# Step I: White Paper Application

## Application Guidelines

1. *The application should be submitted electronically per requirements via the web site of any of the NIAID Genome Sequencing Centers for Infectious Diseases. Include all attachments, if any, to the application.*
2. *There are no submission deadlines; white papers can be submitted at anytime.*
3. *GSC personnel at any of the three Centers can assist / guide you in preparing the white paper.*
4. *Investigators can expect to receive a response within 4-6 weeks after submission.*
5. *Upon approval of the white paper, the NIAID Project Officer will assign the project to a NIAID GSC to develop a management plan in conjunction with the participating scientists.*

# White Paper Application

## Project Title: Molecular Epidemiology of Enteric Caliciviruses in Ethiopia

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**Primary Investigator Contact:**

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### 1. Executive Summary *(Please limit to 500 words.)*

*Provide an executive summary of the proposal.*

Enteric caliciviruses (norovirus – NoV and sapovirus – SaV), particularly NoVs are responsible for most food-borne gastroenteritis outbreaks worldwide. Information on NoV prevalence, strain diversity and disease burden is lacking in Ethiopia. The major objectives of this project are: Detection and characterization of human and animal NoVs and SaVs from human and animal (domestic and wild ruminants or pigs) fecal specimens from gastroenteritis outbreaks or sporadic cases in Ethiopia adjacent or nearby monitoring sites for water viral contamination, from drinking water and water sources for irrigation, and from unprocessed leafy greens (lettuce) using the water irrigation sources. To attain the above objectives, fecal (animal-domestic free range or wild ruminants or pigs and humans), water and lettuce samples will be collected and processed from the same regions to access common point outbreaks or possible contamination sources. As viral loads in water and food specimens are much lower than those found in fecal samples, standard virus concentration methods will be used (ultracentrifugation and ultrafiltration methods) (Girones *et al.*, 1995). Reverse transcription (RT)-PCR (both conventional and real-time) will be performed for the detection of viral RNA, followed by partial sequence analysis, which allows the molecular identification of circulating strains. Representative strains will be selected and sent to JCVI for full genomic sequencing. The proposed studies will add new knowledge to enteric calicivirus databases regarding potential virus transmission routes and strain diversity, which could be critical for the design of preventive strategies, the development of better diagnostics and future vaccine design. New strains may grow in cell culture and animal models providing research tools for pathogenesis and immunological studies.

### 2. Justification

*Provide a succinct justification for the sequencing or genotyping study by describing the significance of the problem and providing other relevant background information.*

*This section is a key evaluation criterion.*

1. *State the relevance to infectious disease for the organism(s) to be studied; for example the public health significance, model system etc.*

Human norovirus (NoV), is the leading cause of foodborne illness in the US, accounting for 58% of all cases (Scallan *et al*., 2011). In the U.S., human NoVs are listed as Category B biodefense pathogens by NIH, and are on the EPA "candidate contaminant list" for the regulation of drinking water. NoVs are highly contagious due to several reasons. The infectious dose is low, with an estimated medium infectious dose of 18 viral particles ( Teunis *et al*., 2008). The virus is shed at high titers during peak shedding, with a load of approximately 10-11 $\log_{10}$ genomic copies per gram/ml in stool samples (CDC. 2011, Chan *et al*., 2006). Virus shedding can persist for weeks after the symptoms have resolved (Takanashi *et al*., 2010). About 20% of NoV-infected individuals do not show clinical signs ( Moe *et al*., 2009), and asymptomatic individuals, such as food handlers, can be important sources of infection (Hall *et al*., 2012). Besides the fecal-oral transmission route, NoV-infected individuals often vomit and the aerosols formed contain infectious NoVs, which is also critical for the rapid spread and high attack rates of NoVs (Cheesbrough *et al*., 1997). These viruses are environmentally stable and resistant to many disinfectants such as the concentrations of chlorine in some drinking water ( Keswick *et al*., 1985) and alcohol solutions (Liu *et al*., 2010 , Park *et al*., 2010). Human NoVs display high antigenic and genetic diversity, with over 25 genotypes within at least three genogroups (GI, II, IV) (CDC 2011, Zheng *et al*., 2006). Because there is little cross-protection among genotypes, susceptible individuals can be serially infected by different genotypes (Donaldson *et al*., 2008, Takanashi *et al*., 2010). Thus, NoV outbreaks often occur in communities where people live in a confined space and consume prepared food, such as cruise ships, military, hospitals, and nursing homes ( Bailey *et al*., 2005, Enserink 2006, Green *et al*., 2002, Johnston *et al*., 2007, Zingg *et al*., 2008). NoV outbreaks can result in facility closings due to the lack of effective disinfectants for NoVs, causing enormous economic impacts. For example, costs associated with a single NoV outbreak in a US tertiary care hospital were estimated to be $657,644 (Johnston *et al*., 2007). In conclusion, NoVs have become a leading enteric pathogen of public health impact worldwide.

The role of NoV as a causative agent of gastroenteritis in Africa is not well studied. However, outbreaks and sporadic cases of NoV associated gastroenteritis have been reported from some African countries (Maslin *et al*., 2008, Sdiri-Loulizi *et al.*, 2008, Silva *et al*., 2008, Kamel *et al.*, 2009, Kiulia *et al.*, 2010, Mans *et al*.,2010, Mattison *et al*., 2010, Page *et al*., 2010, Ayukekbong *et al.*,2011). No published data is available about the epidemiology of NoV infection in Ethiopia.

SaVs are important enteric pathogens that can cause diarrhea in humans and animals. SaV infects both children and adults and have been found to cause outbreaks of gastroenteritis in day-care centres, healthcare facilities and elementary schools. SaV also causes sporadic cases of acute gastroenteritis requiring hospitalization as well as symptomatic and asymptomatic infections not requiring hospitalisation (Farkas *et al.*, 2000). Human SaVs but not animal SaVs have also been reported in some African countries (Sdiri-Loulizi *et al*., 2011, Mans *et al*., 2011, Kiulia *et al*., 2010; Maslin *et al*., 2008, Dove *et al*., 2005). So far no data is available about the detection of SaV in Ethiopia from humans or animals.

Enteric viruses, including NoVs and SaVs have been isolated from and linked to outbreaks originating from contaminated drinking water sources, recreational and urban rivers, and shellfish harvested from contaminated waters (reviewed in Fong and Lipp 2005). They have been reported to survive and remain infectious for up to 120 days in freshwater and sewage, and for up to 100 days in soil at 20-30°C (Jiang *et al*., 2001). Additionally they are generally more resistant than bacterial indicators during conventional wastewater treatment such as chlorination and filtration and are able to withstand lipid solvents (Jiang *et al*., 2001). This could make water bodies a potential vehicle for water borne transmission of the viruses to the population.

Several molecular epidemiological studies of enteric viruses in developed countries revealed that the incidence is decreasing related to the development of vaccines and improved sanitary conditions. However, in developing countries, including Ethiopia the disease burden is increasing, associated with poor hygiene and sanitary conditions, limited access to safe/clean water, malnutrition and lack of high quality food, immunodeficiencies including HIV and lack of vaccines. To date, there have been few epidemiological surveys to determine the prevalence and genetic characterization of enteric viruses in Ethiopia.
The proposed study will add new knowledge to enteric calicivirus databases regarding potential virus transmission routes and strain diversity, which could be critical for the design of preventive strategies, the development of better diagnostics and future vaccine design. New strains may grow in cell culture and/or in animal models, providing research tools for pathogenesis and immunological studies.

2. *Are there genome data for organisms in the same phylum / class / family / genus? What is the status of other sequencing / genotyping projects on the same organism including current and past projects of the NIAID GSC? Provide information on other characteristics (genome size, GC content, repetitive DNA, pre-existing arrays etc.) relevant to the proposed study. Have analyses been performed on the raw data already generated/published? If additional strains are proposed for a species, please provide a justification for additional strains?*

NoV is genetically diverse and currently classified into genogroup I (GI) to GV by molecular characterization based on the complete capsid sequence (Zheng *et al*., 2006). Genogroup I (GI), GII, and GIV viruses are primarily human pathogens, although there are three porcine specific genotypes within the GII genogroup. The GIII and GV viruses infect bovine and murine species, respectively, and are genetically distinct from human strains (Smiley *et al*., 2003; Karst et al., 2003) while porcine strains are related to human strains genetically and antigenically, with a possibility of zoonotic transmission (Wang *et al*., 2005a; Wang *et al*.,2007). NoVs have also been detected in several animals, including lions, cats and dogs (Martella *et al*., 2007, Martella *et al*., 2008b; Pinto et al., 2012). Each genogroup is further subdivided into genotypes, based on phylogenetic analysis of the complete VP1 sequences. Sapoviruses like NoVs have high genetic variability and are constantly evolving. On the basis of capsid gene (ORF1), SaVs have been classified into five distinct genogroups (GI to GV) (Farkas *et al*., 2004). Each genogroup can be further divided into genetically diverse genotypes. Human sapoviruses belong to genogroups GI, GII, GIV, and GV, whereas porcine sapovirus belongs to genogroup GIII. New porcine sapovirus genogroups (GVI, GVII, GVIII, IX and X) were also proposed (Martella *et al.*, 2008a, Wang *et al.*, 2005a). Recombinant sapoviruses have also been described in both

human and swine hosts (Hansman *et al*., 2005; Wang *et al*., 2005b). In this study, we will conduct molecular biological surveys of the caliciviruses from clinical specimens, animal feces, food and water to identify the current prevalence and genetic diversity of NoVs and SaVs in Ethiopia. To our knowledge no study has been done in Ethiopia to genetically characterize the full genome caliciviruses. While most phylogenetic studies of these enteric viruses are based upon a small segment of the viral genes, full-length sequences of NoVs and SaVs from developing countries like Ethiopia, might provide new insights in relation to the classification and characterization of these viruses and could result in identification of novel genogroups or genotypes. This would advance epidemiologic studies, intervention strategies and vaccine design, which will improve public and animal health.
JCVI already has a pipeline established for full genome sequencing of GI and GII human noroviruses. The inclusion of noroviruses from other genogroups as well as sapoviruses should easily be implemented into the pipeline.

3. *If analyses have been conducted, briefly describe utility of the new sequencing or genotyping information with an explanation of how the proposed study to generate additional data will advance diagnostics, therapeutics, epidemiology, vaccines, or basic knowledge such as species diversity, evolution, virulence, etc. of the proposed organism to be studied.*

Although enteric caliciviruses (NoV and SoV) are the leading cause of gastroenteritis worldwide, many challenges need to be addressed, including understanding reasons for the genetic diversity, their pathogenesis and immunity. The great diversity of NoV/SaV strains is attributed both to the accumulation of point mutations associated with error-prone RNA replication and to recombination. Recombination could result in generation of new viruses with unknown pathogenic potential and altered species tropism for both animals and humans. Full genome sequencing and characterizing sequence variation and diversity is important in understanding calicivirus evolution (including recombination), since emerging human caliciviruses associated with new epidemic strains of unknown origin may evolve from animal sources. This makes surveillance and characterization of caliciviruses from humans, animals as well as the environment, critical.

Immunity to human caliciviruses in general, including human NoVs, is complex and not completely understood (Souza *et al.*, 2008). Multiple factors may be involved in susceptibility or resistance to infection and in the development of immunity to human NoVs (Hutson *et al.*, 2002). However, since NoVs do not grow in cell culture and there is no small animal model for human NoVs, pathogenesis and immunological studies have been hampered. Full-length genome sequencing can identify new strains that may be able to grow in cell cultures and/or replicate in animal models. This will advance in understanding viral pathogenesis and immune responses. Moreover, full genome sequencing could lead to the development of better molecular diagnostics to aid in a better understanding of their epidemiology.

In general, the important aspect of this study is that it adds new knowledge to enteric calicivirus databases regarding potential genetic variability and strain diversity as well as similarities with animal strains, providing new and much needed data for a developing country. Knowledge of the circulating outbreak and environmental enteric viruses and genotypes is critical for vaccine design, implementation and monitoring of vaccine effectiveness, which can advance improvements in public and animal health. Importantly,

viral strains from asymptomatic patients will be detected by screening and sequencing variants from water bodies.

## 3. Rationale for Strain Selection

4. *Provide the rationale behind the selection of strains and the number of strains proposed in the study. The focus of the program is on potential agents of bioterrorism or organisms responsible for emerging or re-emerging infectious diseases. Non-select agents or non-pathogenic organisms will be considered when they can provide insight into these scientific areas.*

Enteric caliciviruses, including noroviruses and sapoviruses that cause food and water borne diarrheal infections, are of public and animal health concern. First they are shed in extremely high numbers in the feces of infected individuals and animals (Farthing 1989). Second, they have a low infectious dose. Third, enteric viruses are very stable in the environment and they can survive under a wide pH range (pH 3 to 10) and at low temperatures for extended periods (Kocwa-Haluch *et al*., 2001). They have been reported to survive and remain infectious for a long time in freshwater, sewage and soil (Jiang *et al*., 2001). They are also more resistant than bacterial indicators during conventional wastewater treatment (Jiang *et al*., 2001). This could make water bodies a potential vehicle of transmission of the viruses to the population. Enteric viruses have been isolated from and linked to outbreaks originating from contaminated drinking water sources, recreational and urban rivers (reviewed in Fong and Lipp 2005). In the U.S., human NoVs are listed as Category B biodefense pathogens by NIH, and are on the EPA "candidate contaminant list" for the regulation of drinking water. Moreover NoVs and SaVs are RNA viruses which could evolve quickly and change disease patterns and host tropisms. They are also considered as emerging pathogens with a high potential of genetic diversity including recombination. These characteristics have important public health significance associated with the emergence of new epidemic strains of unknown origin either from human or animal sources.

Positive samples from humans and animals (pigs and calves) from Ethiopia will be characterized briefly by sequencing the RT-PCR products. Representative strains of NoV and SaV will be selected based on the partial sequences for full-genomic sequencing at JCVI. We estimated that >40 calicivirus samples will be from Ethiopia. Dr. Saif's lab has historical human and animal NoV and SaV strains whose complete genomes have not been characterized. For comparison of strains from developing and developed countries, 36 such US samples will be sequenced at JCVI. Complete genome sequencing of both US and Ethiopian samples will improve our understanding of recombination frequency among caliciviruses and identify possible epistatic interactions between viral genes.

## 4a. Approach to Data Production: Data Generation

5. *State the data and resources planned to be generated. (e.g draft genome sequences, finished sequence data, SNPs, DNA/protein arrays generation, clone generation etc.)*

Fecal, water and food samples will be collected from the same regions to access common point outbreaks or possible contamination sources in Ethiopia. A cross sectional study will be conducted. Water samples from different sites (river, drinking and irrigation) and feces from human and domestic free range or wild ruminants or pigs will be collected and

processed. As viral loads in water and food samples are much lower than those found in fecal samples, detection of enteric viruses in water requires methods of concentrating the virus (Queiroz, *et al.*, 2001). In this study we will use ultracentrifugation and ultrafiltration method to concentrate the viruses (Girones *et al*., 1995). The viral nucleic acid will be extracted from fecal suspensions, concentrated viruses from water and food samples by using a commercial kit for RNA extraction (RNeasy mini kit, Qiagen, USA) according to the manufacturer's instructions. The RT-PCR assays (both conventional and real-time) using specific primer pairs are the primary assays for detection of enteric viruses. The PCR products will be sequenced to determine variations in viral genetic sequences, if present. Each nucleotide sequence will be analyzed using Basic Local Alignment Search Tool (BLAST) program to determine which virus sequence it is. Virus sequences will be compared with those of published strains deposited in the NCBI (National Center for Biotechnology Information) GenBank database (http:\\www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment will be performed by using Clustal W software. A phylogenetic tree of aligned sequences will then be constructed by the neighbor-joining method using MEGA4 software (Tamura et al., 2007). The nucleotide sequences will be translated to deduce amino acid sequences, which will also be compared to detect changes at the amino acid level, if any. Based on preliminary characterization (RT-PCR and partial sequencing), representative strains of NoV and SaV will be selected for full-genomic sequencing by the JCVI.

## 4b. Approach to Data Production: Data Analysis

6. *Briefly describe the analysis (value-add) envisioned to be performed subsequently by the community and the potential to develop hypotheses driven proposals given the datasets and resources produced by this work.*

Viral gastroenteritis is a major cause of morbidity and mortality worldwide. To date, there have been few epidemiological surveys that aimed to determine the circulation and genetic characterization of enteric viruses in Ethiopia. While most phylogenetic studies of these enteric viruses are based upon a small segment of the viral genes, full-length sequences of NoVs and SaVs from developing countries like Ethiopia, will provide different insights on the classification and characterization of these viruses and could result into identification of novel genogroups or genotypes**.** Furthermore, we can compare viral strains from outbreaks with those of the strains from environmental and food specimens, similar (identical) strains that could have been linked with the infecting strains from clinical samples can be traced in fecally contaminated foods and water, indicating links and possible evidence of viral contamination of water bodies by infected persons. We also can compare the strains from developing and developed countries (Ethiopia vs the US), in the former where humans and animals contact closely. The results from this proposed project will also give base line data for future studies. Moreover, the information generated from this proposed work can be utilized by various governmental and non-governmental bodies to improve health care efforts in the country and future vaccine development. Government policy makers can use the outcome of this research to take steps in making irrigation water for planting vegetables clean and safe by developing new techniques to minimize the pollution of water and hence contamination of foods.

## 5. Community Support and Collaborator Roles:

7. *Provide evidence of the relevant scientific community's size and depth of interest in the proposed sequencing or genotyping data for this organism or group of*

*organisms. Please provide specific examples.*

In this proposed project prominent and relevant scientists in the areas are involved. Dr. Linda Saif, a Distinguished University Professor, who is known nationally and internationally for her work on enteric viral diseases including caliciviruses, is the coordinator of this project. Dr. Saif detected for the first time porcine sapovirus–Cowden strain (porcine enteric calicivirus) in the USA (Saif et al., 1980). The other virologist involved in the study is Dr. Qiuhong Wang, who has contributed to the molecular epidemiology of enteric caliciviruses and on the development of methods for their detection (Wang et al., 2007). Molecular epidemiologists like Dr. Wondwossen Gebreyes and Dr. Appolinaire Djikeng are also involved in the project to contribute to overall study design and sampling. Because few calicivirus genomes are available from few African countries and especially ones from diverse sources including humans, food, animals and the environment.

8. *List all project collaborators and their roles in the project*

a. LJ Saif (Distinguished University professor), Project coordinator, Research advisor
b. Qiuhong Wang (MD, PhD), Research advisor
c. Zufan Sisay Worku (Bsc, MSc), PhD student
d. Dr. Nega Berhe (MD, PhD), Research advisor
e. Dr. Wondwossen Gebreyes, DVM, PhD, Research advisor
f. Dr. Appolinaire Djikeng (PhD), Kenyan collaborator and supervisor

**Institutes**:

g. Aklilu Lemma Institute of Pathobiology, Addis Ababa, Ethiopia
**Role**:
   - Home Institute for the PhD student
   - Facilitates sample collection (fecal samples from animals, obtaining human stool specimens from clinics and collecting water samples and leafy vegetables from river and irrigation sites in Ethiopia)
   - Granting permits as needed

h. Department of Biology, Addis Ababa University, Ethiopia
**Role**:
   - Graduate school of the PhD student
   - Facilitates sample collection (fecal samples from animals, obtaining human stool specimens from clinics and collecting water samples and leafy vegetables from river and irrigation sites in Ethiopia)
   - Granting permits as needed

i. Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia
**Role**:
   - Laboratory site in Ethiopia
   - Processing (RNA extraction, virus concentration) of fecal, water and food samples collected from Ethiopia
   - Preliminary characterization of caliciviruses (RT-PCR and partial sequencing) of clinical specimens collected

j. The Biosciences eastern and central Africa – International Livestock Research Institute Hub (BecA-ILRI Hub), Nairobi, Kenya
**Role**:
- Laboratory training site
- Preliminary characterization of caliciviruses (RT-PCR and partial sequencing) from concentrated viruses (water and food) or RNA extracted from animal fecal specimen

k. Food Animal Health Research Program, Dept. of Veterinary Preventive Medicine, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691.
**Role:**
- Drs. L.J. Saif and Q. Wang's laboratories
- Laboratory training site for the PhD student, Ms. Zufan Sisay Worku
- Preliminary characterization of US caliciviruses (RT-PCR and partial sequencing)

l. JCVI, USA
**Role**:
- Based on preliminary characterization (RT-PCR and partial sequencing), full genome sequencing of selected representative animal and human caliciviruses (NoV and SaV), from Ethiopia and the US.

9. *List availability of other funding sources for the project.*

-NIH-Fogarty
-We have applied for various funding sources and are waiting for their responses.

## 6. Availability & Information of Strains:

10. *Indicate availability of relevant laboratory strains and clinical isolates. Are the strains/isolates of interest retrospectively collected, prepared and ready to ship?*

*Note: If samples are prospectively prepared the GSC can provide protocols and recommendation based on the Centers past experiences. The samples must however meet minimum quality standards as established by the Center for the optimal technology platform (sequencing/ genotyping) to be used in the study.*

The relevant reference strains are available from Dr. Saif's lab and the US CDC. Sample collection in Ethiopia currently is ongoing and will continue till May 2013. Samples will be kept frozen before testing and shipping to JCVI. The US samples were retrospectively collected from humans and animals and are ready to be shipped to JCVI. Filled metadata file of the US samples is attached.

11. *Attach relevant information, if available in an excel spreadsheet for multiple samples: e.g*
    - *Name*
    - *Identifier*
    - *Material type (DNA/RNA/Strain)*
    - *Genus*
    - *Species*

- *Specimen / Strain*
- *Isolation source*
- *Isolated from*
- *Select agent status*
- *International permit requirement*
- *BEIR/ATCC repository accession number*
- *Other public repository location*
- *Other public repository identifier*
- *Sample provider's name*
- *Sample provider's contact*

12. *What supporting metadata and clinical data have been collected or are planned on being collected that could be made available for community use?*

- For the samples collected in Ethiopia, patients' clinical history such as the presence of watery diarrhea, nausea, vomiting, abdominal cramps, headache, muscle pain and/or fever will be included in the study.
- The necessary parameters, including water temperature and pH, will be taken during water sample collection.

**References:**


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**7. Compliance Requirements:**

**7a. Review NIAID's Reagent, Data & Software Release Policy:**

*NIAID supports rapid data and reagent release to the scientific community for all sequencing and genotyping projects funded by NIAID GSC. It is expected that projects will adhere to the data and reagent release policy described in the following web sites.*

*http://www3.niaid.nih.gov/LabsAndResources/resources/mscs/data.htm*

*http://grants.nih.gov/grants/guide/notice-files/NOT-OD-08-013.html*

*Once a white paper project is approved, NIAID GSC will develop with the collaborators a detailed data and reagent release plan to be reviewed and approved by NIAID.*

Accept ☐ Decline ☐

**7b. Public Access to Reagents, Data, Software and Other Materials:**

13. *State plans for deposit of starting materials as well as resulting reagents, resources, and datasets in NIAID approved repositories. Sequencing projects will not begin until the strain is deposited into NIAID funded BEI repository (http://www.beiresources.org/). This includes web based forms are completed by the collaborator and received by the NIAID BEI (http://www.beiresources.org/).*

**7c. Research Compliance Requirements**

*Upon project approval, NIAID review of relevant IRB/IACUC documentation is required prior to commencement of work. Please contact the GSC Principal Investigator(s) to ensure necessary documentation are filed for / made available for timely start of the project.*

**Investigator Signature:**

**Investigator Name:** **Date**