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APPENDIX I

Estimation of the LD_{50} of the Challenge Strain by Method of Reed and Muench (144)

LD_{50} of the challenge strain C_s in the mice gave the following results

TABLE 6 LD_{50} of the challenge strain C_s in the BALB/cJ mice

Dilution Rate	Mortality	Death	Survivor	Total		Percent Mortality
				Death	Survivor	
10^7	8/8	8	0	31	0	100
10^6	8/8	8	0	23	0	100
10^5	8/8	8	0	15	0	100
10^4	6/8	6	2	7	2	78 *
10^3	1/8	1	7	1	9	10
10^2	0/8	0	8	0	17	0

In this example , the dilution factor was 10 and 50 % endpoint dilution (ED_{50})* of the challenge strain C_s was the figure between 10^4 and 10^3 dilution .

The % mortality at dilution next below was 10 % and the % mortality at dilution next above was 78 % .

Calculate the " proportional distance " from the formula :

$$\text{Proportional distance} = \frac{\text{50 percent-(mortality at dilution next below)}}{\text{(mortality next above)-(mortality next below)}}$$

$$= \frac{50 - 10}{78 - 10}$$

$$= \frac{40}{68}$$

$$= 0.5882$$

Calculate the " 50 % endpoint dilution (ED_{50})" or the " 50 % lethal dose (LD_{50}) " from the formula :

$$\text{Log } LD_{50} = \text{log of dilution titer lower than 50 \% mortality + (proportional distance x log of dilution factor)}$$

$$= 3 + (0.5882 \times 1)$$

$$= 3 + 0.5882$$

$$= 3.5882$$

$$LD_{50} = \text{Anti - log } 3.5882$$

$$= 3874 \text{ . } 3602 \text{ organisms}$$

$$= 3.87 \times 10^{3} \text{ organisms}$$

APPENDIX II

MEDIA AND REAGENTS

1. Media

1.1 Brain Heart Infusion Broth

The broth was used to culture S. typhimurium C_s and G_{30} . One liter of the broth was prepared as follows :

Dissolved powder of Brain heart infusion broth (BBL , Cockeysville , MD.) 37 g in distilled water 1 liter and sterilized by autoclaving at 15 pounds pressure (121° C) for 15 min .

For S. typhimurium G_{30}, added Ø.ØØ2 % w/v galactose in this broth before autoclaving .

1.2 Nutrient Agar Plates

Dissolved powder of nutrient agar (DIFCO Laboratories , Detroit , Michigan , USA) 23 g in 1 liter distilled water and heat to boiling to dissolve completely . Sterilized in the autoclave for 15 min at 15 pounds pressure (121°C) .

This medium was used to culture S.typhimurium C_s and G_{30} .

1.3 Soft Agar

Dissolved Ø.7 % of nutrient agar (DIFCO Laboratories , Detroit , Michigan , USA) in distilled water and heat to boiling to dissolve completely . Sterilized in the autoclave for 15 min at 15 pounds pressure 121° C) .

This medium was used to keep S. typhimurium C_s and G_{30} as the stock cultures .

1.4 Tetrazolium - Galactose Medium

Nutrient agar	23	g
Galactose	10	g
Sodium sulphite	2.5	g
Distilled water to	1	liter

The medium was sterilized by autoclaving for 15 min at 15 pounds pressure (121° C) .

Before pouring plates , held melted agar at 56°C and added 5 ml of a sterilized solution (by membrane filtration a 1 % w/v solution of Tetrazolium (BDH Chemicals LTD. Poole England) in distilled water per liter .

This medium was differential for S. typhimurium G_{30} and C_s . The strain G_{30} which was unable to ferment galactose should grow with a red center on this medium but strain C_s grew without a red center .

2. Cell Culture Media

2.1 RPMI 1640 Medium

Power of RPMI 1640 medium with L - glutamine without antibiotics and sodium bicarbonate in a package of one liter, was purchased from GIBCO Laboratories .

The preparation of 1 x liquid medium :

2.1.1 Measured out 5 % less distilled water than

desired total volume of medium , using a mixing container that was as close to the final volume as possible .

2.1.2 Added powdered medium to 15 - 30° C (room temperature) water with gentle stirring . (Did not heat water)

2.1.3 Rinsed out inside of package to remove all traces of powder . Added 2.0 g of $NaHCO_3$ per liter of medium . The medium would appear cloudy because of incomplete solubilization .

2.1.4 Diluted to a desired volume with water .

2.1.5 Adjusted pH of medium to 0.2 - 0.3 below desired final working pH* (7.3) ; using either 1 N NaOH or 1 N HCL . Medium should be clear . After pH had been adjusted , kept container closed until medium was filtered .

2.1.6 Sterilized immediately by membrane filtration.

2.1.7 Aseptically dispensed into sterile containers.

2.1.8 Labeled and stored at 4° C .

2.2 Hanks' Balanced Salt Solution (HBSS)

Powder of HBSS without sodium bicarbonate in a package of one liter was purchased from GIBCO Laboratories .

The preparation of 1 x liquid medium :

---

* PH unit would usally rise 0.1 - 0.3 upon filtration .

2.2.1 Measured out 5 % less distilled water than desired total volume of medium , using a mixing container that was as close to the final volume as possible .

2.2.2 Added powdered medium to distilled water at room temperature with gentle stirring .

2.2.3 Rinsed out inside of package to remove all traces of powder .

2.2.4 Added Ø.35 g of $NaHCO_3$ per liter of medium .

2.2.5 Diluted to a desired volume with water . Stirred until dissolved . (Did not over - mix)

2.2.6 Adjusted pH of medium to Ø.2 - Ø.3 below desired final working pH* (7.3) ; used of 1 N NaOH or 1 N HCL was recommended . (Added slowly with stirring)

After pH had been adjusted , kept container closed until medium was filtered .

2.2.7 Sterilized immediately by membrane filtration .

2.2.8 Aseptically dispensed into sterile containers.

2.2.9 Labeled and stored at 4° C .

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* PH unit would usually rise Ø.1 - Ø.3 upon filtration .

2.3 **Complete Medium** (with 10 % heat - inactivated FCS)

The medium was used to culture the PP cell suspension . One hundred millilters of the medium were prepared as follows :

Heat - inactivated FCS	10	ml
Gentamycin , 10,000 ug/ml	0.1	ml
HEPES , 1 M	1.5	ml
L - glutamine , 1 M	100	ml
RPMI 1640 to	100	ml

The pH of medium was adjusted to 7.3 by using 1 M $NaHCO_3$, sterilized by membrane filtration and stored at 4° C .

2.4 **Incomplete Medium**

The medium was used to store the isolated PP after dissecting from the small intestines . One hundred milliliters of the medium were aseptically prepared as follows :

Gentamycin , 10,000 ug/ml	0.1	ml
RPMI 1640 to	100	ml

The medium should be stored at 4° C .

2.5 **Enzyme Solution**

The medium was used to extract the PP cells . One hundred milliliters of the medium were freshly prepared as follows :

Neutral protease (Sigma , Chemical Co , St - Louis ,

Mo)	Ø.15	g
RPMI 1640 to	1ØØ	ml

The medium was sterilized by membrane filtration and kept at 37° C prior to use .

2.6 **Gelatine - Hanks Balanced Salt Solution** .
(gelatin - HBSS)

The medium was used for intracellular killing assay. One hundred milliliters of the medium were aseptically prepared as followed :

Dissolved 1ØØ mg gelatine (DIFCO Laboratories , Detroit , Michigan) in 5 ml sterile HBSS with gentle heating and added this to 95 ml sterile HBSS .

Gelatine was added to protect the microorganisms since HBSS alone was bactericidal .

3. **Reagents**

3.1 **Reagents for Oral Immunization**

3.1.1 **Ø.85 % Normal Saline Solution** (NSS)

One liter of NSS was prepared as follows :

NaCL	8.5	g
Distilled water to	1,ØØØ	ml

The solution was sterilized by autoclave at

15 pounds pressure (121°C) for 15 min and stored at room temperature.

3.1.2 50 % Saturated Sodium Bicarbonate

(50 % sat $NaHCO_3$)

One hundred milliliters of 50 % sat $NaHCO_3$ were prepared as follows :

Added excess $NaHCO_3$ to distilled water and stirred until saturated removed excess $NaHCO_3$ and then mixed saturated $NaHCO_3$ 50 ml with distilled water 50 ml .

The solution was sterilized by autoclave at 15 pounds pressure (121° C) for 15 min .

3.2 Reagent for Cell Cultures and Intracellular Killing Assay

3.2.1 0.4 % Trypan Blue

This solution was used to examine viability of cells . One hundred milliliters of the solution were prepared as follows :

Trypan Blue	0.4	g
Distilled water to	100	ml

3.2.2 HEPES Buffer , 1 M

The buffer was an organic buffer used to control the physiological pH range of the cell culture media . One molar solution of the HEPES buffer was prepared as follows :

HEPES (Sigma Chemical Co ,St . Louis , Mo)		
	235.3	g
Distilled water to	1000	ml

The solution was sterilized by membrane filtration , and stored at 4° C . HEPES buffer was usually employed in cell culture media at concentrations of 15 mM .

3.2.3 Sodium Bicarbonate , 1 M

This was a buffering solution added , at the time of use , to balanced cell culture media to provide proper buffering capacity . One molar solution of the sodium bicarbonate was prepared as follows :

$NaHCO_3$	84.01	g
Distilled water to	1000	ml

The solution was sterilized by membrane filtration , and stroed at 4° C .

3.2.4 Fetal Calf Serum (FCS)

The fetal calf serum (CSL , Melbourne , Australia) was heat - inactivated at 56° C (water bath) for 30 min and then was stored at - 20° C .

3.2.5 Gentamycin , 10,000 ug/ml

The stock solution was prepared by dissolving 80 mg/2 ml gentamycin sulfate (General Drugs House CO , LTD ,

Bangkok) in 6 ml of distilled water to a concentration of 10,000 ug/ml and stored at -20° C . It was employed in complete media at concentration of 10 ug/ml .

3.2.6 L - Glutamine , 1 M

One molar solution of L - glutamine (Sigma Chemical CO, St Louis , MO) was prepared as follows :

L - glutamine	146.1	g
Distilled water to	1000	ml

It was stored at -20° C and was employed in complete media at concentration of 2 mM.

3.2.7 Bovine Serum Albumin Solution (0.01% w/v in Distilled Water

This solution , used for disrupting the macrophages in the intracellular killing assay , was prepared by adding 10 mg bovine serum albumin (CSL ,Melbourne , Australia) to 100 ml sterile distilled water ; the pH was adjusted to 7.3 with 0.1 N NaOH . Bovine serum albumin was added to protect the bacteria against the bactericidal effect of distilled water .

3.2.8 Hydrochloric Acid , 1 N

HCL	10	ml
Distilled water to	100	ml

Used to adjust pH .

3.2.9 Sodium Hydroxide , Ø.1 N

NaOH	Ø.4	g
Distilled water to	1ØØ	ml

Used to adjust pH .

3.3 Reagents for Nonspecific Esterase Staining

3.3.1 Fixative Agent

$Na_2 HPO_4$	2Ø	mg
$KH_2 PO_4$	1ØØ	mg
Distilled water	3Ø	ml
Acetone	45	ml
Formalin	25	ml

The agent was adjusted to 6.6 pH and stored at 4° C .

3.3.2 Phosphate Buffer , 1/15 M , 7.6 pH

Solution A

$KH_2 PO_4$	1.82	g
Distilled water to	2Ø	ml

Solution B

$Na_2 HPO_4 . 2H_2 O$	14.91	g
Distilled water to	1ØØ	ml

Mixing 13 ml of solution A with 87 ml of solution B .

3.3.3 4 % Sodium Nitrite Solution

$NaNO_2$	1	g
Distilled water to	25	ml

3.3.4 Pararosaniline Solution

Pararosaniline hydrochoride (Sigma Chemical CO , ST Louis , MO)	1	g
Distilled water	20	ml
HCL (conc)	5	ml

The solution was filtered after cooling and stored at room temperature .

3.3.5 Hexazotized Pararosaniline

Pararosaniline solution	2	ml
4 % $NaNO_2$ (fresh)	2	ml

3.3.6 Incubation Medium

The medium was freshly prepared by diluting 4.5 ml of the buffer with distilled water up to 45 ml and adding 3 ml of Hexazotized pararosaniline and 0.05 g of - napthyl acetate (Sigma Chemical CO, ST Louis , MO) in 2.5 ml of ethylenglycolmonomethylether (Merck , Germany) .

The mixture was adjusted to 6.1 pH with 1 N NaOH , then filtered .

3.3.7 1 % Methyl Green Solution

Methyl green	1	g
Distilled water	100	ml

3.3.8 Sodium Hydroxide , 1 N

NaOH	4	g
Distilled water to	100	ml

It was used to adjust pH .

3.4 Reagents for Indirect Immunofluorescent Antibody Test

3.4.1 Phosphate Buffer Saline , 7.2 pH

NaCl	8.0	g
KCl	0.2	g
$Na_2 HPO_4$	1.15	g
$KH_2 PO_4$	0.196	g
Distilled water to	1000	ml

This PBS was adjusted to 7.2 pH and stored at 4° C . It was used to dilute serum and wash slides .

3.4.2 Methanol , AR Grade

Methanol (BDH Chemicals LTD . Poole England) was fixative for bacterial antigens .

3.4.3 Conjugate

Fluorescein -labeled swine anti - rabbit immunoglobulin (DAKOPATTS , Denmark) was kindly given by the Virology Unit , Department of Microbiology , Faculty of Medicine , Chulalongkorn University , Bangkok .

3.4.4 Buffered Glycerol Mounting Medium

To prepare 100 ml of PBS - buffered glycerol solution , used as mounting medium , combined 90 ml of glycerol solution (DIFCO Laboratories ,Detroit , Michigan , USA) with 10 ml of PBS 7.6 pH .

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