Chapter II

MATERIALS AND METHODS

1 Horseshoe crabs *Tachypleus gigas* (Muller) and *Carcinoscorpius rotundicauda* (Latreille), which were called in Thai as " Mangda jan" and " Mangda tuey" respectively, were purchased mainly from Samutphrakarn's market, and one time each from Samutsakorn's and Chonburi's markets. The crabs arrived at the Microbiology section, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University alive and two lots were kept in sea water for the purpose of haemolymph recollection. The horseshoe crabs were divided into groups according to sex and species. Prior to starting the experiment, the crabs were cleaned with tap-water to remove dirt and then blotted dried. The number and size of each lot of the crabs used in this study were recorded as indicated in table 6.

2 Treatment of glassware and plastic-ware (3,18,72) All glassware (see appendix) were washed with "Colox-Lypon F" mixture solution (see appendix) in warm water with the help of brushes, and the detergent was removed by washing with water for three times. The glassware was then soaked in warm distilled water for one hour and rinsed with pyrogen-free water for injection (see appendix) which had been tested by LAL test using the tube method (Marine Biological Ltd. lot no. 45693). The glassware was dried at 75°C for one hour and the internal surfaces were sprayed evenly with silicone (Zep-par, U.S.A.). The treated glassware were packed with aluminium foil to prevent

bacterial contamination after depyrogenation. The glassware was then depyrogenated in an oven at 200°C for two hours.

Plastic-ware (see appendix) was washed as above for the glassware, but after washing the plastic-ware was soaked in 10% formalin for 30 minutes. Then the plastic-ware was washed twice with distilled water and soaked in distilled water for one hour and then rinsed with pyrogen-free water for injection which had passed LAL test. The plastic-ware was dried at 50°C before the inside was sprayed with silicone. Then it was packed for steam sterilization and subjected to autoclave (121°C for 15 minutes).

3 Preparation of amoebocyte lysate (2,3,5,18,72,73) All glassware and plastic-ware which was to come in contact with haemolymph, amoebocyte or the lysate had been treated pyrogen-free as described.

3.1 Haemolymph collection The crab dorsal joint area was cleaned with 70% alcohol. A pyrogen-free, disposable, 18-gauge, one and a half inches long needle was inserted into the cardiac chamber by way of the dorsal junction of the cephalothorax of the crab as shown in figure 5. The haemolymph was allowed to flow directly into pyrogen-free, siliconized 500-mL polypropylene centrifuge bottle or 50-mL polycarbonate centrifuge tube containing 125 or 25 mL 0.125% sodium ethylenediaminetetraacetate (EDTA) or 0.025 M EDTA or 0.05 M EDTA (see appendix) with or without 5 mM Tris buffer pH 7.4 (see appendix) in 3% sodium chloride solution (see appendix) in the early

experiments. After the conditions of the preparation had been established, then only 0.025 M EDTA in 3% sodium chloride solution was used throughout the study. The ratio of haemolymph and anti-clotting solution was 1:1.

Figure 5. Haemolymph collection.

Table 6. Types of the crabs, sex, lot no., and sources.

S.N.	LOT NO.	TYPE OF CRABS	SEX	NUMBER	SOURCES
1	P-01	*T. gigas*	FEMALE	2	Samutphrakarn
2	P-02	*T. gigas*	FEMALE	2	Samutphrakarn
3	P-03	*T. gigas*	FEMALE	2	Samutphrakarn
4	P-04	*T. gigas*	FEMALE	2	Samutphrakran
5	P-05	*T. gigas*	FEMALE	2	Samutphrakarn
	P-06	*C. rotundicauda*	MALE	6	Samutphrakarn
6	P-07	*T. gigas*	FEMALE	2	Samutphrakarn
	P-08*	*T. gigas*	MALE	3	Samutphrakarn
	P-09*	*C. rotundicauda*	FEMALE	10	Samutphrakarn
7	P-10	*T. gigas*	MALE	2	Samutphrakarn
8	P-11	*T. gigas*	MALE	2	Samutphrakarn
9	TF-01	*T. gigas*	FEMALE	3	Samutsakorn
	TM-01	*T. gigas*	MALE	5	Samutsakorn
	CF-01	*C. rotundicauda*	FEMALE	10	Samutsakorn
	CM-01	*C. rotundicauda*	MALE	15	Samutsakorn
10	TF-02	*T. gigas*	FEMALE	2	Samutphrakarn
	TM-02	*T. gigas*	MALE	5	Samutphrakarn
	TM-03	*T. gigas*	MALE	6	Samutphrakarn
	CF-02	*C. rotundicauda*	FEMALE	20	Samutphrakarn
11	TF-03	*T. gigas*	FEMALE	3	Samutphrakarn
	TM-03	*T. gigas*	MALE	8	Samutphrakarn
	CF-03	*C. rotundicauda*	FEMALE	14	Samutphrakarn
	CM-02	*C. rotundicauda*	MALE	12	Samutphrakarn
12	CM-03	*C. rotundicauda*	MALE	18	Chonburi
	CM-04	*C. rotundicauda*	MALE	10	Chonburi
	CF-04	*C. rotundicauda*	FEMALE	12	Chonburi
	CF-05	*C. rotundicauda*	FEMALE	11	Chonburi
13	TM-05	*T. gigas*	MALE	10	Samutphrakarn
	TM-06	*T. gigas*	MALE	7	Samutphrakarn
	CM-05	*C. rotundicauda*	MALE	19	Samutphrakarn
	CF-06	*C. rotundicauda*	FEMALE	25	Samutphrakarn
14	TF-04	*T. gigas*	FEMALE	3	Samutphrakarn
	CM-06	*C. rotundicauda*	MALE	10	Samutphrakarn
15	TM-07	*T. gigas*	MALE	4	Samutphrakarn
	CF-07	*C. rotundicauda*	FEMALE	7	Samutphrakarn
16	TF-05	*T. gigas*	FEMALE	5	Samutphrakarn
	CM-07	*C. rotundicauda*	MALE	7	Samutphrakarn

* The crabs had been kept in sea water for second haemolymph collection after 24-hour from the first collection and were found that there was no activity.

3.2 Preparation of amoebocytes The above mixture of haemolymph and the solution was subjected to centrifugation at 80 g, 25°C for 10 minutes (Mistral 3000, MSE, England) in order to assist sedimentation of the cells. The blue supernate was discarded or used for testing osmolarity as described below. The loosely-packed cells so obtained were then resuspended in 3% sodium chloride in the same container. When the container was the centrifuge bottle, a suspended cells were transferred aseptically to a centrifuge tube or when freeze and thaw technic was used. The cells were transfer to polypropylene centrifuge tubes in a closed cabinet. The cells were loosely-packed with the same condition of centrifugation and resuspended with 3% sodium chloride solution for three times in order to remove EDTA. The volume of pack cells was recorded.

3.3 Test for osmolarity Three experiments were carried out to determine osmolarity (with the help of Quality Control section of Queen Saovabha Memorial Institute using Micro Osmometer Model 3 MO, Advanced Instruments, Inc.,U.S.A.).

3.4 Lysis of the amoebocytes After cells were washed as described, pyrogen-free distilled water with or without 5mM Tris buffer pH 7.4 was added at a 1:3 ratio of packed cells to the water. Together with, five glass beads (4 mm. in diameter) were added and the mixture was vortex-mixed for 30 seconds three times, with five minute intervals or without adding of glass beads the mixture was subjected to freeze and thaw 10 times in acetone-dry ice and 37°C water bath. Then it was centrifuged at 2020 g (3000 R.P.M.) at 25°C for 30

minutes. After investigation, only Water for Injection and the freeze-thaw method were used. The clear solution was pipetted into vials or test-tubes and stored at -20°C or subjected to lyophilization.

The overall preliminary trial methods for each lot, are tabulated in table 7.

Table 7. Detail of preliminary experiments

Lot no.	Anti-clotting solution	Method of lysis
P-01	0.125% EDTA 3% NaCl in 5 mM Tris buffer	Tris buffer and vortexing
P-02	0.025 M EDTA 3% NaCl in 5 mM Tris buffer	Tris buffer and vortexing
P-03	0.05 M EDTA 3% NaCl in 5 mM Tris buffer	Tris buffer and vortexing
P-04	0.025 M/0.05 M EDTA 3% NaCl in 5 mM Tris buffer	Tris buffer and vortexing
P-05	0.025 M EDTA 3% NaCl in 5 mM Tris buffer	Water and beads
P-06	0.025 M EDTA 3% NaCl in 5 mM Tris buffer	Water and beads
P-07	0.025 M EDTA 3% NaCl in 5 mM Tris buffer	Water and beads
P-08	0.025 M EDTA 3% NaCl in 5 mM Tris buffer	Water and beads
P-09	0.025 M EDTA 3% NaCl in 5 mM Tris buffer	Water and beads
P-10	0.025 M EDTA 3% NaCl in Water for injection	Water and beads
P-11	0.025 M EDTA 3% NaCl in Water for injection	Freeze and thaw

Each lot of the lysate was standardized for protein content and gelation with endotoxin.

4 Protein content determination Each lot of the lysate was subjected to total protein determination by using Lowry protein

determination method (74).

Standard protein Bovine Albumin lot no. 18F-0409, Sigma, U.S.A. provided by Research and Development section (R&D) of Queen Soavabha Memorial Institute (QSMI) was used as standard protein. Twenty-five milligrams of the protein was dissolved in 5 mL distilled water to give a concentration of 500 ug/mL. The standard protein solution was prepared in three sets of different concentrations. Each set was composed of 250, 125, 100, 50, and 25 ug/mL.

Reagents Solution A (2% Na_2CO_3 in 0.1 N NaOH) and solution B (0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate) were provided by R&D of QSMI which there were used in routine research. The solution C, was prepared freshly before use by mixing 50 mL of solution A and 1 mL of solution B. Folin-Ciocalteu Reagent, lot no. 64016090, E. Merck, was diluted with distilled water in double amount prior to use. This diluted reagent was assigned as Solution D.

Performance of the test 0.5 mL of either standard protein solution or unknown protein lysate solution which had a protein concentration not more than 500 ug/mL; otherwise diluted with distilled water to appropriate concentration. To this solution, 3mL of solution C was added and mixed well. The mixture was allowed to stand for 10 minutes at room temperature. Then 0.3 mL of solution D was rapidly added with prompt mixing. After 30 minutes of standing at room temperature, the absorbance (ABS) was read at 650 nm using Shimadzu spectrophotometer model UV-260 from the Quality Control section of

QSMI. The total protein concentration of the unknown was obtained by reading from calibrated curve of ABS against concentration or calculating from a formula :- conc. = K*ABS + B. The protein content of the lysate from each species and sexes of the horseshoe crabs was compared statistically using Student's T test from Epistat software program.

5 Endotoxins In this study, there were 3 types of endotoxin used.

5.1 Working standard endotoxin (WSE) This was lipopolysaccharide (LPS) extracted from *Escherichia coli* 055:B5 by Westphal method, control no. 749885 and was purchased from Difco Laboratory, U.S.A.. A vial contained 25 mg. of LPS was opened under vertical laminar flow. The powdered LPS was immediately weighed approximately 1 mg. into depyrogenated centrifuge tubes using the digital analytical balance, Satorius Model 1602 NP-1. The tubes were capped and stored -20°C. When the endotoxin was required for testing one of the centrifuge tubes containing LPS was diluted to exactly 1 mg/mL in screw-cap glass tube with 5mM Tris buffer. 0.1 mL of 1 mg/mL endotoxin was mixed by the help of vortex for 20 minutes with the buffer to obtain 10 mL of 10 ug/mL of endotoxin. 0.1 mL of 10 ug/mL was again mixed with 9.9 mL of the buffer to obtain 100 ng/mL of the endotoxin by vortexing 10 minutes. This 100 ng/mL endotoxin was divided into 1 mL in screw-cap tubes and stored at -20°C. The shelf-life period was one to two months.

5.2 Reference endotoxin (RE) was purchased from Sigma,

U.S.A. lot no. 124F-6842 having an activity as 2 ug *E. coli* O55:B5 equivalent to 7,600 E.U. (Endotoxin Unit). One mL of 5mM Tris buffer was added to the lyophilized reference endotoxin with vortexing for 20 minutes. 0.1 mL of 2 ug/mL endotoxin was diluted with 1.7 mL Tris buffer to give 100 ng/mL of endotoxin. This 100 ng/mL reference endotoxin was diluted to 2, 1, 0.5, 0.1, and 0.05 ng/mL as described below. This reference endotoxin was used in parallel with tests as indicated, for comparison with working standard endotoxin.

5.3 Standard endotoxin (SE) was purchased from Wako, Japan as LPS from *E. coli* O55:B5 lot no. DDL 9217. It was instructed to dilute whole vial with 5 mL diluent. Therefore 5 mL Tris buffer was added to give 100 ng/mL endotoxin, and it was used in some unknown parenteral fluid tests as indicated hereunder.

In sensitivity tests with known endotoxin concentrations, 100 ng/mL of endotoxin was diluted one ten-fold to obtain 10 ng/mL which further diluted one five-fold to give 2 ng/mL endotoxin. This 2 ng/mL endotoxin was again diluted two times to give 1 ng/mL and in turn, was diluted two times and ten times to obtain 0.5 ng/mL and 0.1 ng/mL of endotoxin respectively. The 0.1 ng/mL of endotoxin was further diluted two times to 0.05 ng/mL. All dilutions were performed with Tris buffer pH 7.4. Hence, there were 2, 1, 0.5, 0.1, and 0.05 ng/mL of endotoxin used for the sensitivity test along with negative control.

6 Commercial Limulus Amoebocyte Lysate (LAL) There were two

commercial types used in this study as :-

1 Marine Biological Ltd., U.S.A. lot no. 45693

2 Wako Ltd., Japan. lot no. LAE 9660.

7 Performance of the amoebocyte lysate endotoxin assay (3,17) There were two methods used in this study, namely Tube Method and Micro-test Method. Both of the methods were performed either in closed cabinet or under vertical laminar flow cabinet with aseptic technic.

Preparation of test material All the test materials either standard endotoxin or working standard endotoxin or unknown endotoxin solutions were subjected to pH determination and would have a pH within 6 to 8 (75). The solution which was not in this range, was adjusted by using either 0.1N HCl or 0.1 N NaOH (see appendix).

7.1 Tube method (3) The assay was performed by adding 0.1 mL of the test material to a 0.1 mL of amoebocyte lysate in 10x75 mm. pyrogen-free glass test tube. Negative control was prepared by adding 0.2 mL of the lysate and 0.1 mL of the Tris buffer pH 7.4 (see appendix). Positive control was the mixture of lysate and standard or working standard endotoxin which had a concentration of 0.1 ng/mL. The mixture was incubated at 37°C for 60 minutes.

7.1.1 Interpretation of result At the end of incubation period the result so obtained was interpreted according to Jorgensen et al 1973 (3) with some modifications as followed :-

4 means firm gel upon 180 degree inversion of the

tube.

3 means soft gel with very viscous upon 180 degree inversion.

2 means weak gel with adhesion of starch-like floccules to sides of the tube when the tube was slanted.

1 means very weak gel with some starch-like floccules adhering to sides of the tube.

0 means no visible increase in viscosity or opacity.

7.2 Micro-test method (46) The assay was done by adding 10 uL each of the test material and 10 uL of the lysate to form a drop in a pyrogen-free sterile petri dish. This was then incubated at 37°C for 30 minutes. Not more than one week old defibrinated sheep blood was then added on the top of each drop in the volume of 5 uL. The assay was carried out with negative and positive control as in the tube method.

7.2.1 Interpretation of result The results were read immediately after the addition of the blood. In case of positive reaction the red cells remained held together within the drop in the shape of a bright red button surrounded by a clear zone. In case of negative one (no gelation) the blood dispersed throughout the drop.

The sensitivity of the lysate from each species of the crabs was statistically compared using Rank test from Epistat software program.

8 Addition of magnesium ions (76) It was done to determine optimum concentration of magnesium chloride used in activation of the lysate reaction with standard endotoxin. Stock solution of 1 M $MgCl_2$ solution was prepared (see appendix) and was tested for endotoxin-free using commercial LAL test. The $MgCl_2$ stock solution was diluted ten times to give 0.5 M concentration with water for injection. The endotoxin stock solution of 100 ng/mL was obtained as previously described and was diluted with ten fold dilution to obtain 0.1 ng/mL. There were four concentrations of $MgCl_2$ used in this test, i.e., 100 mM, 50 mM, 25 mM, and 12.5 mM. In order to obtain 0.1 ng/mL of endotoxin with different $MgCl_2$ concentrations, they were prepared as shown in table 8. Each solution was tested with different lots of the lysate using the tube method as previously described. The test was done three times in each lot of the lysate. The result was analyzed by statistical Rank test using Epistat computer software.

Table 8. Preparation of Magensium Chloride concentration.

0.1 ng/mL Endotoxin(WSE) with $MgCl_2$ conc.	components		
	1 ng/mL Endotoxin(WSE)	0.5 M $MgCl_2$	Water for Injection
100 mM	0.2 mL	0.4 mL	1.4 mL
50 mM	0.2 mL	0.2 mL	1.6 mL
25 mM	0.2 mL	0.1 mL	1.7 mL
12.5 mM	0.2 mL	0.05 mL	1.75 mL

9 Optimum incubation period of the lysate assay. In order to determine the optimal incubation period, five sets of the lysate sensitivity tests were prepared for both tube method and micro method.

Each set of the test consisted of 2, 1, 0.5, 0.1 ng/mL of endotoxin. For the tube method, 0.1 mL of endotoxin, 0.1 mL of the lysate and 0.01 mL of 1 M $MgCl_2$ solution were used. For the micro method, 10 uL of the same endotoxin concentrations and 10 uL of the lysate along with 1 uL of 1 M $MgCl_2$ which added to endotoxin solution with the help of microsyringe before the addition of the lysate. The incubation temperature was 37°C and incubation time was varied from 0, 15, 30, 45, 60 minutes in case of tube method and from 0, 15, 20, 25, 30 minutes in case of micro method. After such incubation periods, the reactions were read and recorded.

10 Specificity of the lysate (77,78) The test was carried out to define the reaction of GAL and CAL to autoclave killed bacterial organisms. The organisms and their sources used in the test were tabulated along with media used for their growth in table 9. Two or three colonies of these organisms were taken from the given specimen and inoculated in 30 mL of corresponding broth containing in 250 mL sterilized conical flasks which were incubated at 37°C overnight prior to inoculation. Each genus of the bacteria were inoculated to the respective broth as indicated in table 9. Subsequently, the inoculated broths were incubated at 35°C with constant rotated shaking at 200 R.P.M. with the help of Incubator and Lab Shaker, Adolf Kuhner AG, Schweiz. The incubation period was overnight except that for *Candida albicans* which was incubated for three days. At the end of the incubation period, the broths were cooled in ice bath and 1 mL of each broth was taken to make ten fold serial dilution with normal saline solution, starting from 10^{-1} to 10^{-9} dilutions, totalling of nine

dilutions for each broth. These dilutions which were in 13 mL screw-cap tubes were also cooled in ice bath. Corresponding to the agar plates used for each organisms (see table 9), 0.1 mL of each ten-fold dilutions were plated on three agar plates separately with the help of pipette and plating glass rod. Then, the agar plates were incubated statically in the incubator (Memmert model B 80) at 37°C for 2 days except that of *Candida albicans* was incubated for 5 days. The purity of the organisms was examined and the colony-count was performed for each plate by using Braun colony counter. The average amount of bacteria from three plates in the broth was calculated using the following formula :-

Table 9. Bacterial organisms, sources, and media used in culture.

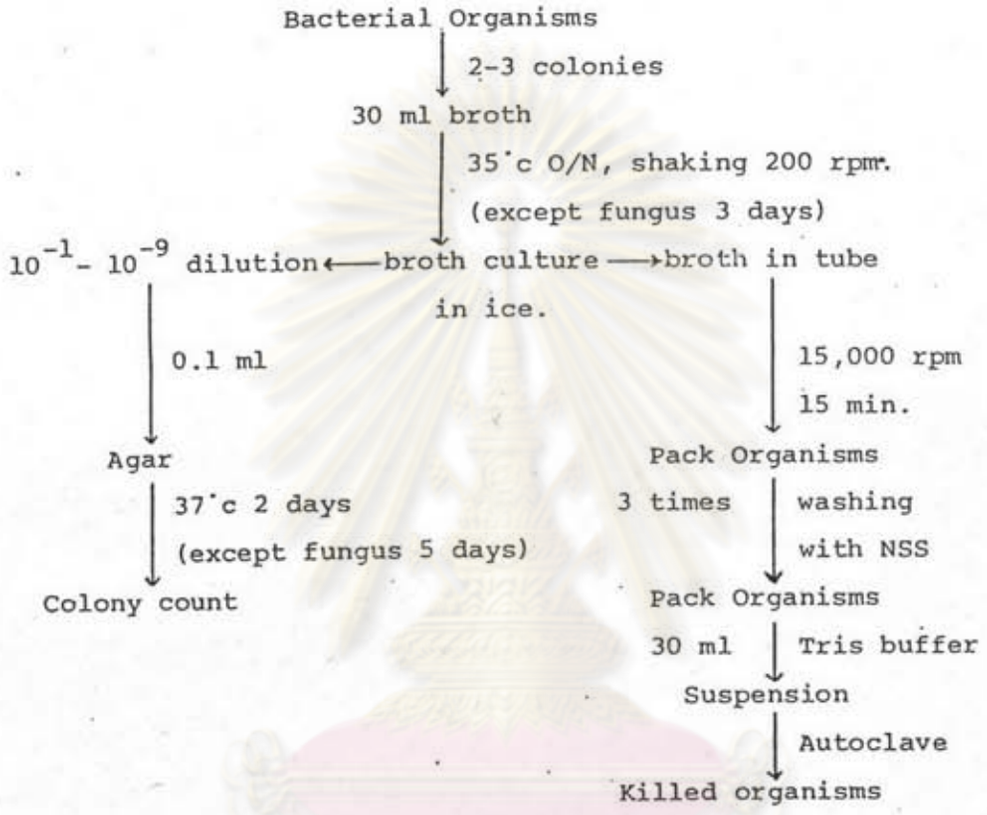
S.N.	ORGANISMS	SOURCES	MEDIA USED	
			BROTH	AGAR
1	*E. coli* ATCC 25922	Microbiology Department Faculty of Medicine Chulalongkorn University	Tryptic Soy Broth (TSB)	Tryptic Soy Agar (TSA)
2	*S. typhi* NCTC 781	-as above-	TSB	TSA
3	*P. aeruginosa* ATCC 27853	-as above-	TSB	TSA
4	*K. pneumoniae*	Clinical specimen	TSB	TSA
5	*S. aureus* ATCC 25923	-as first organism-	TSB	TSA
6	*S. marcescens* DMS 0300	N.I.H., Thailand	TSB	TSA
7	*Streptococcus* C203S	National Streptococcus Center, Thailand.	Todd-Hewett Broth (THB)	Blood Agar (BA)
8	*C. albicans* CDC 85-000000	-as first organism-	Sabouraud Dextrose Broth(SDB)	Sabouraud Dextrose Agar(SDA)

Bacteria/mL = Average bacterial colonies x dilution factor x 10

The amount of bacterial per mL was represented the amount of live bacterial organisms in the broth. These calculations were recorded.

The broths so obtained were centrifuged at 15,000 R.P.M. at 4°C for 15 minutes using Beckman J2-21 centrifuge. The supernate discarded whereas the packed organisms were resuspended in 30 mL normal saline solution and then centrifuged at the same conditions. The washing with normal saline solution was repeated three times. The packed organisms were resuspended in 30 mL Tris buffer pH 7.4 which were contained in 100 mL conical flasks. The obtained suspensions were subjected to autoclave. Each killed organism suspension was diluted to ten fold dilution Tris buffer in order to possess a range of 10^7 to 10^3 organisms. To each genera of the bacteria, the endotoxin assay was performed by using 0.1 mL of the lysate and 0.01 mL 1 M $MgCl_2$ solution. The mixture was incubated for 60 minutes at 37°C and at the end of incubation period the gelation reaction was observed and recorded. Overall experimental design was depicted in figure 6.

Figure 6. Experimental design of LPS. preparation.



11 Lyophilization of the lysate (79) The lysate was divided into four lots having a volume of two mL each. Each lot of the lysate was mixed with either 2% or 4% dextran (molecular weight approximately 40,000 daltons, Fluka, West Germany lot no. 246362) solutions to give 1% or 2% dextran content of each lot of lysate respectively. At this stage, 0.1 mL of the mixture was taken for sensitivity test. The mixture were pipetted into 3 mL vials, each contained 0.2 mL of the mixture lysate, and were rapidly frozen in acetone and dry-ice and 0.1 mL of the frozen mixture was rapidly thawed and then tested for sensitivity. The frozen lysate mixtures were placed on heat-exchanger plate which initially maintained temperature at -45°C. Then evacuation of air was started to implement sublimation at pressure approximately 1 to 3 x 10^{-1} mbar using Lybold GT-2 lyophilizer. The rate of heat exchange was increased approximately 3°C per hour, and at -5°C, the temperature was kept constant for three hours before further increased the temperature at the rate of 5°C per hour, until the final temperature was 25°C. The rubber closure was done under vacuum and Aluminum caps were used to close the vials. The product was stored at approximately 4°C.

12 Shelf life test The four lots of lyophilized lysate were assayed with 2, 1, 0.5, 0.1, 0.05 ng/mL endotoxin, and negative control as described in sensitivity test, in different period of time, i.e., day 0, 7, 14, 30, 60, and 90. Other set of experiment was set up for testing the lysate which stored at -20°C by performing the test at day 0, 15, 30, 45, 60, 75, and 90.

13 Detection of endotoxin in parenteral fluids or drugs (80)

The twenty-five unknown samples from three sources were coded from

Table 10. Types and codes of unknown samples.

S.N.	CODE NO.	TYPES OF SAMPLE	REMARK
1	S-01	WATER FOR INJECTION	
2	S-02	WATER FOR INJECTION	
3	S-03	WATER FOR INJECTION	
4	S-04	WATER FOR INJECTION	
5	S-05	WATER FOR INJECTION	FROM MARKET SOURCE
6	S-06	WATER FOR INJECTION	FROM MARKET SOURCE
7	S-07	NORMAL SALINE SOLUTION	
8	S-08	NORMAL SALINE SOLUTION	
9	S-09	NORMAL SALINE SOLUTION	
10	S-10	NORMAL SALINE SOLUTION	
11	S-11	NORMAL SALINE SOLUTION	FROM MARKET SOURCE
12	S-12	5% DEXTROSE 1/2 SALINE	
13	S-13	5% DEXTROSE 1/2 SALINE	
14	S-14	5% DEXTROSE 1/2 SALINE	
15	S-15	5% DEXTROSE 1/2 SALINE	
16	S-16	5% DEXTROSE 1/2 SALINE	
17	S-17	5%DEXTROSE NORMAL SALINE	
18	S-18	5%DEXTROSE NORMAL SALINE	
19	S-19	5%DEXTROSE NORMAL SALINE	
20	S-20	5%DEXTROSE NORMAL SALINE	
21	S-21	5%DEXTROSE NORMAL SALINE	
22	S-22	5%DEXTROSE NORMAL SALINE	FROM PRIVATE FIRM
23	S-23	PARACETAMOL FOR INJECTION	FROM PRIVATE FIRM
24	S-24	PARACETAMOL FOR INJECTION	FROM PRIVATE FIRM
25	S-25	PARACETAMOL FOR INJECTION	FROM PRIVATE FIRM

S-01 to S-25 as shown in table 10. The test was so arranged that the samples were grouped according to their types, and were tested in triplicate. Prior to the test, pH of the samples were determined whether with the range of 6 and 8 or otherwise were adjusted with 0.1N HCl or 0.1N NaOH. The samples were thoroughly shaken for two minutes. Each series of tests consisted of samples, negative control, positive control and inhibition testing as shown in table 11. The inhibition test was included in the series was to determine whether test samples

contained factor which inhibited the gelation reaction. Therefore, 0.01 mL of 10 ng/mL endotoxin was added, in addition of test sample and lysate mixture, to obtain 0.1 ng/mL concentration of the endotoxin in the mixture.

Table 11. Arrangement of the unknown samples testing.

Components	Samples (mL) 1	2	3	Negative control (mL)	Inhibition test (mL)	Positive control (mL)
Samples	0.1	0.1	0.1	-	0.1	-
Water for Injection	-	-	-	0.1	-	-
1 M MgCl2	0.01	0.01	0.01	0.01	0.01	0.01
10 ng/mL endotoxin	-	-	-	-	0.01	-
0.1 ng/mL endotoxin	-	-	-	-	-	0.1
Lysate	0.1	0.1	0.1	0.1	0.1	0.1