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APPENDIX

APPENDIX I

CULTURE MEDIA

1. Blood Agar

Add 50 to 70 ml of defibrinated sheep blood or citrated blood (aseptically withdrawn) to 1,000 ml of melted Trypto-Soy Agar (Eiken Chemical Co., Ltd., Tokyo, Japan) which has been brought to a temparature of 50°C. Mix well and pour in Petri dishes.

Trypto-Soy Agar (Eiken) (83) :

Ingradients per liter

Tryptone "Eiken"	15 g
Soypeptone "Eiken"	5 g
Sodium Chloride	5 g
Agar "Eiken"	15 g

pH 7.3 ±

To rehydrate the medium, suspend 40 grams of the medium in 1,000 ml of distilled water, and carefully heat to dissolve the medium completely after thorough mixing. Distribute in tubes or flasks and sterilize in the autoclave for 15 min at 15 lb pressure (121°C).

2. Maintenance Medium (modified from the method of Josephine and Bohnhoff) (82)

This medium is aseptically prepared by mixing Todd Hewitt broth (see Appendix I-3) with heat-inactivated fetal bovine serum (Flow Laboratories, U.K.) in proportion of 1 : 1 (V/V).

3. Todd Hewitt Broth (84)

Dissolve 30 g of Bacto Todd Hewitt broth (Difco Laboratories, Detroit, Michigan, U.S.A.) in 1,000 ml distilled or deionized water. Distribute as desired in tubes, flasks or bottles and sterilize in the autoclave for 15 min at 15 lb pressure (121°C).

Ingredients per liter

Beef Heart, Infusion form	500 g
Neopeptone, Difco	20 g
Bacto Dextrose	2 g
Sodium Chloride	2 g
Disodium Phosphate	0.4 g
Sodium Carbonate	2.5 g

final pH 7.8 ± 0.2 at 25°C

Storage

Bacto Todd Hewitt Broth : Below 30°C

Prepared medium : 15 - 30°C

APPENDIX II

REAGENTS

1. Anticoagulant Citrate Dextrose (ACD) Solution : Solution A (86)

Ingredients per liter

Citric acid ($C_6H_8O_7$)

anhydrous 7.3 g or monohydrate 8.0 g

Sodium Citrate ($C_6H_5Na_3O_7$)

anhydrous 19.3 g or dihydrate 22.0 g

Dextrose ($C_6H_{12}O_6$)

anhydrous 22.4 g or monohydrate 24.5 g

Water for Injection to 1,000 ml

pH 4.5 - 5.5

Dissolve the ingredients and mix. Filter the solution until clear, place immediately in suitable containers and sterilize in the autoclave for 15 min at 15 lb pressure (121°C).

15 ml of ACD Solution A is required for each 100 ml of whole blood.

2. 1 % Merthiolate

Dissolve 10 g Thimerosol N.F. (L.P. Standard Laboratories Co., Ltd.) in 1,000 ml distilled water.

Store at room temperature.

3. 0.85 % Normal Saline Solution (NSS)

Dissolve 8.5 g NaCl in 1,000 ml distilled water. Distribute as desired in tubes, flasks or bottles and sterilize in the autoclave for 15 min at 15 lb pressure (121°C).

Store at room temperature.

4. Reducing Agent (68, 69)

Add 0.4 ml 25 % NaOH (see Appendix II-6) drop by drop to 0.2 ml thioglycolic acid (E. Merck, Darmstadt, Germany) in 4 ml distilled water ; then make up to 10 ml with SLO Buffer (see Appendix II-5) (final pH 6.7).

Reduction of SLO :

Add 5 ml of reducing agent to 20 ml of undiluted SLO about 15 min before use.

SLO reduced with thioglycolic acid will retain its activity for 2 - 3 days but it is preferable to prepare a fresh batch each day.

5. SLO Buffer (68)

Ingredients per liter

NaCl	7.4 g
KH_2PO_4	3.17 g
Na_2HPO_4	1.81 g
Distilled Water to	1,000 ml

pH 6.5 - 6.7

Store at 2 - 6°C

6. 25 % Sodium Hydroxide Solution

Dissolve 25 g NaOH in 100 ml distilled water in 100 ml volumetric flask.

7. Standard ASO Serum

Sera which must be clear, nonhemolyzed, nonchylous and showed ASO titers higher than 166 Todd units, were collected from serological laboratories (Chulalongkorn Hospital, Ramathibodi Hospital and Children's Hospital). They were then pooled together in a 125 ml Erlenmeyer flask. After gently stirring (in order to avoid formation of foam) with a magnetic bar for about an hour at $4^{o}C$, the sera were distributed into clean dry test tubes (13 x 100 mm) in 0.5 ml aliquots, tightly sealed with parafilm and stored at $-70^{o}C$ until used.

Twenty of these sera were sampled randomly to determine ASO titers by using Streptolysin O Reagent (F. Hoffmann - La Roche & Co, Ltd., Diagnostica, Basle, Switzerland) according to the method described in the test procedure (see Appendix III). Eighteen of the twenty sera showed ASO titers of 200 Todd units whereas the other two showed 250 Todd units. Thus the working titers of these sera were 200 Todd units and they would be used as a standard ASO serum for the titration of SLO throughout this study.

8. Standardized Suspension of Erythrocytes

Fresh human "O" cells, treated with 1 part of ACD Solution (see Appendix II-1) to 10 parts of whole blood, are obtained from the blood bank. Blood obtained from a human donor suspected of having a streptococcal infection should not be used.

After being stored at $4^{o}C$ for 3 days, the erythrocytes are washed in SLO Buffer (see Appendix II-5) and centrifuged at 2,000 rpm for 3 - 5 min. The red tinged supernate is syphoned off and the process repeated until the supernate is colorless. It is essential that the last centrifugation yield a colorless supernate. The final centrifugation should be at 2,000 rpm for 15 min. Three washings are usually required. Cells requiring more than 5 washings should not be used.

From these erythrocytes, the suspension is prepared and standardized by adding SLO Buffer or erythrocytes mass so that after it has been diluted 1 : 12 with distilled water it gives a transmittance of 20 % at the wave length of 520 nm (Junior II spectrophotometer, model 6/35, Coleman, Maywood, Illnois, U.S.A.) SLO Buffer is used to adjust the spectrophotometer to the zero point. The suspension so diluted is roughly 6 % (48).

It is noted that cell suspension cannot be stored overnight because aged cells are more resistant to lysis by SLO (42).

APPENDIX III

QUANTITATIVE DETERMINATION OF ASO

Principle

Antibodies to streptolysin O, present in the serum of patients during the course of infection with beta-hemolytic streptococci, are determined quantitatively by inhibition of hemolysis. This depends on the fact that streptolysin O homolyzes erythrocytes, and this hemolysis can be prevented by the neutralizing action of antibodies to streptolysin O (antigen-antibody reaction).

Reagents

1. Streptolysin O (F. Hoffmann - La Roche & Co, Ltd., Diagnostica, Basle, Switzerland)
2. SLO Buffer (see Appendix II-5)
3. Red Cell Suspension

After washing according to the method described in Appendix II-8, 5 % cell suspension is made in SLO Buffer (see Appendix II-5) for the test.

Method

1. Streptolysin O solution

The contents of one vial of Streptolysin O (Roche) are dissolved in 15 ml distilled water. The solution should be used within 20 min.

2. Serum dilution series (15)

Solution 1 : 10 ; 0.5 ml serum + 4.5 ml SLO Buffer

Solution 1 : 100 ; 1 ml solution 1 : 10 + 9 ml SLO Buffer

Solution 1 : 500 ; 2 ml solution 1 : 100 + 8 ml SLO Buffer

Various amounts of these dilutions are pipetted into tubes according to Table 9.

Controls

1. Red Cell Control (tube No. 12)

The supernate after centrifugation or 2 hr standing at $4^{o}C$ should display no hemolysis. This tube may be used to aid in determining slight amounts of hemolysis in other tubes.

2. Serum Control (tube No. 13)

The supernate after centrifugation or 2 hr standing at $4^{o}C$ should display no hemolysis.

3. SLO Control (tube No. 14)

This tube must be completely hemolyzed.

Result

The ASO titers, expressed in Todd units, is the reciprocal of the highest ditution of serum showing no hemolysis.

For example, if tubes No. 1 - 3 display no hemolysis, while tube No. 4 displays weak hemolysis and the next tube (No. 5) complete hemolysis, the ASO titer of this serum is 125 Todd units (tube No. 3).

Table 9 Methodology for the Determination of ASO Titers

	Serum Dilutions											Controls		
	1:10	1 : 100						1 : 500				Red Cell	Serum	SLO
Reagent \ Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Diluted Serum (ml)	0.2	1	0.8	0.6	0.5	0.4	0.3	1	0.8	0.6	0.4	-	0.5	-
SLO Buffer (ml)	0.8	0	0.2	0.4	0.5	0.6	0.7	0	0.2	0.4	0.6	1.5	1.0	1.0
SLO Solution (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-	-	0.5
Shake gently to mix Incubate at 37°C for 15 min														
5 % Red Cell suspension (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Shake gently Incubate at 37°C for 45 min, shaking after first 15 min Centrifuge tubes for 5 min at 1,500 rpm														
Unit Value	50	100	125	166	200	250	333	500	625	833	1250	Controls		

APPENDIX IV

CALIBRATION OF SPECTROPHOTOMETER FOR SPECTROPHOTOMETRIC METHOD OF SLO TITRATION

In the present investigation, a spectrophotometer was used to ascertain the 50 % hemolytic end point. It was therefore necessory to make a special calibration for the spectrophotometer in order to set the table for converting the transmittance values into homolysis values.

The method was as follows (48) :

Hemoglobin solution prepared by diluting standardized erythrocyte suspension (see Appendix II-8) 1 : 12 with distilled water, i.e., 1 part of standardized erythrocyte suspension and 11 parts of distilled water, represented a 100 % hemolysis sample.

From this sample additional ones with 90 %, 80 %, 70 %,... down to 10 % hemolysis were prepared by dilution with distilled water. The transmittance corresponding to these samples was determined and expressed as the function of the degree of hemolysis. It was most convenient, accurate and rapid to tabulate this relation. For this purpose, however, it was not the transmittance but the optical density that should be measured. From the values found the linear regression was calculated using the method of least squares. This regression made it possible to convert every optical density value (and thus also transmittance) into a homolysis value.

The exact procedure to pursue in this calibration was as follows : samples of 10 %, 20 %, 30 %,... 100 % homolysis were prepared as described, the corresponding optical density values at the wave length of 520 nm were noted and this procedure was repeated three times with blood from different donors. For an example of the results of this procedure, see Table 10

Table 10 Tabulated Results of Spectrophotometric Readings of Hemolysis Samples

% Hemolysis	OD	OD	OD
10	0.086	0.100	0.092
20	0.164	0.163	0.160
30	0.233	0.240	0.237
40	0.301	0.310	0.308
50	0.368	0.382	0.374
60	0.435	0.448	0.441
70	0.490	0.511	0.503
80	0.560	0.579	0.565
90	0.614	0.640	0.625
100	0.696	0.696	0.696

Values from 30 measurements would thus be obtained. Calculations for the regression coefficients formula were tabulated (see Table 11)

Table 11 Tabulated Calculations for the Regression Coefficient Formula

X = the hemolysis percentage or the hemolysis values

Y = the observed optical density multiplied by 1,000

X	Y	XY	X^2
10	86	860	100
10	100	1 000	100
10	92	920	100
20	164	3 280	400
20	163	3 260	400
20	160	3 200	400
30	233	6 990	900
30	240	7 200	900
30	237	7 110	900
40	301	12 040	1 600
40	310	12 400	1 600
40	308	12 320	1 600
50	368	18 400	2 500
50	382	19 100	2 500
50	374	18 700	2 500
60	435	26 100	3 600
60	448	26 880	3 600
60	441	26 460	3 600
70	490	34 300	4 900
70	511	35 770	4 900
70	503	35 210	4 900
80	560	44 800	6 400
80	579	46 320	6 400
80	565	45 200	6 400
90	614	55 260	8 100
90	640	57 600	8 100
90	625	56 250	8 100
100	696	69 600	10 000
100	696	69 600	10 000
100	696	69 600	10 000
1 650	12 017	825 730	115 500

Cosidering the optical density to be a function of hemolysis, the regression coefficient (b) for this function was calculated by using the following formula :

$$b = \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

where x is the hemolysis percentage

y is the observed optical density multiplied by 1,000

n is the number of measurements

$$b = \frac{825,730 - \frac{(1,650)(12,017)}{30}}{115,500 - \frac{(1,650)^2}{30}}$$

$$= \frac{164,795}{24,750}$$

$$= 6.6584$$

The values for optical density corresponding to each value for transmittance (each value separately) were divided by coefficient b (see Table 12)

Table 12 Calculation of Hemolysis Values

$$\% \text{ Hemolysis} = \frac{OD}{b} \times 1{,}000$$

T	OD	% Hemolysis	T	OD	% Hemolysis
100	0.000		60	0.220	33.0
99	0.005	0.7	59	0.230	34.5
98	0.010	1.5	58	0.235	35.2
97	0.015	2.2	57	0.240	36.0
96	0.020	3.0	56	0.250	37.5
95	0.020	3.0	55	0.260	39.0
94	0.025	3.7	54	0.270	40.5
93	0.030	4.5	53	0.275	41.3
92	0.035	5.2	52	0.280	42.0
91	0.040	6.0	51	0.290	43.5
90	0.045	6.7	50	0.300	45.0
89	0.050	7.5	49	0.301	45.2
88	0.055	8.2	48	0.320	48.0
87	0.060	9.0	47	0.330	49.5
86	0.065	9.7	46	0.340	51.0
85	0.070	10.5	45	0.350	52.5
84	0.075	11.2	44	0.360	54.0
83	0.080	12.0	43	0.370	55.5
82	0.085	12.7	42	0.380	57.0
81	0.090	13.5	41	0.390	58.5
80	0.095	14.2	40	0.400	60.0
79	0.100	15.0	39	0.410	61.5
78	0.110	16.5	38	0.420	63.0
77	0.115	17.2	37	0.430	64.5
76	0.120	18.0	36	0.440	66.0
75	0.125	18.7	35	0.455	68.3
74	0.130	19.5	34	0.470	70.5
73	0.135	20.2	33	0.480	72.0
72	0.140	21.0	32	0.500	75.0
71	0.150	22.5	31	0.510	76.5
70	0.155	23.2	30	0.520	78.0
69	0.160	24.0	29	0.540	81.1
68	0.165	24.7	28	0.550	82.6
67	0.175	26.2	27	0.570	86.6
66	0.180	27.0	26	0.585	87.8
65	0.190	28.5	25	0.600	90.1
64	0.195	29.2	24	0.620	93.1
63	0.200	30.0	23	0.640	96.1
62	0.210	31.5	22	0.660	99.1
61	0.215	32.2	21	0.680	102.1
			20	0.700	105.1

The hemolysis values thus obtained were inserted in a matrix table (see Table 13).

Table 13 Complete Inversion Table for Transmittance-Hemolysis Values

% Transmittance	0	1	2	3	4	5	6	7	8	9
20			99	96	93	90	88	87	83	81
30	78	76	75	72	70	68	66	64	63	61
40	60	58	57	55	54	52	51	49	48	45
50	45	43	42	41	40	39	37	36	35	34
60	33	32	31	30	29	28	27	26	25	24
70	23	22	21	20	19	19	18	17	16	15
80	14	13	13	12	11	10	10	9	8	8
90	7	6	5	4	4	3	3	2	1	1

% Hemolysis

% Hemolysis

It was necessary to make a special calibration for every spectrophotometer and a new calibration is required at each change of a lamp.

BIOGRAPHY

Flying Officer Yingwon Thumthranon, WRTAF, was born on March 31, 1960 in Nakornrajsima, Thailand. She graduated with the degree of Bachelor of Science in Microbiology from the Faculty of Science, Chulalongkorn University in 1982.

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