Annex A
Diagnostic Supplies Needed for 1 Year for Laboratory Confirmation of Outbreaks and for Laboratory-Based Surveillance for *Vibrio cholerae* O1/O139 Antimicrobial Susceptibility

Assumptions:

- Supply each district with materials to collect and transport 50 specimens
- Supply each regional laboratory with materials to process 100 specimens
- Supply each national reference laboratory with materials to confirm 500 isolates

District Level

Supplies needed for each district

50 cotton swabs
50 bottles or tubes of Cary-Blair (or other) transport medium
Transport for specimens to regional laboratory

Regional Level

Supplies needed for each regional laboratory

100 sterile cotton or polyester swabs
500 g Cary-Blair medium
500 g TCBS medium
25 g sodium desoxycholate
Glass slides for serologic testing and string test
5 g *N*,*N*,*N*′,*N*′-tetramethyl-D-phenylenediamine dihydrochloride (oxidase reagent)
Filter paper for oxidase test
Sterile wooden sticks or platinum inoculating loops for oxidase test
500 g nonselective agar* (e.g., tryptone soy agar, heart infusion agar)
4 x 2 ml polyvalent *V. cholerae* O1 diagnostic antiserum
500 g Bacto-peptone medium
500 g NaCl
NaOH
pH paper or pH meter
500 petri dishes (9 cm)
1000 test tubes (e.g., 13 x 100 mm or 16 x 125 mm)
Transport for specimens to reference laboratory
Materials and postage for production and dissemination of reports

*Do not use nutrient agar because some formulations have no added salt and do not allow optimal growth of *V. cholerae*.

National Reference Laboratory

Supplies needed by each national reference laboratory for confirmation

5 x 100 sterile cotton or polyester swabs
5 x 500 g Cary-Blair medium
5 x 500 g TCBS medium
5 x 25 g sodium desoxycholate
Glass slides for serologic testing and string test
5 x 5 g *N,N,N',N'*-tetramethyl-D-phenylenediamine dihydrochloride (oxidase reagent)
Filter paper
Sterile wooden sticks or platinum inoculating loops
5 x 500 g nonselective agar* (e.g., tryptone soy agar, heart infusion agar)
20 x 2 ml polyvalent *V. cholerae* O1 diagnostic antiserum
5 x 2 ml *V. cholerae* O139 diagnostic antiserum
5 x 2 ml *V. cholerae* O1 serotype Ogawa diagnostic antiserum
5 x 2 ml *V. cholerae* O1 serotype Inaba diagnostic antiserum
5 x 500 g Bacto-peptone medium
5 x 500 g NaCl
NaOH
pH paper or pH meter
5 x 500 petri dishes (9 cm)
5 x 1000 test tubes (e.g., 13 x 100 mm or 16 x 125 mm)

Antimicrobial susceptibility test supplies (disk diffusion method)

5 x 500 g Mueller-Hinton agar
200 disks of the following antibiotics
- Trimethoprim-sulfamethoxazole
- Chloramphenicol
- Furazolidone (if furazolidone is being considered for use in cholera treatment)
- Tetracycline

Control strain *Escherichia coli* ATCC 25922
0.5 McFarland turbidity standard
Sterile cotton swabs
Sterile saline
Forceps and 95% alcohol for flaming
Zone size criteria chart
Materials and postage for production and dissemination of reports

*Do not use nutrient agar because some formulations have no added salt and do not allow optimal growth of *V. cholerae.*

Annex B
Supplies Needed for Laboratory Identification of *Shigella dysenteriae* 1 During an Outbreak

Assumptions:

- Supply each district with materials to collect and transport 50 specimens
- Supply each regional laboratory with materials to process 100 specimens
- Supply each national reference laboratory with materials to confirm 500 isolates

District Level (Materials to collect and transport 50 specimens)

Supplies needed for each district

100 cotton swabs
50 bottles or tubes of Cary-Blair or other transport medium
Transport for specimens to regional laboratory

Regional Level (Materials to process 100 specimens)

Supplies needed for each regional laboratory

200 sterile cotton or polyester swabs
100 bottles or tubes of Cary-Blair (or other) transport medium
500 g XLD medium
500 g MacConkey medium
500 g Kligler iron agar
500 g motility agar
500 g nonselective agar (e.g., tryptone soy agar, heart infusion agar)
Diagnostic antisera:
  4 x 2 ml monovalent *S. dysenteriae* serotype 1 (not Group A polyvalent)
  2 x 2 ml polyvalent *S. flexneri* (Group B)
  2 ml polyvalent *S. sonnei* (Group D)
Glass slides for serologic testing
500 disposable petri dishes (9 cm)
1000 disposable test tubes (e.g., 13 x 100 mm or 16 x 125 mm)
Transport for specimens to reference laboratory
Materials and postage for production and dissemination of reports

National Reference Laboratory (Materials to confirm 500 isolates)

Supplies needed by each national reference laboratory for confirmation

500 sterile cotton or polyester swabs
5 x 500 g Cary-Blair medium or other transport medium
5 x 500 g XLD medium
5 x 500 g MacConkey medium
3 x 500 g Kligler iron agar
3 x 500 g motility agar
3 x 500 g nonselective agar (e.g., tryptone soy agar, heart infusion agar)
Diagnostic antisera:
- 20 x 2 ml monovalent *S. dysenteriae* serotype 1 (not Group A polyvalent)
- 10 x 2 ml polyvalent *S. flexneri* (Group B)
- 5 x 2 ml polyvalent *S. sonnei* (Group D)

Glass slides for serologic testing
5 x 500 disposable petri dishes (9 cm)
5 x 1000 disposable test tubes (e.g., 13 x 100 mm or 16 x 125 mm)

Antimicrobial susceptibility test supplies for 100* Shigella *isolates

2 x 500 g Mueller-Hinton Agar
200 disposable petri dishes (9 cm)
200 disks of the following antibiotics:
- Trimethoprim/sulfamethoxazole
- Chloramphenicol
- Ampicillin
- Nalidixic acid
- Ciprofloxacin (1 cartridge only)

Control strain *Escherichia coli* ATCC 25922
0.5 McFarland turbidity standard
Sterile cotton swabs
Sterile saline
Forceps and 95% alcohol for flaming
Zone size criteria chart
Materials and postage for production and dissemination of reports

Annex C
Guidelines for Establishing a Public Health Laboratory Network for Cholera Control

Purpose

- To establish a routine system for confirming the presence of *Vibrio cholerae* O1 and O139.
- To monitor the antimicrobial susceptibility patterns of *V. cholerae* O1 isolates from throughout the country.
- To provide feedback to guide development of appropriate antimicrobial treatment policies for cholera.

Overview

When outbreaks of a cholera-like illness occur, there is a need for accurate data to confirm the presence of *V. cholerae* O1. In addition, data about the antimicrobial susceptibility patterns of *V. cholerae* O1 isolates from throughout the country are needed to develop an effective antimicrobial treatment policy. Following is an outline of a system involving regional and reference laboratories to carry out these activities. Laboratories at different levels have corresponding degrees of responsibility for collection and transport of specimens, identification and confirmation of isolates, and feedback of the results to the appropriate levels. The roles and responsibilities of each level are outlined below, along with the basic supplies needed to carry out these activities. A full listing of the supplies needed for a 1-year period can be found in Annex A.

A. Surveillance

The laboratory-based surveillance will consist of two parts:

- Initial confirmation of the outbreak
- Ongoing surveillance for antimicrobial susceptibility of the *V. cholerae* isolates.

1. Initial confirmation of the outbreak

In areas currently not experiencing a cholera outbreak, cholera should be suspected if a patient older than 5 years develops severe dehydration or dies from acute watery diarrhea, or if there is a sudden increase in the daily number of patients with acute watery diarrhea. If such events are noted, 5 to 10 stool specimens should be sent to the regional laboratory for confirmation. Specific instructions for collecting and transporting stools can be found in the WHO "Guidelines for Cholera Control" and in Chapter 2 in this manual. A stool specimen data sheet to send with the specimens is found in Annex F.

Once the outbreak is confirmed, it is not necessary to collect specimens from additional patients for diagnosis. The diagnosis for treatment purposes can be made on clinical criteria. Collecting and processing an excessive number of stool specimens can quickly deplete scarce laboratory resources.

In areas where cholera is known to be present, confirmation of additional outbreaks is not necessary.

2. Surveillance for antimicrobial susceptibility of *Vibrio cholerae* isolates

Every 3 months, the regional laboratories in areas that are affected by cholera should each send 10 to 20 *V. cholerae* isolates to the national reference laboratory for susceptibility testing. The affected districts in the region should each send sufficient specimens for the regional laboratory to achieve this number. The regional laboratory should send these isolates to the reference laboratory for confirmation. A representative sample of isolates from each reference laboratory (a total of 10 to 20) should periodically be sent to an international reference laboratory for confirmation of the antimicrobial susceptibility pattern and possibly for additional studies, such as subtyping by ribotyping, pulsed-field gel electrophoresis or other molecular studies. Arrangements can be made through WHO for sending these isolates to an international reference laboratory on a regular basis.

B. Roles of District, Regional, and Reference Laboratories

1. District level

When an outbreak begins in a district, it should be confirmed by collecting 5 to 10 stool specimens and sending them to the regional laboratory for confirmation of the presence of *V. cholerae.* The basic materials needed at the district level to collect the stool specimens are as follows:

- Cary-Blair (or other) transport medium in tubes
- Sterile swabs

Each district should have sufficient supplies to send 50 stool specimens to the regional laboratory. In addition, the district will need to develop a rapid and reliable means of sending the specimens to the regional laboratory.

2. Regional level

The regional laboratory receives the specimen from the district and performs the initial isolation of *V. cholerae*. Each region should have sufficient supplies to identify at least 100 isolates of *V. cholerae* O1 and to send the isolates to the national reference laboratory for additional testing. The basic materials needed at the regional level are as follows:

- Thiosulfate citrate bile salts sucrose agar (TCBS) medium
- Ingredients to prepare alkaline peptone water
- Polyvalent O1 *V. cholerae* diagnostic antisera

- Heart infusion agar (HIA) or other nonselective medium
- Petri dishes
- Tubes for transport (HIA used as transport medium for isolates)

3. Reference level

The regional laboratory sends isolates to a national reference laboratory for confirmation and antimicrobial susceptibility testing. Each reference laboratory will need sufficient materials to confirm at least 500 isolates sent from the regional level throughout the year. The basic materials needed are as follows:

- TCBS medium
- Ingredients to prepare alkaline peptone water
- Polyvalent O1 and O139 group *V. cholerae* diagnostic antiserum
- Monovalent Ogawa diagnostic antiserum
- Monovalent Inaba diagnostic antiserum
- HIA or other nonselective medium
- Petri dishes
- Tubes for transport and storage of isolates (HIA medium used for this)
- Supplies for antimicrobial susceptibility testing (see Annex B)

4. Referral to international reference laboratories

As part of the laboratory-based surveillance process, isolates should periodically be sent to an international reference laboratory for confirmation of the antimicrobial susceptibility patterns. This is especially important if strains exhibit a new or unusual antimicrobial susceptibility pattern. Arrangements can be made through WHO for sending such specimens to an international reference laboratory and to provide for the rapid feedback of the results.

5. Feedback of Results

Regional laboratory to district, confirmation of outbreak

When the regional laboratory confirms the presence of *V. cholerae* O1 in stool specimens received from the district, it should contact the district as quickly as possible to inform the health authorities that *V. cholerae* O1 has been identified from the district.

Reference laboratories to regional laboratories

The reference laboratory should regularly communicate the results of the studies carried out on isolates submitted from the regional laboratory. The results should be sent to the regional laboratory and to the Ministry of Health. This includes the results of isolates sent both for confirmation of outbreaks and for the routine surveillance for *V. cholerae* carried out every 3 months. These results can serve as an internal quality control for the regional laboratories. In addition, every 3 months summaries of the results from all national reference laboratories should be distributed to the regional laboratories and appropriate persons in the Ministry of Health for further distribution to all relevant parties.

International reference laboratory to reference laboratory

The international reference laboratory should provide timely feedback of results to the national reference laboratory that is coordinating the shipping of the specimens. These results will be shared with the other reference laboratories and will serve as an external quality control for identification of *V. cholerae* O1 and O139 and for determining the antimicrobial susceptibility of the these strains.

6. Additional components for network

This system could be expanded to include other bacterial pathogens, such as those causing dysentery. For instance, periodic surveillance of isolates from patients presenting with bloody diarrhea could be done to determine the prevalence of various organisms causing dysentery and their antimicrobial susceptibility patterns.

Annex D
International Reference Laboratories

WHO Collaborating Centre for Research, Training, and Control in Diarrhoeal Diseases
International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B)
G.P.O. Box 128
Dhaka 100
BANGLADESH

WHO Collaborating Centre for Diarrhoeal Diseases Research and Training
National Institute of Cholera and Enteric Diseases
P-33, CIT Road Scheme XM
Beliaghata
P.O. Box 177
Calcutta 700 016
INDIA

WHO Collaborating Centre for Phage-Typing and Resistance of Enterobacteria
Central Public Health Laboratory
61 Colindale Avenue
London NW9 5HT
UK

International *Escherichia* and *Klebsiella* Centre (WHO)
Department of Clinical Microbiology
Statens Seruminstitut
Artillerivej 5, 2300 Copenhagen S
DENMARK

WHO Collaborating Centre for *Shigella*
National Reference Laboratory for *Escherichia coli* and *Shigella*
Foodborne and Diarrheal Diseases Laboratory Section
Centers for Disease Control and Prevention
1600 Clifton Rd., N.E., MS C03
Atlanta, GA 30333
USA

WHO Collaborating Centre for Global Monitoring of Antimicrobial Resistant Bacteria
Nosocomial Pathogens Laboratory Branch
Centers for Disease Control and Prevention
1600 Clifton Rd., N.E., MS G-08
Atlanta, GA 30333
USA

National Reference Laboratory for *Vibrio cholerae* O1 and O139
Epidemic Investigations and Surveillance Laboratory
Foodborne and Diarrheal Diseases Laboratory Section
Centers for Disease Control and Prevention
1600 Clifton Rd., N.E., MS C03
Atlanta, GA 30333
USA

Annex E
Designing a Survey to Examine Antimicrobial Susceptibility of Organisms Causing Epidemic Diarrhea

Rationale

In many places where cholera and epidemic dysentery caused by *Shigella dysenteriae* serotype 1 occur, laboratory resources are scarce. In addition, the characteristics of the patient population may make it necessary to begin treatment and provide a full treatment course of an antimicrobial agent when the patient is first seen. The clinician cannot wait for test results. Often the test results and final antimicrobial susceptibility pattern take up to a week to determine.

One method of overcoming these shortcomings of laboratory testing of individual patients is to design and carry out a survey to determine the organisms causing epidemic diarrhea and their susceptibility patterns, use the information to choose an appropriate antimicrobial agent for treatment, then develop a treatment policy based on the syndrome (i.e., dysentery or watery diarrhea). This method will conserve resources and improve the case management of diarrhea.

Below is a basic outline of how to carry out such a survey that can be adapted to local conditions. It is important for the laboratory and epidemiology departments to work together on studies of this sort. Doing so fosters cooperation, shares the workload, and brings in additional expertise.

Methods

Location

Do the survey in locations that are representative of the population. Include some urban and some rural health centers. The sites chosen should be easily accessible so that specimens can be quickly transported to the laboratory doing the survey. In addition, they should have sufficient patients with diarrhea to allow the health care worker to collect 10-20 specimens in a few days.

Timing

If possible, the survey should be done at the beginning of the cholera or dysentery season when there are adequate numbers of patients with diarrheal disease at clinics and when the information gained will help establish treatment policies and drug purchase for the coming year.

How many patients

Enough patients should be sampled to provide 40 to 50 isolates. Isolation rates range from 25% to 75%. Thus 100 patients is a reasonable target. Select enough sites with high enough patient flow to reach this target in 1 to 2 weeks.

Logistics

Patients should be selected systematically, such as the first 5 patients in the morning; every third patient; or, in the case of clinics with fewer patients, all patients presenting with bloody diarrhea or watery diarrhea that particular day. The number of specimens collected should not overwhelm the laboratory.

If the survey is being carried out for dysentery, patients should, if possible, currently have diarrhea with visible blood, been ill for fewer than 4 days, and not have received an antimicrobial agent before a stool specimen is collected. Patients should be given a cup to collect a stool sample. Examine the stool for blood. If blood is visible, take a swab of the stool and place it in refrigerated (4°C) transport medium (see Chapter 2 for instructions on transport of specimens). Dispose of the stool cup so as to minimize the chance of infecting other persons.

If the survey is being carried out for presumptive cholera, the patients should have acute watery diarrhea with illness for fewer than 4 days, and should not have received an antimicrobial agent before a stool specimen is collected. For cholera, it is preferable to focus on adults and children over age 2 (younger children have many other causes of watery diarrhea that would reduce the yield of *V. cholerae* O1).

Transport the specimens to the laboratory. Examine specimens and test the antimicrobial susceptibilities of *S. dysenteriae* 1 and *V. cholerae* O1/O139 isolated (see Annexes A and B for necessary laboratory supplies).

What to Do with Results

Share the results with other health workers in the country, especially those involved in developing treatment policies or purchasing drugs. If the country has a health bulletin, use it to publish and disseminate the results. It is helpful to share the results with neighboring countries and with the country WHO office, or with the WHO Inter-country or Regional Office, so that they can be easily and quickly shared with the other countries in the area.

What to Do with the Isolates

Keep the isolates if possible. Methods to do so are described in Chapter 10, "Storage of Isolates." Any unusual isolates or those with novel antimicrobial susceptibility patterns should be sent to a national or international reference laboratory for confirmation.

Annex F
Stool Specimen Data Sheet for Epidemic Diarrhea

STOOL SPECIMEN DATA SHEET – EPIDEMIC DIARRHEA

Country _____ Region _____

District _____ Village/Town _____

Specimen Number	Date collected	Date of illness onset	Name	Age	Sex *(M/F)*	Blood in stool? *Yes / No*	Appear-ance*	Taken antibiotics *Yes / No* * *

* Formed (F); Soft (S); Watery (W); Bloody-mucus (BM)
**Type of antibiotic, dose and number of days taken.

Collected by: Name & Title _____

Transmit results to: Name & Title _____

Address _____

Phone/Fax/ Telex _____

Annex G
Most Frequently Encountered Reactions in Screening Biochemicals[a]

Test	Organism				
	Shigella	*Escherichia coli*	*Salmonella*	*Salmonella* ser. Typhi	*Vibrio cholerae*
Kligler iron agar[b]	K/A-	A/AG-	K/AG+	K/A(+)	K/A-
Triple sugar iron agar[b]	K/A-	A/AG-	K/AG+	K/A(+)	A/A-
Lysine iron agar[b]	K/A-	K/K -	K/K+	K/K(+)	K/K-
Lysine decarboxylation[c,d]	-	+	+	+	+
Motility[c]	-	+	+	+	+
Urea hydrolysis[c]	-	-	-	-	-
Indole production[c,d]	+ or -	+	-	-	+
Oxidase production[c]	-	-	-	-	+

[a] For each of these organisms, variable reactions may occur.
[b] Reactions expressed as "slant/butt"; K = alkaline; A = acid; G = gas produced; + = hydrogen sulfide (H_2S) produced; (+) = weakly positive for H_2S production; - = no H_2S produced.
[c] + = positive reaction; - = negative reaction.
[d] For *V. cholerae*, 1% salt (NaCl) added to biochemical formulation.

References

World Health Organization. Manual for the laboratory investigations of acute enteric infections. Geneva: World Health Organization, 1987; publication no. WHO/CDD/83.3 rev 1.

Bopp CA, Brenner FW, Wells JG, Strockbine NA. *Escherichia, Shigella,* and *Salmonella*. In: Murray PR, Pfaller MA, Tenover FC, Baron EJ, Yolken RH, ed. Manual of Clinical Microbiology, 7th ed. Washington, DC: ASM Press; 1999: 459-474.

Centers for Disease Control and Prevention. Laboratory methods for the diagnosis of *Vibrio cholerae.* Atlanta: CDC; 1994.

Annex H
Diagnostic Laboratory Supplies for Isolation and Presumptive Identification of *Escherichia coli* O157:H7 During an Outbreak (Sufficient for 100 Specimens)

100 sterile cotton or polyester swabs
500 g Cary-Blair or other transport medium
500 g sorbitol MacConkey agar
500 g nonselective agar (e.g., tryptone soy agar, heart infusion agar)
O157 latex agglutination kit for 100 tests
200 petri dishes (9 cm)
200 test tubes (e.g., 13 x 100 mm or 16 x 125 mm)

Suggested Citation

Centers for Disease Control and Prevention. Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera. Atlanta, Georgia: CDC, 1999.

Additional copies of this manual can be obtained from:

Foodborne and Diarrheal Diseases Laboratory Section
Centers for Disease Control and Prevention
Mailstop C03
1600 Clifton Road, N.E.
Atlanta, GA 30333 USA
Fax 404-639-3333