot until the pot is half full. The remaining sample will be discarded. A preservation buffer solution will then be added to the sample pot and the pot will be shaken to fully submerge the soil particles in the solution. A new kit will be used to collect each composite sample.

Once collected, soil samples and eDNA samples on water filters will be returned to BCOMING's partner, Nature Metrics, for analysis. This will provide us with a list of species whose DNA is present at the site and will allow us to standardise the assessment of the species richness across sites. These data will also be compared to other samples collected (1.2.2.) in order to calibrate the eDNA data.

1.1.8. Trophic stable isotopes sampling

Stable isotope analysis will be conducted on all invertebrates and vertebrates from all sites for the stable isotope signatures of d13C and d15N. This will cover the majority of the taxa present at all sites. For invertebrate analysis, a limb of the organism will be used, or if it is too small and many are present, whole organisms will be used. For fish, caudal fin clippings will be used. In most cases, three samples of each taxonomic group will be analyzed for each site. However, because of the limited number of available organisms one or two samples will be used in some cases. For any taxa that will not be analyzed due to abundance limitations, stable isotope data from a closely related and functionally highly similar taxon will be recorded for use in the statistical analysis. The material will be dried at 40°C for 8 hours to remove all traces of moisture. After drying, the material will be homogenized, and the stable isotope signatures of d13C and d15N will be identified using a Finnigan MAT Delta Plus isotope ratio mass spectrometer.

Terrestrial sampling

In BCOMING, terrestrial sampling will be conducted in Cambodia, Guinea and Ivory Coast. For Guadeloupe, the aquatic sampling will be matched to former terrestrial sampling implemented under the Insula project which data will be available for BCOMING.

At each study site in Cambodia, Guinea, and Ivory Coast, terrestrial sampling will include at least 1 session of bat and rodent capture. At Stung Treng in Cambodia and Madina Oula in Guinea, longitudinal terrestrial sampling will be implemented. Additionally, humans will be sampled in Cambodia and Guinea at all sites, and domestic chickens will be sampled at all sites in Cambodia to compare the structure and diversity of their microbiota.

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Host (e.g., age, sex, and reproductive status) and site (e.g., geographic coordinates, habitat) metadata will be systematically recorded during pathogen sampling sessions. All animal sampling will be conducted in accordance with national and international guidelines and regulations (OIE animal health code, WHO guidelines for personal protection, CITES, and Nagoya protocol). Biological samples will be stored in solutions that allow for the preservation of RNA and DNA at room temperature in the field for subsequent molecular analysis (Trizol, RNA later, or ethanol) and in virus transport medium for a subset of samples for virus isolation. Whenever possible, samples will be placed in liquid nitrogen, transported to the laboratory, and immediately frozen at -80°C for later use. All biological samples collected during the project will be stored in existing biobanks managed by the consortium partners.

Protocols involving bats

Bat captures will be conducted in four areas in Cambodia (Phnom Penh, Battambang, Stung Treng, and Mondulkiri) and three areas in West Africa (Conakry and Kindia in Guinea and the Taï forest in Ivory Coast). The targeted species will be those of the genus Rhinolophus, host of coronaviruses close to SARS-CoV. A one-week mission will be organized in each area, and the captures will take place every evening in different capture sites, with a maximum of 3 consecutive nights on the same site.
For two study areas (Stung Treng in Cambodia and Kindia in Guinea), a longitudinal follow-up will be set up with 6 capture sessions per year during 2 years in these areas in order to study the dynamics of coronavirus circulation in these species.
During each session, bats will be captured, sampled, and released during the night in sites that will be chosen according to the information (presence, species, human/bat interface) received from the Veterinary Services and the local communities (sites sheltering the bat during the day, sites where they feed at night, …). Biological data (species, age, sex, reproduction), morphometric data, and biological samples (blood, saliva, feces, urine, parasites, semen) will be collected from captured specimens before they are released at night. A maximum of 50 bats can be collected per night to allow for optimal handling of the animals.
Nets of 3, 6, or 12 meters in length with 30 mm mesh will be set up before nightfall in the sites where they rest during the day (tree, house housing a bat colony, cave, ...). The nets will be checked every hour from nightfall (around 7 pm) until they are folded (at the latest before dawn) in order to detach the trapped bat. These bats will be placed separately in cotton containment bags while waiting to be sampled and released as soon as possible a few hours later.
The following biological data will be collected for each specimen: species, age, sex, physiological status, pregnancy, and lactation. The following morphometric data will be collected using rulers, metric tapes, calipers, and weights by handling the bats gently: total length, forearm length, metacarpal length, and tail length.

The following biological samples will be collected. Staff members will follow the safety measures detailed in deliverable D9.6 (EPQ).

- Blood

Whole blood drops will be collected by puncture of the propatagal or brachial vein with a 26-gauge needle. Bleeding will be stopped by compression with a cotton ball. The animal will be released only when the bleeding has stopped. Blood drops will be collected directly on blotting paper, preserved dry in sealed bags with silica gel (dried blood spots (DBS)). They will be stored at -80°C in the laboratory.

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The volume of blood of bats is about 9 to 11 ml/100gr. Less than 10% of this volume will be collected in order not to weaken the bat (1ml/100gr).

- Saliva
Saliva will be collected with a cotton swab gently inserted in the throat. The cotton swab will then be preserved in a tube containing RNAlater.

- Stool
Stool will be collected with a rectal swab. The cotton swab will then be preserved in a tube containing RNAlater.
Stools will also be collected non-invasively by placing a plastic sheet under the colonies in the morning at dawn.

- Urine
Urine will be collected opportunistically with a plastic pipette if the bat urinates during the manipulation. It will be preserved on blotting paper.

- Skin and hair
For bat DNA analysis, tissue should be collected by making a small puncture in the wing, which heals quickly, using a biopsy device (Acu-Punch sterile, disposable 2-mm skin biopsy punches, Acuderm, Inc.). The biopsy should not touch any of the large blood vessels in the wing and should not be close to the wing margin. Bat often naturally pierce their wing with branches while flying, and this type of injury has been shown to heal completely within 30 days (Faure et al. 2009). Again, the animal will be released only when the bleeding has stopped. The piece of tissue will be preserved in 96% ethanol. Hair will also be preserved in 96% ethanol.

Bats will be released overnight at the capture site and just after data and sample collection.

Protocols involving Rodents

Rodent captures will be conducted in parallel with bat captures in four areas in Cambodia (Phnom Penh, Battambang, Stung Treng, and Mondulkiri) and three areas in West Africa (Conakry and Kindia in Guinea and the Taï forest in Côte d'Ivoire). The targeted species will be those present in bat caves and species of the genus *Rattus* in villages near the caves.
During the one-week missions organized in each zone, the captures will take place every night in different capture sites, with a maximum of three consecutive nights on the same site.
As for the CS, in two study areas (Stung Treng in Cambodia and Kindia in Guinea), a longitudinal follow-up will be set up with 6 capture sessions per year during 2 years in these areas, in order to study the dynamics of coronavirus circulation in these species.
The rodents will be captured with Sherman traps and wire traps (for *Rattus* sp.) which have already proven their efficiency for the capture of African and Asian rodents. Capture sessions will last no more than three days per site to avoid adversely impacting the rodent population at a given site.
Traps will be baited with local resources consumed by rodents during the capture period. Cotton will also be placed in the traps to allow the rodents to protect themselves from the cold for the night. The traps will then be armed before nightfall and collected at daybreak. The frequency of trap collection can be adapted according to the weather conditions, especially in case of very hot weather.
On the longitudinal monitoring sites, rodents will be marked with identification nano-chips (Biolog-ID) injected subcutaneously between the shoulder blades. Blood will be collected on filter paper using

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primarily lateral tail vein or saphenous vein incisions. In case of difficulty with these methods, the retro-orbital sinus sampling technique will be used only if there is an experimenter present in the team who is experienced in this particular method; otherwise, the blood collection will be abandoned. Oral-buccal and rectal swabs will also be collected. The rodents will then be released at the capture sites. At other sites, rodents will be euthanized to allow for the collection of organs where viruses of interest can be detected. Euthanasia can be done by two methods. The method used primarily for all animals will consist of anesthetizing the individuals with a cotton ball soaked in liquid isoflurane. The handling of the isoflurane will require great care and the presence of two people to avoid any accident for the manipulator. Then, when the muscular relaxation of the individual is satisfactory, the animal is euthanized by cardiac puncture, which also allows the collection of the blood sample. The autopsy of the euthanized animals is then performed to allow the collection of other biological samples.
In case of a problem with the isoflurane supply during a field mission, euthanasia will be performed by cervical dislocation allowing the separation of the marrow from the brain. This technique will only be performed on animals with low muscle mass and rodents weighing less than 150g. Thus, in the absence of isoflurane, individuals weighing more than 150g will be released without being harvested. The autopsy will be performed according to the safety measures outlined in deliverable D9.6 (EPQ) of this protocol and will allow the collection of the following organs: liver, spleen, kidney, lungs. These samples will be preserved in a tube containing RNA later and placed, if possible, in liquid nitrogen until their storage in the laboratory in a -80°C freezer.

Protocols involving poultry

Domestic chicken sampling will be limited to the four Cambodian study areas of the BCOMING project to analyze the link between biodiversity and microbiota. The main objectives of the microbiota analysis in the BCOMING project are 1) to characterize the influence of biodiversity on microbial communities, providing additional measures of microbial diversity, and 2) to identify potential interactions between bacteria and/or microbiota structure and the prevalence of zoonotic pathogens. Indeed, our knowledge is limited on how the microbiota may impact the ability of viruses to thrive in a new host and how biodiversity and the urbanization gradient may impact microbial diversity and richness.
The analysis of gut microbiota will focus on three groups of animals (bats, rodents, domestic chickens) and humans along the urbanization gradient using metagenomic approaches. Using bacterial association network analysis, we will investigate the relationship between the composition and structure of microbiota and the prevalence of zoonotic pathogens in wildlife and assess whether bacterial consortia or network structures associated with zoonotic pathogens are found in farm animals and humans.

In this study, 400 domestic chickens will be collected, 100 from each study area in Cambodia (Phnom Penh, Battambang, Stung Treng, and Mondulkiri). During the field surveys with the populations living in these areas, the study will be presented, and a request for authorization to collect samples will be made to the local veterinary authorities and the owners of the animals.

Two types of samples will be collected from domesticated hens: droppings and blood. Droppings will be collected via two rectal swabs, and samples will be preserved in ethanol to preserve bacterial DNA and later RNA for virological analysis.
Blood will be collected from the wing vein. The bird is placed in lateral recumbency and the wing is stretched to expose the wing vein. Using a 3 cc syringe and a 25G needle, approximately 1 to 2 cc of blood is collected and stored in a dry tube. After centrifugation, the serum is extracted for serological analysis. Serum should be stored at -80°C if possible, otherwise, at -20°C.

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Protocols involving humans

The procedures for the human biological sampling are also detailed in the ethics deliverable D9.1 relating to humans. The details of the recruitment of participants and the informed consent procedures are provided in deliverable 9.1.
Participants will volunteer approximately one hour of their time for participation in the study, including providing biological samples and completing the questionnaire. Venous blood sample will be collected by standard venipuncture from the right or left antebrachium using a vacutainer dry tube (3-5 mL) using a sterile, disposable needle. Rectal swabs will be collected by gently inserting sterile, flexible, nylon-tipped swabs into the anal canal and rotating while removing. One swab will be collected in nucleic acid stabilization solution (i.e. RNAlater) and another in a viral transport medium (VTM). Nasopharyngeal swabs will be collected by gently inserting sterile, flexible, nylon-tipped swabs into the nasopharynx and rotating for a few seconds before removing it. Similarly to rectal swabs, one swab will be stored in nucleic acid stabilization solution and another in VTM. All specimens will be stored in adequate storage media and stored at +4°C in a cool box, then shipped on a daily basis to IPC to be stored as soon as possible at -80°C until further analysis.
Systematic random sampling will be implemented by selecting a random starting point (household) to initiate the questionnaire and sample collection. Upon completion of the study requirements, study staff will then move to the next household (unit = 1). All eligible person from one unit will be invited to participate in the study.

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