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 This is the protocol used for analysing the samples from 2015-2016 Bangladesh National Serosurvey

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Bangladesh National Serosurvey Vibriocidal Protocol (96-well plate, OD600, mAb)

Forked from a private protocol

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ABSTRACT

This protocol provides the details on how vibriocidal assays were performed on serum from the nationally representative serosurvey for *V. cholerae* O1.

MATERIALS

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- ✕ Petri Dish **P212121 Catalog #LI-PD01100**
- ✕ micropipettors; Sterile tips and serological pipettes **Contributed by users**
- ✕ 15 ml sterile falcon tubes and rack **Contributed by users**
- ✕ 25 ml Reservoir **Contributed by users**
- ✕ brain Heart Infusion Broth **Contributed by users Catalog #Oxoid CM1135-U**
- ✕ NaCl 0.9% **Contributed by users**
- ✕ Tissue Culture Plate, 96 Well **Bio Basic Inc. Catalog #TCP20-96.SIZE.1**
- ✕ 1.5 ml micro-centrifuge tube **Ambion Catalog #AM12450**
- ✕ Multichannel pipette P1-10, P200 and their corresponding tips **Contributed by users**
- ✕ Ethanol 70% **Contributed by users**
- ✕ Centrifuge 5810 swinging bucket **Eppendorf Centrifuge Catalog #02262500**
- ✕ Blood Agar Plate (5% sheeps blood in Tryptic Soy Agar base) **Contributed by users**
- ✕ 50 ml Falcon Tubes **Contributed by users**
- ✕ Biotek Microplate Asorbance Reader **Contributed by users**
- ✕ Orbital Shaker-Incubator **Contributed by users**

STEP MATERIALS

- ✕ brain Heart Infusion Broth **Contributed by users Catalog #Oxoid CM1135-U**

- 1 Streak *V. cholerae* (O1 Ogawa or Inaba) onto blood agar and incubate at 37 °C overnight (approx 12:00:00)

- 2 Fill 15 mL culture tube with 10 mL sterile brain heart infusion broth (BHI)
brain Heart Infusion Broth **Contributed by users Catalog #Oxoid CM1135-UK**
 that has been left at (22 °C - 25 °C) for at least 1 hour

- 3 Inoculate a loopful of bacteria from the blood agar plate (1-2 colonies) into 15mL culture tube from previous step

- 4 Incubate in an orbital shaker-incubator at 37 °C and 220 rpm for 03:00:00 - 04:00:00

- 5 While the bacterial culture is growing in the orbital shaker/incubator, aliquot 20 µL of the serum samples to be tested into Eppendorf tubes and place them in a 56 °C water bath for 00:30:00 to heat-inactivate the native complement system proteins

- 6 Dilute the heat-inactivated sample sera 1:10 (15 µL serum + 135 µL 0.9% NaCl saline) before beginning the assay (this is the starting titer of 10), and keep on ice until ready to add samples to 96 well plate (below)

- 7 Take guinea pig complement serum aliquot (300 μ L plate) out of the -20°C freezer and let it begin thawing at 4°C (refrigerator)
- 8 When the bacterial culture has finished growing, transfer it into a 15 mL centrifuge tube
- 9 Centrifuge at 3000 rpm and 22 °C - 25 °C (room temperature) for 00:10:00
- 10 Discard supernatant into 1:10 bleach solution or biohazard waste

Wash 1

- 11 Wash cells by resuspending the cell pellet in 10 mL saline
- 12 Centrifuge again at 3000 rpm and 22 °C - 25 °C (room temperature) for 00:10:00
- 13 Discard supernatant into 1:10 bleach or biohazard waste

Wash 2

- 14 Wash cells by resuspending the cell pellet in 10 mL saline

15 Centrifuge again at 3000 rpm and 22 °C - 25 °C (room temperature) for 00:10:00

16 Discard supernatant into 1:10 bleach or biohazard waste

Resuspending cells post-wash

17 After washing cells 2 times, resuspend them in 1 mL 0.9% NaCl.

18 Transfer 200 µL solution into 96 well plate and measure Optical density (OD) at 600 nm, OD600. Based on OD600 reading prepare 1 ml of *V. cholerae* working dilution diluted to approximately OD600 0.3 with 0.9% NaCl. (Ex. if OD600 =1.0, add 300 µL bacterial solution + 700 µL 0.9% NaCl).

19 Prepare a 96 well plate by first adding 25 µL of saline to all columns, except column 2 (See 96 Well Plate Layout below)

		1	2	3	4	5	6	7	8	9	10	11	12
		Controls	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240
A	Growth Ctl	Sample 1											
B	Growth Ctl	Sample 1											
C	Growth Ctl	Sample 2											
D	Growth Ctl	Sample 2											
E	Negative Ctl	Sample 3											
F	Negative Ctl	Sample 3											
G	Negative Ctl	mAb (1 ug/ml)	0.5 ug/ml	0.125 ug/ml	0.0625 ug/ml	0.03125 ug/ml	0.0156 ug/ml	0.00787 ug/ml	0.0039 ug/ml	0.00195 ug/ml	0.00098 ug/ml	0.00049 ug/ml	
H	Negative Ctl	mAb (1 ug/ml)	0.5 ug/ml	0.125 ug/ml	0.0625 ug/ml	0.03125 ug/ml	0.0156 ug/ml	0.00787 ug/ml	0.0039 ug/ml	0.00195 ug/ml	0.00098 ug/ml	0.00049 ug/ml	
		2-fold Dilutions											

Plate layout

- 20 Add 50 µL of 1:10 diluted serum samples from Step 5 to wells in column 2 (for 96 Well Plate Layout go to step #19). Each sample should be done in duplicate; e.g. Sample 1 in 2A and 2B, Sample 2 in 2C and 2D, and Sample 3 in 2E and 2F.
- 21 Add 50 µL of *V. cholerae*, Inaba or Ogawa monoclonal antibody in PBS (1 µg/ml) into wells 2G and 2H, which will serve as the assay control.
- 22 Use a multichannel pipettor to perform 2-fold serial dilutions of the samples starting from column 2 and going to column 12, i.e. transfer 25 µL of the sample from column 2 into column 3, gently pipette up and down a few times, then transfer 25 µL from column 3 into column 4, and so on down the rows, removing 25 µL g from the last column (12) and discarding. *Check the volume of the sample/dilution wells after you are finished to make sure they are uniform before proceeding*
- 23 Prepare the Growth Indicator solution as follows for a single 96-well plate:
 - 2.85 mL saline
 - 150 µL diluted bacteria (working dilution as determined in Step 15).
 - 300 µL guinea pig complement serum
- 24 Pour the Growth Indicator solution into a 25 mL reagent reservoir, add 25 µL of it to wells 1A, 1B, 1C, and 1D (Growth Controls), and 25 µL to all sample/2-fold dilution wells (columns 2-12; for plate layout go to step #19).

Be careful not to cross-contaminate any of the wells. Mix wells by gently pipetting up and down with the multichannel pipettor, be sure to change tips between columns to avoid cross-contamination!

- 25 Add 25 μL saline to 1E, 1F, 1G, and 1H (Negative Controls)
- 26 Incubate plate on orbital shaker-incubator at 37 °C and 50 rpm for 01:00:00
- 27 Take the plate out of the shaker/incubator and add 150 μL BHI to all wells (including controls), being careful not to cross-contaminate.
- 28 Incubate at 37 °C without shaking for 02:00:00 and then measure the OD₅₉₅. Make sure the Growth Control wells are between 0.20 and 0.28. If below, continue to grow and measure OD₅₉₅ every half hour (can take up to 4 hrs total).
- 29 Read the OD of the entire plate at 595 nm with a plate reader. Subtract the average OD of 4 negative control wells from all wells to get a 'blanked' value (if this brings any values below 0, the value should be set to zero).
- 30 Take the average of OD of the four Growth Controls and divide by 2 to get the *50% kill OD*.
- If ≥ 2 Growth control wells are out of range (0.2-0.28) the plate should be rejected. If only one growth control well is out of range, that well will be rejected and the other three averaged to get the *50% kill OD*.**
- 31 Determine the titer for each sample/row by taking the reciprocal of the dilution factor for which the OD₅₉₅ reading is less than the *50% kill OD*. If this is not achieved by a sample, assign a titer value of 5 to indicate below limit of detection.

Example: If the average Growth Control OD₅₉₅ = 0.24, and the sample in row B (above) has an

OD595 of 0.11 in column 7, then the titer is reported as 320.

- 32** Check to make sure monoclonal antibodies are within acceptance range. If mAb assay control readings are not in range (50% Kill titer for Ogawa of 0.0625-0.03125 (Col 5 or 6), and for Inaba of 0.125-0.0625 (Col 4 or 5), plate should be rejected.
- 33** Check to make sure technical replicates for each sample have a titer within one 2-fold dilution of one another.
- If the technical replicate titers exceeds more than one 2-fold dilution, the sample should be rejected.
 - If the technical replicates titers differ by one 2-fold dilution, the sample should be assigned the lower of the two titers