Interim Technical Note

Introduction of DNA-based identification and typing methods to public health practitioners for epidemiological investigation of cholera outbreaks

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Objective

The objective of this note is to provide information summarizing the added value of monitoring *Vibrio cholerae* strains associated with diarrheal disease using DNA-based techniques as part of comprehensive cholera prevention and control.

Specific objectives:

- To provide a brief technical overview of the molecular techniques to be applied to cholera samples
- To provide operational information on how to access DNA-based identification, characterization and typing methods and how to store and send biological samples to testing laboratories depending on the questions to be answered.

Target audience

Public health practitioners in cholera-affected countries needing information on how molecular diagnostic testing using DNA-based methods is useful for cholera control.

Background

Laboratory-based microbiological identification and monitoring of strains is the mainstay of infectious disease surveillance. Discriminating between epidemiologically related and unrelated strains, determining their genetic relationship and detecting the emergence of new subtypes is essential for understanding the source of infection and improving cholera surveillance.
Effective surveillance entails detection of the earliest cases, identification of the source(s) of infection (, and an understanding of the dynamics of transmission, so as to quickly implement appropriate control measures. As the clinical presentation of most cholera cases is not specific to the disease, the identification of the etiologic agent from stool samples is needed for confirmation of the diagnosis and outbreak declaration.

Historically, culture and use of species-specific anti-sera has been the mainstay of *Vibrio cholerae* identification. Molecular tests, using more technologically advanced techniques, have been developed in recent years permitting the traditional identification information while delving much deeper into genomic features that expand the epidemiologic analyses possible[1, 2, 3, 4].

DNA-based molecular tests increase the quantity of specific DNA sequences or uncover the entire genome to facilitate pathogen detection and provide a unique genomic signature for the pathogen being analysed. They have a wide spectrum of indications, from the detection and identification of cholera strains, to the monitoring of their geographic expansion during and between outbreaks and the evolution of key *Vibrio* genetic elements over time. In addition to etiologic identification, molecular typing techniques are able to answer a wide range of questions related to the global investigation of cholera outbreaks by allowing

comparison of strains from diverse origins (isolated in different countries, in different years, from clinical and environmental sources), and data on strains' characteristics can be exchanged and recorded in large and open access databases of characterized organisms.

Molecular typing techniques require sophisticated equipment and trained laboratory staff. Hence, their use has so far been restricted to research institutes, and less-resourced cholera endemic countries have not yet fully benefited from these technological advances. In such settings, having access to a regional or international reference laboratory through well documented shipping and reporting procedures should be considered a priority.

An improved engagement of endemic countries and control programs in molecular testing is expected to better inform the public health practitioners and enhance the management of cholera outbreaks.

Molecular methods applicable to cholera samples

The main indications for molecular methods applied to cholera samples are:

- Rapidly identifying toxigenic *Vibrio cholerae* in stool specimens of suspect cases.
- Tracking the genetic evolution of *V. cholerae* strains and detecting the emergence of new clones.
- Detecting the presence of known antimicrobial resistance encoding genes[1].
- Establishing the genetic relatedness of strains between the current and previous outbreaks (similar or different sources of infection, related or independent events).
- Understanding the localized geographical spread of strains in a given country or between neighbouring countries and mapping the origin and expansion of transmission.
- Conducting phylogenetic analyses to enable the visualization of world-wide circulation.

Tests and technologies: all techniques presented here for the detection, characterization and genotyping of *Vibrio cholerae* strains are based on the extraction of genetic material, either directly from biological/ environmental samples or from isolated bacterial strains, followed by genetic analysis of either targeted cholera specific DNA sequence(s) or the entire bacterial genome.

The recommended timeline for testing is at the beginning of a suspected outbreak (for strain identification and characterization) and then periodically during the outbreak until its conclusion to monitor the circulating strains (see WHO "Guidance document on cholera surveillance").

1. DNA-based techniques for identification and characterization of cholera *Vibrio* strains: PCR tests

Polymerase Chain Reaction tests (PCR), based on DNA-specific combinations of sequences unique to the pathogen, are an alternative to culture and biochemical analysis for the identification of *Vibrio cholerae* strains.

[1] It is important to consider that unexpressed resistance genes may occur. In addition, detection of antimicrobial resistance genes by PCR or WGS does not allow the detection of resistance mechanisms which are not known. It remains necessary to determine the antimicrobial resistance phenotype of isolated strains at the beginning of the outbreak and regularly along the course of the epidemic (see WHO "Guidance document on cholera surveillance").

PCR tests can provide the following information for the identification and additional characterization of *Vibrio cholerae* strains:

- **Identification:**
  - Species (*V. cholerae)*
  - Serogroup (O1, O139)

- **Characterization:**
  - Presence of cholera toxin genes (*ctxA*, *ctxB*)[2]
  - Biotype (El Tor or Classical)
  - Presence of known antimicrobial resistance encoding genes

Advantages: PCR can be performed quickly as it does not require pure cultures or even viable organisms as it amplifies target DNA directly from stool, food, or environmental samples without culturing steps. PCR does not require the use of a biosafety cabinet. Results can be delivered quickly (3 to 4 hours). Material collected on dry filter papers is easier to ship to testing sites (see transport regulations below). PCR is feasible on samples collected from patients having recently started an antibiotic treatment.

Additional needs: PCR requires specialized equipment (but not specific to cholera testing), dedicated training and some level of standardization to ensure reliable results, access to supplies and specific infrastructure.

2. DNA-based techniques for advanced genotyping *of Vibrio cholerae* strains

Following the identification (species, serogroups) and the characterization of the cholera strains (biotypes and presence of toxin genes) by PCR, a more refined level of information is obtainable through the investigation of the genotypic features of the strains.

The strength of a typing method depends on its capacity to distinguish between strains, its reproducibility, ease of performance, cost, rapidity, as well as the potential to generate sharable data through standardized reports and consultation of an open-access database.

Various molecular typing techniques have been used over time for cholera epidemiological investigations (e.g PFGE, MLST). Two main genotyping methods are currently recommended as the most powerful tools for understanding the epidemiology of cholera, Multiple Loci VNTR Analysis (MLVA)[5,6,7] and Whole Genome Sequencing (WGS)[8,9]. The choice of which of these methods essentially depends on the purpose and scale of the investigations. Both methods can be applied to human, food, and environmental samples. They complement the information obtained by the techniques used for identification by culture and/or PCR. Nevertheless, they are not intended to be used in a stand-alone approach.

Due to their specific outcomes, it may be recommended to combine the two techniques to gather comprehensive information sets, again depending on the purpose of the investigation.

The main features of the two methods are outlined in Table 1 below.

[2] The detection of the Cholera Toxin (CTX) genes may be necessary to confirm the identification of toxigenic *V. cholerae* in new suspect case(s) in previously non-affected countries or in those that have eliminated the disease.

Table 1: Overview of indications and limitations of main molecular techniques used for Cholera strain characterization and monitoring of outbreaks

Method	Multiple Loci VNTR Analysis (MLVA)	Whole Genome Sequence (WGS)
Indications	Local analysis over a restricted period (*i.e.* **within the same outbreak**) Provides insights into outbreak relatedness at a micro-evolutionary level.	Identification of the geographic origin of infection and follow-up of spread of bacteria, complete characterization and tracking of the cholera strains **between different epidemics across time and space**. Allows both short and long-term analysis of trends of genetic evolution.
Advantages	Can provide rapid answer and allow delivery of timely information. Easier to set up than WGS	The sequence data offer the ultimate resolution in strain typing and produce definitive answers about strains' characteristics.
Additional needs	Need for specific equipment and dedicated training	Need for sophisticated equipment and dedicated training including bio-informatic skills. Requires reliable high speed internet collection for transmission of data files. Available in few reference centres only.

Storage and shipment of samples

The ability to properly collect and transport samples is a critical component of the investigation. As the attribute of the results is highly dependent on the quality of the samples to be tested, health practitioners should pay attention to the following requirements for the collection, the storage of the samples, and their shipment to the recipient laboratory.

Table 2: Best practice for storage and shipment of cholera samples

	How to store the samples on site of collection?
Stools	• Kept at ambient temperature up to 4 hours after the collection OR • Refrigerated if the delay between the time of collection and testing is expected to exceed 4 hours OR • Transferred to Cary Blair transport medium at ambient temperature for longer term on-site storage or until testing can begin. OR • Deposited on moistened filter paper placed in a screw-cap microtube with a few drops (around 200 µl) of saline (0.9% NaCl) to prevent the sample from drying[10].

	Stored at ambient temperature OR • Deposited on dry filter paper[11,12] and stored at ambient temperature NOTE: • Stool samples stored on dry filter paper do not allow the recovery of viable strains (*i.e.* for culture confirmation and antimicrobial susceptibility testing), only DNA analysis. • Stool samples stored on moistened filter paper and Cary Blair medium maintain viable organisms for further culture in addition to DNA analysis. • Shipping regulations are less strict for dried samples than the moistened, still-viable samples (see "How to send the samples" below)	
Isolated strains (from culture)	• Kept in the laboratory on solid non-selective culture medium in test tubes for a few days at room temperature OR • In Stock Culture Agar inoculated with a fresh culture for longer periods (several years) at room temperature. Inoculation in Stock Culture Agar must be followed by an incubation step at 37°C to ensure bacterial growth but this step is not necessary if the ambient temperature is sufficient to allow a good growth of the culture (between 20°C and 37°C). • In both cases, tubes must be tightly capped to reduce evaporation and dehydration. **For MLVA and WGS testing isolated strains are preferred to stool samples**	
How to send the samples?		**Transport Regulations**
1. Within the same country by <u>ROAD</u>: use primary and secondary leak proof containers and triple packaging (UN 3373)[13] **2. Shipment within the country or abroad by <u>AIR</u>: use primary and secondary leak proof containers and triple packaging (UN 3373). Strictly follow IATA regulations for <u>biological material category B.</u>[13]**		
Stool	• In Cary-Blair transport medium (if available) per the manufacturer's recommendations (rectal swab or swab dipped into the non-chlorinated liquid stool specimen are immersed in the transport medium). Shipment at ambient temperature OR • Deposited on a moistened filter paper[10] placed in a screw-cap microtube with few drops (around 200 µL) of saline (0.9% NaCl) to prevent the sample from drying. Shipment at ambient temperature OR • Deposited on a dry filter paper[11,12]. Shipment at ambient temperature	Inoculated Cary Blair media are regulated materials (UN 3373). Moistened filter papers are regulated materials (UN 3373). Dry filter papers are NOT regulated

		(Exempt human specimens); they can be sent by routine postal services.
Isolated strains (from culture)	• In stock culture agar; shipment at ambient temperature OR • Deposit a bacterial suspension in liquid medium on filter paper, and moistened or dried, with the same limitations as mentioned above	Isolated strains are regulated material (UN 3373).

Regulatory and Administrative Requirements for collection and transportation of samples[13]

The following documents may be required depending on the local regulation of the recipient labs:

- Request form of the recipient laboratory
- Authorisation for export and import from relevant authorities in countries
- Ethical approval from an institutional review board (IRB) of the exporting country on an ad hoc basis in the case of clinical research projects (not required for diagnosis purposes)
- Material Transfer Agreement

In any case, it is mandatory to:

- Inform the recipient laboratory about the arrival of the samples
- Identify a focal point person (names and contact details) to whom the recipient laboratory can send the results

Where to send the samples?

When capacity for a microbiological and genotyping reference laboratory is not available at the national level, access to these services in another country should be facilitated. National laboratories and cholera control programmes are invited to approach international reference laboratories for information and support.

Cholera endemic countries should develop protocols and standard operating procedures for sample collection and shipment in collaboration with partners having fully equipped testing facilities abroad to increase their access to molecular testing of *Vibrio cholerae*. It is expected that scientific collaboration between countries at risk of cholera and international teams will mutually benefit from these partnerships which can be opportunities for training and providing a better understanding of the epidemiology and transmission patterns of cholera, both globally and at the local level.

A cholera strain data bank is being developed to optimize exchange of information in real time and transparency and to facilitate data sharing and analysis.

Testing facilities should communicate test results to the sampling sites or to the team in charge of cholera surveillance as soon as possible. Closing the communication loop between the time any testing is prescribed and the time the result is delivered to the relevant partners is critical. The identification of focal point persons (responsible for communication flow between the surveillance team and laboratory staff) is vital for the quality and the timeliness of information exchange.

The following reference laboratories and scientific teams have already joined the network. The table below describes their molecular testing capacities and provides details for contact persons.

Table 3: Information related to availability of DNA-based tests within the Cholera network. Open list to be up-dated on an ad-hoc basis

Institutions	Available methods	Contact person
Pasteur Institute, France	MLVA, WGS	Marie-Laure Quilici quilici@pasteur.fr
Sanger Institute, UK	WGS	Nicholas Thomson nrt@sanger.ac.uk
University of Maryland, USA	MLVA, WGS	Colin Stine CSTINE@epi.umaryland.edu
NICD, South Africa	MLVA, WGS	Anthony Smith anthonys@nicd.ac.za Dr Karen Keddy karenk@nicd.ac.za
CDC Atlanta, USA	WGS	Maryann Turnsek hud4@cdc.gov
Centre for Human Microbial Ecology Translational Health Science and Technology Institute, Faridabad India	WGS	T. Ramamurthy tramu@thsti.res.in

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