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## APPENDIX I

Other materials :

Plastic culture flask or culture bottle (sterile) 25-50 $cm^2$

Sterile beakers 10, 50, 250 ml

Forceps, scissors, blade (scalpel) (sterile)

Trypsinize flask or erlenmeyer flask (sterile) 125, 250 ml

Sterile petri dish.

Magnetic stirer and sterile teflon-coverd stering bar.

Conical centrifuge tube (sterile) 50 ml with screw cap.

Microplate (96 well), Leighton tube, pipette (sterile) 1, 5, 10 ml

Reagents

1. 50 % Household "Clorox"

| | | |
|---|---|---|
| Household "Clorox" | 50 | ml |
| Distilled water | 50 | ml |

Freshly preparated before use.

2. Phosphate buffer saline (PBS), pH 7.4 (10x)

| | | |
|---|---|---|
| NaCl | 40.0 | gm |
| KCl | 1.0 | gm |
| $Na_2HPO_4$ | 5.75 | gm |
| $K_2HPO_4$ | 1.0 | gm |
| Distilled water to make | 500.0 | ml |

pH adjusted with 1 N NaOH or 1 N HCl

Ingredients dissolved in enough water to make 500 ml. Sterilized by autoclave at 121°C for 15 minutes and store at 4°C.

3. Phosphate buffer saline pH 7.4 (1x)

| | | |
|---|---|---|
| PBS pH 7.4 (10x) | 50 | ml |
| Sterile distilled water to make | 500 | ml |
| P/S ($10^5$ IU/ml) | 1 | ml |

Stored at 4°C or Room temperature.

4. PBS pH 7.4 (1x) with kanamycin 500 ug/ml.

| | | |
|---|---|---|
| PBS pH 7.4 (10x) | 50 | ml |
| Sterile distilled water to make | 500 | ml |
| Kanamycin ($10^5$ ug/ml) | 2.5 | ml |

Stored at 4°C.

5. Antibiotic stock :

5.1 Combination stock sodium penicillin and streptomycin (P/S)

Sodium penicillin G "DUMEX" $5x10^6$ IU

Streptomycin "DUMEX" 5 gm

Sterile distilled water is added to penicillin and streptomycin 50 ml and mixed well. This solution will give a final concentration of $10^5$ IU/ml of penicillin and $10^5$ ug/ml of streptomycin. This solution was distributed in to small bottles and store at -20 °C.

5.2 Kanamycin $10^5$ ug/ml

Kanamycin 1 gm was added to 10 ml of sterile distilled water. Mixed well, this solution give a final concentration of $10^5$ ug/ml. It was stored at -20 °C.

6. L-15 "FLOW" (1x)

| | | |
|---|---|---|
| L-15 (power) | 1 | pack |
| Distilled water to make | 1000 | ml |

Ingredient was dissolved and sterilized by Millipore filter and stored at 4 °C.

7. Growth medium.

| | | |
|---|---|---|
| L-15 (1x) | 90 | ml |
| Fetal bovine serum"FLOW" | 10 | ml |
| P/S | 0.1 | ml |

pH adjusted with 1N HCl to 7.2-7.4 and stored at 4 °C.

8. Maintenance medium.

| | | |
|---|---|---|
| L-15 (1x) | 95 | ml |
| Fetal bovine serum"FLOW" | 5 | ml |
| P/S | 0.1 | ml |

pH adjusted with 1 N HCl to 7.2-7.4 and stored at 4 °C.

9. Trypsin diluent of glucose

| | | |
|---|---|---|
| NaCl | 8.0 | ml |
| KCl | 0.4 | gm |
| $Na_2HPO_4$ | 0.06 | gm |
| $KH_2PO_4$ | 0.06 | gm |
| glucose | 50.0 | gm |
| Distilled water to make | 1000 | ml |

Ingredients dissolved in water to make 1000 ml. Fresh preparation before use.

10. 10 % Trypsin

| | | |
|---|---|---|
| Trypsin (Difco 1:250) | 50.0 | gm |
| Trypsin diluent | 450 | ml |

Trypsin was added to trypsin diluent and shaken untill dissoved for 30 minutes at 4 °C. It was spun 10000 rpm for 30 minutes at 4 °C and supernatant were collected. This solution was sterilized by millipore filter and dispensed in 100 ml aliquots into sterilized bottles and stored at -20 °C.

11. Versene 10x (stock solution 1:5000)

| | | |
|---|---|---|
| Versene | 2.0 | gm |
| NaCl | 80.0 | gm |
| KCl | 2.0 | gm |
| $KH_2PO_4$ | 2.0 | gm |
| $Na_2HPO_4$ | 11.5 | gm |
| Distilled water to make | 1000 | ml |

Each salt was weighed separately and dissolved in sequence. It was then dispensed in 100 ml amounts. Autoclaved at 15 pounds for 15 minutes. Stored at room temperature.

12. Trypsin-versene solution

| | | |
|---|---|---|
| Trypsin (10 %) | 5.0 | ml |
| Versene stock 10x | 100.0 | ml |
| Sterile distilled water to make | 1000 | ml |

Trypsin-versene and water were mixed using sterile procedures. It was then dispensed in 100 ml amounts into sterile bottle and stored at -20°C.

13. Thioglycollate broth for bacteriological sterility test

While stirring 29.8 gm thioglycollate broth (Difco) powder was added to 500 ml distilled water. Then 5 gm of dextrose was added, followed by sufficient distilled water to make 1000 ml. This solution was dispensed in 5 ml amounts into tube and autoclaved at 15

pounds for 15 minutes. It was stored at room temperature in the dark.

14. $NaHCO_3$ 7 %

| | | |
|---|---|---|
| $NaHCO_3$ | 7.0 | gm |
| Distilled water | 100 | ml |

Sodium hydrogen carbonate was dissolved into 100 ml of sterile distilled water. It was then dispensed in 10 ml amounts into sterile tubes and stoppered tightly. It can be stored in a refrigerator or at room temperature.

15. 1 N NaOH

| | | |
|---|---|---|
| NaOH | 4.0 | gm |
| Distilled water to make | 100 | ml |

NaOH was dissolved in water and dispensed in 10 ml amounts into tubes. It was then sterilzed by autoclaving at 121°C for 15 minutes and stored at 4°C.

16. 1 N HCl

| | | |
|---|---|---|
| HCl (conc. 36 % ) | 9.2 | ml |
| Distilled water to make | 100 | ml |

This solution was mixed well and dispensed in 10 ml amounts tubes. It was sterilized by autoclaving at 121°C for 15 minutes and stored at 4 °C.

17. 0.01 % neutral red

One gram of nautral red is dissolved in 1000 ml of double distilled water and solution steriled by Millipore filteration.

18. Sabouraud agar

To redehydrate sabouraud dextrose agar "difco" 65 grams in distilled water 1000 ml and heat to boiling to dissolve the medium completely. Sterilize in the autoclave for 15 minutes at 15 pounds pressure (121 °C).

19. Dimethylsulfoxide (DMSO)

DMSO is sterilized for use as a cell protective agent by autoclave at 121 ° C for 10 minutes and stored at 4 ° C.

## APPENDIX II

Stock IPNV :

Calculation of infectious pancreatic necrosis virus titer was as follow:

| virus dilution | number dead | number surviving | total dead | total surviving | mortality ratio | percent mortality |
|---|---|---|---|---|---|---|
| $10^{-6}$ | 5/5 | 0/5 | 8 | 0 | 8/8 | 100 |
| $10^{-7}$ | 2/5 | 3/5 | 3 | 3 | 3/6 | 50 |
| $10^{-8}$ | 1/5 | 4/5 | 1 | 7 | 1/8 | 13 |
| $10^{-9}$ | 0/5 | 5/5 | 0 | 12 | 0/12 | 0 |

50 % end point or the proportionate distance between the two dilutions is $10^{-7}$ and $10^{-8}$

Applying the following formula =

$$\frac{\text{\% mortality above 50 \% - 50 \%}}{\text{\% mortality above 50 \% - \% mortality below 50 \%}}$$

$$= \frac{50 - 50}{50 - 13} \qquad \frac{0}{13}$$

$$= 0$$

Negative log of titer = negative log of dilution above 50 % mortality plus proportionate distance

$$= 7.0 + 0$$

$$TCID_{50} = 10^{-7}$$

Stock CV :

Calculation of CV titer was as follow :

| virus dilution | number dead | number surviving | total dead | total surviving | mortality ratio | percent mortality |
|---|---|---|---|---|---|---|
| $10^{-4}$ | 5/5 | 0/5 | 13 | 0 | 13/13 | 100 |
| $10^{-5}$ | 3/5 | 2/5 | 8 | 2 | 8/10 | 80 |
| $10^{-6}$ | 4/5 | 1/5 | 5 | 3 | 5/8 | 63 |
| $10^{-7}$ | 1/5 | 4/5 | 1 | 7 | 1/8 | 13 |
| $10^{-8}$ | 0/5 | 5/5 | 0 | 12 | 0/12 | 0 |

50 % end point or the proportionate distance between the two dilution is $10^{-6}$ and $10^{-7}$

Applying the following formula =

$$\frac{\text{\% mortality above 50 \% - 50 \%}}{\text{\% mortality above 50 \% - \% mortality below 50 \%}}$$

$$= \frac{63 - 50}{50 - 13} \qquad \frac{13}{37}$$

$$= 0.35$$

Negative log of titer = negative log of dilution above 50 % mortality plus proportionate distance

$$= 6.0 + 0.35$$

$$= 6.4$$

$$TCID_{50} = 10^{-6.4}$$

Stock SHV :

Calculation of SHV titers was as follow :

| virus dilution | number dead | number surviving | total dead | total surviving | mortality ratio | percent mortality |
|---|---|---|---|---|---|---|
| $10^{-3}$ | 5/5 | 0/5 | 13 | 0 | 13/13 | 100 |
| $10^{-4}$ | 4/5 | 1/5 | 8 | 1 | 8/9 | 89 |
| $10^{-5}$ | 3/5 | 2/5 | 4 | 3 | 4/7 | 57 |
| $10^{-6}$ | 1/5 | 4/5 | 1 | 7 | 1/8 | 13 |
| $10^{-7}$ | 0/5 | 5/5 | 0 | 12 | 0/12 | 0 |

50 % end point or the proportionate distance between the two dilutions is $10^{-5}$ and $10^{-6}$

Applying the following formula=

$$\frac{\text{\% mortality above 50 \% - 50 \%}}{\text{\% mortality above 50 \% - \% motality below 50 \%}}$$

$$= \frac{57 - 50}{50 - 13} \qquad \frac{7}{47}$$

$$= 0.148$$

Negative log of titer = negative log of dilution above 50 % mortality plus proportionate distance

$$= 5.0 + 0.148$$

$$= 5.2$$

$$TCID_{50} = 10^{-5.2}$$

By the same method, we can caculate the titers of IPNV CV SHV which multiplied in freshwater fish cell cultres as seen in table 10.

# BIOGRAPHY

Miss Sumittra Vattanodorn was born on October 5, 1955 in Suratthani, Thailand. She was graduate student from department of Science in the year 1978. She has been awearded a Bachelor' s degree in Science (Biology) from Ramkhamhang University.

Since 1979, she has been employed as scientist at Facultry of Veterinary of science, Chulalongkorn University. She started to work on sero-immunology. Three years later, while she has being worked on virus, she was trained in laboratory diagnosis of flavivirus infection for 2 months from United States Army Medical Component Armed Forces Research Instutude of Medical Sciences.