# CHAPTER III

## Results

### Preparation of amoebocyte lysate

Preliminary trials At the beginning of the study, there were mainly two factors, the anti-clotting solution concentration and choice of cell lysis method to be considered. The first experiment (P-01) was carried out by using 0.125% (0.0034 M) EDTA 3% NaCl in 5 mM Tris buffer pH 7.4 as the anti-clotting solution and followed by using 5 mM Tris buffer alone for cell lysis. The obtained lysate was tested with endotoxin but it was not able to give gelation even at a level of endotoxin of 100 ng/mL. In P-02 experiment, the anti-clotting EDTA was 0.025 M and the rest of conditions were set constant. It was found that the lysate could form gel at the endotoxin level of 1 ng/mL. In experiment P-03, 0.05 M EDTA was used and it was found that there was no gelation even with endotoxin at 100 ng/mL. Experiment P-04 was carried out to repeat the conditions of P-02 and P-03 using one crab each. The gelation could only be achieved with the lysate prepared from 0.025 M EDTA as expected from P-02.

In order to increase volume yield of lysate, expressed in ratio of lysate volume and pack cell volume, five glass beads having diameter of 4 mm. were added to the pack cells before the addition of water and vortexing. The results P-05 to P-10, were observed

separately and the lysate from these six experiments could give gelation at 1 ng/mL level for P-05, P-06, and 0.1 ng/mL level of endotoxin for P-07, P-08, P-09, and P-10 with addition of 0.01 mL of 1 M $MgCl_2$ solution. These results produced an increased volume yield of 7.8%. The difference of P-10 and the rest of this group was that P-10 did not contain Tris buffer in the anti-clotting solution and it was replaced by water for injection, but the result remained the same.

Increase of the volume yield was again attempted using the freeze and thaw technic in P-11 experiment, the yield was increased by 3.6%, with similar clotting activity. The yield and sensitivity was shown in table 12.

Table 12. Volume of pack cell and lysate per crab with their ratio and sensitivities.

| Lot no. | Pack cells ( mL/ crab) | Lyaste ( mL/ crab) | 0.01 mL 1 M $MgCl_2$ | Sensitivities[a] ng/mL | Ratio Lysate/Pack cell |
|---|---|---|---|---|---|
| P-01 | N/D | N/D | - | >100 | N/D |
| P-02 | N/D | N/D | - | 1* | N/D |
| P-03 | N/D | N/D | - | >100 | N/D |
| P-04 | 3.0/2.8 | 7.2/6.0 | - | >100/1 | 2.4/2.14 |
| P-05 | 2.8 | 6.5 | - | 1 | 2.32 |
| P-06 | 0.5 | 1.0 | - | 1 | 2.00 |
| P-07 | 2.9 | 7.0 | + | 0.1 | 2.41 |
| P-08 | 2.3 | 5.4 | + | 0.1 | 2.35 |
| P-09 | 0.6 | 1.3 | + | 0.1 | 2.17 |
| P-10 | 2.1 | 4.9 | + | 0.1 | 2.33 |
| P-11 | 2.8 | 7.0 | + | 0.1 | 2.50 |

N/D = Not Determined. - = Not Incorporated. + = Incorporated
* = Test was repeated twice with the same result.
a = 100, 10, 2, 1, 0.5, 0.1, 0.05 ng/mL of endotoxin and water for negative control.

Actual preparation of amoebocyte lysate After the preliminary trials had been performed to establish the optimal

conditions for preparation, the actual preparation of the lysate was carried out using 0.025 M EDTA 3% NaCl in water for injection as the anti-clotting solution, water for injection as lysis solution and freeze and thaw technic for lysis procedure. The volume of both pack cells and lysate per crab, and ratio of lysate to pack cells per crab were tabulated in table 13.

Table 13. Volume of pack cell and lysate per crab with their ratio.

| Lot no. | Pack cells ( mL/ crab) | Lysate ( mL/ crab) | Ratio Lysate/Pack cell |
|---|---|---|---|
| TF-01 | 2.5 | 6.5 | 2.60 |
| TM-01 | 2.1 | 5.0 | 2.38 |
| CF-01 | 0.7 | 1.5 | 2.14 |
| CM-01 | 0.4 | 0.9 | 2.25 |
| TF-02 | 2.7 | 6.9 | 2.56 |
| TM-02 | 2.3 | 5.3 | 2.30 |
| TM-03 | 2.4 | 5.6 | 2.33 |
| CF-02 | 0.6 | 1.4 | 2.33 |
| TF-03 | 2.8 | 6.9 | 2.46 |
| TM-04 | 2.2 | 5.3 | 2.41 |
| CF-03 | 0.8 | 1.8 | 2.25 |
| CM-02 | 0.5 | 1.1 | 2.20 |
| CM-03 | 0.4 | 0.9 | 2.25 |
| CM-04 | 0.6 | 1.5 | 2.50 |
| CF-04 | 0.5 | 1.3 | 2.60 |
| CF-05 | 0.8 | 1.8 | 2.25 |
| TM-05 | 2.3 | 5.7 | 2.48 |
| TM-06 | 2.0 | 4.9 | 2.45 |
| CM-05 | 0.3 | 0.7 | 2.33 |
| CF-06 | 0.7 | 1.6 | 2.29 |
| TF-04 | 2.7 | 6.8 | 2.52 |
| CM-06 | 0.6 | 1.3 | 2.17 |
| TM-07 | 2.5 | 5.6 | 2.24 |
| CF-07 | 0.9 | 1.6 | 1.78 |
| TF-05 | 2.5 | 7.0 | 2.80 |
| CM-07 | 1.0 | 1.2 | 1.20 |

The average ratio of lysate and pack cells per crab of female and male *T. gigas* and that of female and male *C. rotundicauda* were 2.59, 2.37,

2.23, and 2.13 respectively. The average lysate per crab of female and male *T. gigas*, and that of female and male *C. rotundicauda* were 6.86, 5.34, 1.57, and 1.09 mL respectively.

Osmolarity test Three lots of amoebocyte lysate were tested for the osmolarity of anti-clotting solution and the mixture of haemolymph and anti-clotting soln. at a volume ratio of 1:1. The osmolarity of the three lots was as shown below.

| lot no. | anti-clotting soln. (mOsm/L) | mixture (mOsm/L) |
|---|---|---|
| P-05 | 908 | 919 |
| P-06 | 910 | 914 |
| P-07 | 904 | 910 |

The values of the mixture from three lots of the lysate were varied due to the fact that haemolymph flowed directly into a bottle containing anti-clotting solution therefore the ratio of the two cannot be accurately kept at 1:1. However, the anti-clotting solution was roughly isotonic for the blood of the crabs.

Protein content of amoebocyte lysate After the preparation of lysate, approximately 0.1 mL of the lysate was kept in refrigerator for protein determination within 24 hours. The lysate was diluted either 50 or 20 times using distilled water as indicated in table 14. Standard protein curve was obtained by plotting standard protein concentration against optical density of that sample as shown in figure 7. The protein concentration was obtained from formula : Concentration = K * ABS + B. The spectrophotometer was calculated the value of K and B from standard protein as:- K = 559.84 and B =

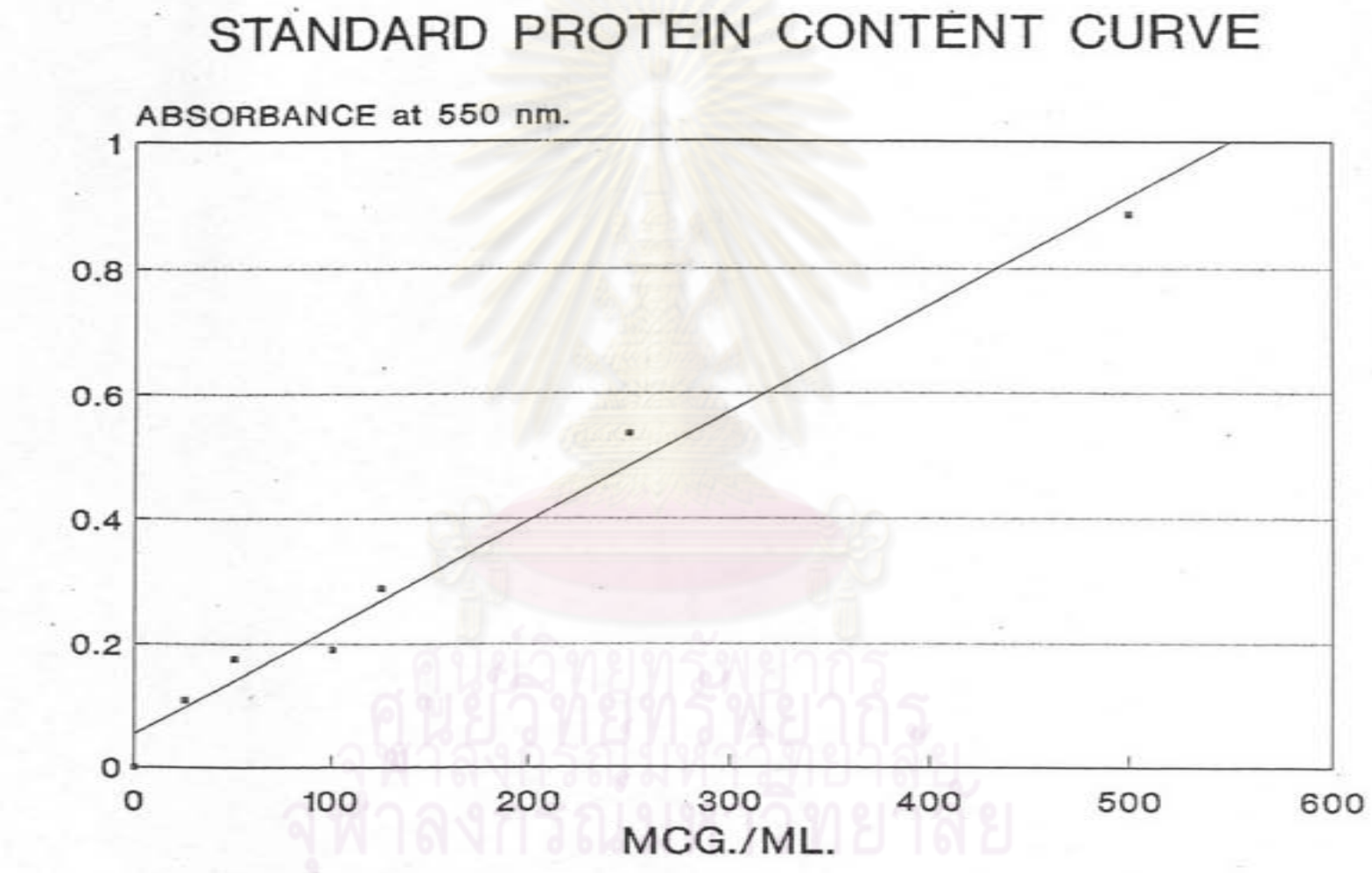


Figure 7. Standard protein curve.

-30.713. This gave a linear regression as 0.9765. The result of protein concentration in each lysate was shown in table 14. The protein content of the lysate was variable. When the data was analyzed by using Student's T test, the protein content of the lysate from both species and sexes of the crabs was not significantly different and when probability of each type of crab was calculated in crosstabulation, the result was P = 0.15 for TF and TM, P = 0.73 for CF and CM, P = 0.31 for TF and CF, P = 0.33 for TF and CM, P = 0.50 for TM and CF, and P = 0.35 for TM and CM.

Table 14. Protein content of amoebocyte lysate.

| Lot no. | Protein content of lysates (ug/mL) |
|---|---|
| TF-01 | 1,292 |
| TM-01 | 1,571 |
| CF-01 | 1,459 |
| CM-01 | 1,054 |
| TF-02 | 1,376 |
| TM-02 | 1,404 |
| TM-03 | 1,020 |
| CF-02 | 797 |
| TF-03 | 1,404 |
| TM-04 | 1,683 |
| CF-03 | 1,348 |
| CM-02 | 998 |
| CM-03 | 1,222 |
| CM-04 | 1,233 |
| CF-04 | 1,515 |
| CF-05 | 987 |
| TM-05 | 1,211 |
| TM-06 | 1,132 |
| CM-05 | 1,459 |
| CF-06 | 1,348 |
| TF-04 | 1,177 |
| CM-06 | 1,233 |
| TM-07 | 1,432 |
| CF-07 | 1,404 |
| TF-05 | 1,320 |
| CM-07 | 1,376 |

Sensitivity of amoebocyte lysate Sets of 2, 1, 0.5, 0.1, 0.05 ng/mL of endotoxin (WSE) were used with 0.01 mL of $MgCl_2$ solution for testing of sensitivity of the lysate. The result are shown in table 15 for GAL and table 16 for CAL.

From the results it was concluded that all the lysate from either *T. gigas* or *C. rotundicauda* could give the gelation reaction with similar level of endotoxin concentration ( 0.1 ng/mL). The rank test indicated the statistical difference between 0.1 ng/mL level and 0.05 ng/mL levels both for GAL ($P < 10^{-6}$) and for CAL ($P<10^{-6}$) , and the reaction at 0.1 ng/mL of endotoxin showed that the preparation of both species was not significantly different. However, both species showed a significant difference at the 0.05 ng/mL of endotoxin. The arrangement of the sensitivity test was shown in figure 8 and the gelation reaction end-point of the lysate was shown in figure 9.

Table 15. Sensitivity test of GAL.

| Lot no. | Endotoxin,WSE ( ng/mL) | | | | | W.I. |
|---|---|---|---|---|---|---|
| | 2 | 1 | 0.5 | 0.1 | 0.05 | |
| TF-01 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 2, 3, 2 | 0, 0, 0 |
| TM-01 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 3, 4 | 2, 2, 3 | 0, 0, 0 |
| TF-02 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 2, 2, 2 | 0, 0, 0 |
| TM-02 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 2, 2 | 0, 0, 0 |
| TM-03 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 2, 2, 2 | 0, 0, 0 |
| TF-03* | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 3 | 3, 3, 3 | 0, 0, 0 |
| TM-04* | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 3, 3 | 0, 0, 0 |
| TM-05 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 3, 2 | 0, 0, 0 |
| TM-06 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 3 | 2, 2, 2 | 0, 0, 0 |
| TF-04 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 2, 2, 2 | 0, 0, 0 |
| TM-07 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 2, 2 | 0, 0, 0 |
| TF-05 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 3, 2 | 0, 0, 0 |

* = One of the test was run in parallel with commercial test.
4 = firm gel; 3 = soft gel; 2 = weak gel; 1 = very weak gel;
0 = no gelation. W.I. = Water for Injection.

Table 16. Sensitivity test of CAL.

| Lot no. | Endotoxin, WSE ( ng/mL) | | | | | W.I. |
|---|---|---|---|---|---|---|
| | 2 | 1 | 0.5 | 0.1 | 0.05 | |
| CF-01 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 4, 4 | 2, 2, 2 | 0, 0, 0 |
| CM-01 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 3, 3 | 0, 0, 0 |
| CF-02 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 3, 3 | 0, 0, 0 |
| CF-03 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 2, 2 | 0, 0, 0 |
| CM-02* | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 3, 3 | 0, 0, 0 |
| CM-03 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 3 | 2, 2, 3 | 0, 0, 0 |
| CM-04 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 3, 2 | 0, 0, 0 |
| CF-04* | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 3, 3 | 0, 0, 0 |
| CF-05 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 3, 4 | 3, 3, 3 | 0, 0, 0 |
| CM-05 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 2, 2, 2 | 0, 0, 0 |
| CF-06 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 3, 2 | 0, 0, 0 |
| CM-06 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 3, 3 | 0, 0, 0 |
| CF-07 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 3, 4, 4 | 2, 2, 2 | 0, 0, 0 |
| CM-07 | 4, 4, 4 | 4, 4, 4 | 4, 4, 4 | 4, 4, 3 | 2, 2, 2 | 0, 0, 0 |

* = One of the test was run in parallel with commercial test.
4 = firm gel; 3 = soft gel; 2 = weak gel; 1 = very weak gel;
0 = no gelation. W.I = Water for Injection.

Optimal Magnesium concentration This was to confirm the use of $MgCl_2$ concentration. The results were shown in table 17.

Table 17. Optimal concentration of Magnesium ions.

| lot no. | Solution of 0.1 ng/mL endotoxin with $MgCl_2$ | | | |
|---|---|---|---|---|
| | 100mM | 50mM | 25mM | 12.5mM |
| TF-03 | 3, 4, 4 | 4, 3, 4 | 2, 4, 3 | 0, 2, 2 |
| TM-04 | 4, 3, 3 | 4, 4, 4 | 2, 0, 3 | 1, 3, 0 |
| CF-03 | 2, 3, 4 | 4, 4, 4 | 3, 0, 2 | 2, 1, 1 |
| CM-02 | 4, 4, 3 | 4, 3, 4 | 1, 0, 4 | 2, 2, 0 |
| CM-04 | 3, 3, 4 | 4, 4, 4 | 2, 2, 3 | 0, 0, 0 |

4 = firm gel; 3 = soft gel; 2 = weak gel; 1 = very weak gel;
0 = no gelation.

Figure 8. Arrangement of sensitivity test.

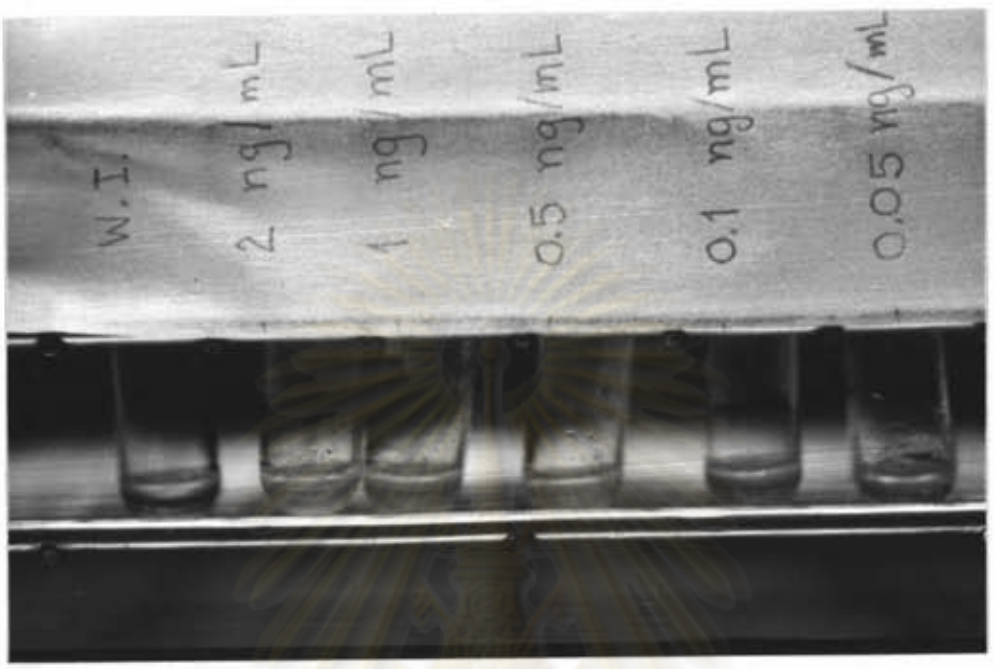


Figure 9. Firm gelation reaction of tube method.

There were statistical differences in the gelation activity at concentrations of $MgCl_2$ between 50 mM and 25 mM $MgCl_2$ (P=0.00061), and between 50 mM and 100 mM (P=0.084) concentration. Therefore the concentration of 50 mM $MgCl_2$ considered to be the optimal concentration and was used for various experiments of amoebocyte lysate test.

Optimal incubation period We used two methods to study the optimal incubation period, i.e., tube and micro-test methods as shown in table 18 and table 19 respectively.

Table 18. Optimal incubation period of tube method.

| Incubation period (minutes) | TM-02 Endotoxin (WSE) (ng/mL) | | | | | CF-04 Endotoxin (WSE) (ng/mL) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 1 | 0.5 | 0.1 | 0.05 | 2 | 1 | 0.5 | 0.1 | 0.05 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 2 | 2 | 0 | 0 | 0 | 3 | 2 | 1 | 0 | 0 |
| 45 | 4 | 3 | 3 | 3 | 0 | 3 | 4 | 4 | 2 | 0 |
| 60 | 4 | 4 | 4 | 4 | 3 | 4 | 3 | 4 | 4 | 2 |

4 = firm gel; 3 = soft gel; 2 = weak gel; 1 = very weak gel;
0 = no gelation.

Table 19. Optimal incubation period of micro-test method.

| Incubation period (minutes) | TF-05 Endotoxin (WSE) (ng/mL) | | | | | CM-07 Endotoxin (WSE) (ng/mL) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 1 | 0.5 | 0.1 | 0.05 | 2 | 1 | 0.5 | 0.1 | 0.05 |
| 0 | N | N | N | N | N | N | N | N | N | N |
| 15 | N | N | N | N | N | N | N | N | N | N |
| 20 | N | N | N | N | N | N | N | N | N | N |
| 25 | P | P | ? | N | N | N | P | N | ? | N |
| 30 | P | ? | P | P | ? | P | P | ? | P | ? |

N = Negative. P = Positive. ? = questionable

At 60 minutes of incubation period from the tube method (table

18), it was found to be maximum number of positive (4 grade) in comparison with other periods. Thirty minutes appeared satisfactory for the micro-test method (table 19).

Specificity of endotoxin test The test was to determine the reaction of GAL and CAL with various bacterial organisms. The data shown in table 20 represented total number of killed bacterial cells by using bacterial colony count. These killed bacterial cells were diluted tenfold and reacted with either GAL (table 21) and CAL (table 22).

Table 20. Bacterial organism cells per mL with their dilutions.

| S.N. | ORGANISMS | Original conc. (cells/mL) |
|---|---|---|
| 1 | *E. coli* ATCC 25922 | $4.50 \times 10^9$ |
| 2 | *S. typhi* NCTC 781 | $3.51 \times 10^9$ |
| 3 | *P. aeruginosa* ATCC 27853 | $2.05 \times 10^9$ |
| 4 | *K. pneumoniae* | $5.20 \times 10^9$ |
| 5 | *S. aureus* ATCC 25923 | $1.29 \times 10^{10}$ |
| 6 | *S. marcescens* DMS 0300 | $1.48 \times 10^{10}$ |
| 7 | *Streptococcus* C203S | $6.00 \times 10^8$ |
| 8 | *C. albicans* CDC 85-000000 | $5.40 \times 10^7$ |

The concentration of killed bacterial cells were restricted to the range of $10^3$ to $10^7$ cells/mL as indicated in table 21 and 22. The firm gelation reaction was found in *E. coli*, *K. pneumonia*, *S. marcescens* at the level of approximately $10^4$ cells/mL whereas *S. typhi* and *P. aeruginosa* were found at the level approximately $10^5$ cells/mL. *S. aureus* and *C. albicans* gave firm gelation at $10^6$ cells/mL.

*Streptococcus* was the only organisms in the test that gave no gelation reaction to both GAL and CAL.

Table 21. Reaction of amoebocyte lysate, TF-03 with killed organisms.

| S.N. | ORGANISMS | Amoebocyte lysate test with approx.$10^x$ cells/mL 7 | 6 | 5 | 4 | 3 |
|---|---|---|---|---|---|---|
| 1 | *E. coli* ATCC 25922 | 3,3,3 | 4,4,4 | 4,4,4 | 4,4,4 | 3,3,2 |
| 2 | *S. typhi* NCTC 781 | 3,4,4 | 4,4,4 | 4,4,3 | 3,4,3 | 2,0,0 |
| 3 | *P. aeruginosa* ATCC 27853 | 4,4,4 | 4,4,4 | 4,4,3 | 2,2,2 | 0,1,1 |
| 4 | *K. pneumoniae* | 4,4,3 | 4,3,4 | 4,4,4 | 4,4,4 | 2,2,3 |
| 5 | *S. aureus* ATCC 25923 | 4,4,4 | 4,4,4 | 3,3,3 | 2,3,0 | 1,0,2 |
| 6 | *S. marcescens* DMS 0300 | 3,3,4 | 4,4,4 | 4,4,4 | 4,4,4 | 3,2,2 |
| 7 | *Streptococcus* C203S | 1,1,0 | 0,0,0 | 2,2,0 | 1,1,2 | 0,0,0 |
| 8 | *C. albicans* CDC 85-000000 | 4,4,4 | 4,4,4 | 2,2,3 | 2,0,0 | 0,1,0 |

4 = firm gel; 3 = soft gel; 2 = weak gel; 1 = very weak gel;
0 = no gelation.

Table 22. Reaction of amoebocyte lysate, CF-04 with killed organisms.

| S.N. | ORGANISMS | Amoebocyte lysate test with approx.$10^x$ cells/mL 7 | 6 | 5 | 4 | 3 |
|---|---|---|---|---|---|---|
| 1 | *E. coli* ATCC 25922 | 4,3,3 | 4,4,4 | 4,4,4 | 4,3,4 | 2,3,2 |
| 2 | *S. typhi* NCTC 781 | 4,4,4 | 4,4,4 | 4,4,4 | 2,3,3 | 3,2,1 |
| 3 | *P. aeruginosa* ATCC 27853 | 4,3,4 | 4,4,4 | 4,4,4 | 3,2,3 | 2,2,2 |
| 4 | *K. pneumoniae* | 3,3,3 | 4,4,4 | 4,4,4 | 4,4,4 | 3,3,2 |
| 5 | *S. aureus* ATCC 25923 | 4,4,4 | 4,4,3 | 3,2,2 | 2,0,0 | 0,0,0 |
| 6 | *S. marcescens* DMS 0300 | 4,3,4 | 4,4,4 | 4,4,4 | 4,4,4 | 2,0,2 |
| 7 | *Streptococcus* C203S | 0,0,0 | 0,0,0 | 0,0,0 | 0,1,0 | 0,0,0 |
| 8 | *C. albicans* CDC 85-000000 | 4,3,4 | 4,4,3 | 0,0,0 | 0,0,0 | 0,0,0 |

4= firm gel;3= soft gel;2= weak gel;1= very weak gel;0= no gelation.

Detection of endotoxin in commercial parenteral products The unknown samples were tested in triplicate and grouped in a set with negative and positive control, and inhibition test. The lysates used for this test were TF-04,CF-05, TF-05, CM-06, TM-06, CF-06, and TM-07.

Table 23. Amoebocyte lysate test with commercial samples .

| S.N. | CODE NO. | Samples 1 2 3 | Amoebocyte lysate reactions Neg. control | Inhibition | Pos. control |
|---|---|---|---|---|---|
| 1 | S-01 | 0 0 0 | | | |
| 2 | S-02 | 0 0 0 | | | |
| 3 | S-03 | 2 2 1 | 0 0 0 | 4 4 4 | 4 4 4 |
| 4 | S-04 | 0 0 0 | | | |
| 5 | S-05 | 0 0 0 | | | |
| 6 | S-06 | 3 2 1 | 0 0 0 | 4 4 4 | 4 4 4 |
| 7 | S-07 | 0 0 0 | | | |
| 8 | S-08 | 0 0 0 | | | |
| 9 | S-09 | 0 0 0 | 0 0 0 | 4 4 4 | 4 4 4 |
| 10 | S-10 | 0 0 0 | | | |
| 11 | S-11 | 0 0 0 | | | |
| 12 | S-12 | 0 0 0 | 0 0 0 | 4 4 4 | 4 3 4 |
| 13 | S-13 | 0 0 0 | | | |
| 14 | S-14 | 4 4 4 | | | |
| 15 | S-15 | 0 0 0 | 0 0 0 | 3 4 4 | 4 4 4 |
| 16 | S-16 | 0 0 0 | | | |
| 17 | S-17 | 0 0 0 | | | |
| 18 | S-18 | 0 0 0 | | | |
| 19 | S-19 | 0 0 0 | 0 0 0 | 4 3 4 | 3 4 4 |
| 20 | S-20 | 0 0 0 | | | |
| 21 | S-21 | 4 4 4 | | | |
| 22 | S-22 | 0 0 0 | 0 0 0 | 4 4 4 | 4 4 4 |
| 23 | S-23 | 0 0 0 | | | |
| 24 | S-24 | 0 0 0 | | | |
| 25 | S-25 | 0 0 0 | 0 0 0 | 0 0 0 | 4 4 4 |

4= firm gel;3= soft gel;2= weak gel;1 = very weak gel;0 = no gelation.

Shelf life of the lysate There were two forms of the lysate

that subjected for shelf-life determination , i.e., lyophilized form and liquid form.

Lyophilized form The lysate TF-02 and CM-01 were rapidly thawing from -20°C and the activity checked which were found to be in the level of 0.1 ng/mL endotoxin. Then 4 mL of each lysate were mixed with 2% dextran solution as stabilizer in the volume ratio of 1:1 and hence the concentration of dextran was 1% in total lysate mixture. These mixtures were then rapidly frozen with acetone-dry ice mixture. The frozen lysate mixtures were thawed and were found to have the activity at 0.1 ng/mL of endotoxin. Then the lysate mixtures were subjected to lyophilization. The activity of lyophilized products were found to possess no activity. The lysate TM-05 and CF-06 were used in repeat experiment and was also found no activity after lyophilization.

Liquid form The lysate lot no. TF-01, CF-01, TM-05, CF-06, TF-05, and CM-07 were separately provided for shelf-life test in -20°C storage and were taken out for activity testing periodically as shown in table 24. The lysate TF-01, CF-01, TM-05, and CF-06 were found to possess the activity only 60 days after the preparation and at 75 days after the preparation, there were no activity even with 100 ng/mL. Four mL of lysate lot no. TF-05 were prepared with tris succinate buffer pH 7.4 and kept at -20°C along with the lysate TF-05 and CM-07 as kept normally in tris buffer pH 7.4. The result of the three batches of lysate were found the same as the previous four lots of the lysate.

Table 24. The activity of the lysate in different period.

| Lysate no. | Shelf-life period (days) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 15 | 30 | 45 | 60 | 75 |
| TF-01 | 4 | 4 | 4 | 4 | 4 | 0 |
| CF-01 | 4 | 4 | 4 | 4 | 4 | 0 |
| TM-05 | 4 | 4 | 4 | 4 | 4 | 0 |
| CM-05 | 4 | 4 | 4 | 4 | 0 | 0 |
| TF-05 | 4 | 4 | 4 | 4 | 4 | 0 |
| CM-07 | 4 | 4 | ND | ND | ND | ND |
| TF-05* | 4 | 4 | 4 | 4 | 4 | 0 |

4 = firm gelation at 0.1 ng/mL.
0 = no gelation at 2 ng/mL.
* = with tris succinate buffer.
ND = not done.