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

Forked from a private protocol

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Protocol status: Working
This is the protocol used for analysing the samples from 2015-2016 Bangladesh National Serosurvey

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Abstract

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

Materials

MATERIALS

- Petri Dish **P212121 Catalog #**LI-PD01100
- micropipettors; Sterile tips and serological pipettes
- 15 ml sterile falcon tubes and rack
- 25 ml Reservoir
- brain Heart Infusion Broth **Catalog #**Oxoid CM1135-UK
- NaCl 0.9%
- 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
- Ethanol 70%
- Centrifuge 5810 swinging bucket **Eppendorf Catalog #**022625004
- Blood Agar Plate (5% sheeps blood in Tryptic Soy Agar base)
- 50 ml Falcon Tubes
- Biotek Microplate Asorbance Reader
- Orbital Shaker-Incubator

STEP MATERIALS

- brain Heart Infusion Broth **Catalog #**Oxoid CM1135-UK

Protocol materials

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- 25 ml Reservoir
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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 **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 µL 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