

Bacterial Endotoxin Test by Gel-Clot Method

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ABSTRACT

The Bacterial Endotoxin Test (BET) is a test to quantify Endotoxin from 'Gram-negative bacteria using 'amoebocyte lysate' extracted from '*Limulus polyphemus* or *Tachypleus tridentatus*' i.e. horseshoe crab. Three techniques are used for the confirmation of endotoxins, those are: the gel-clot technique, the turbidimetric technique, and the chromogenic technique. Endotoxins are toxic complexes which is invariably associated with the cell-wall of the Gram-negative bacteria irrespective of bacterial pathogenicity. Endotoxins are rarely fatal, but they cause fever and hence Endotoxin carrying bacteria are known as 'Pyrogen'.

The test can be done by any of these three techniques. Among all, the gel-clot method is chosen to be superior. The test is carried out in a manner to avoid endotoxins contamination. Mostly bacterial Endotoxin test are performed for the inject-able pharmaceutical products, e.g. SWFI (Sterile Water for Injection), Normal Saline (Sodium Chloride Sterile) and other injections like Amikacin sulphate, Ascorbic acid, etc. Endotoxin had been authenticated as inflammatory agent, onset clinical diabetes, obesity, nausea, vomiting, diarrhea, fever, etc. Endotoxins are thus very necessary to get removed out from the inject-able pharmaceutical products.

Keywords: Endotoxin, Gram-negative bacteria, Amoebocyte lysate, Lysate validation, Lysate reconstitution, Maximum Valid Dilution, Endotoxin Limit, Gel-clot technique

INTRODUCTION

Endotoxins are toxic complexes which is invariably associated with the cell-wall of the Gram-negative bacteria irrespective of bacterial pathogenicity. Endotoxins are rarely fatal, but they cause fever and hence Endotoxin carrying bacteria are known as 'Pyrogen'. Endotoxin is toxic substances which is bound with the bacterial cell wall and are released when the bacterium disintegrates. Endotoxin composed of lipopolysaccharide and lipoprotein complexes. The components that are protein determine its antigenic property; and the polysaccharide component determines the antibody type that can react with the Endotoxin molecule. The reaction of polysaccharide components with the Endotoxin when allowed produces an immune reaction. Endotoxin is rarely fatal, although they often cause fever.

Thus, Bacterial Endotoxin test is the confirmatory test that assures the presence or absence of the Endotoxin in the medicine sample. Mostly bacterial Endotoxin test are performed for the inject-able pharmaceutical products, e.g. SWFI (Sterile Water for Injection), Normal Saline (Sodium Chloride Sterile) and other injections like Amikacin sulphate, Ascorbic acid, Esmoprazole sodium, Methylprednisolone, Iron sucrose, Ondansetron, Ranitidine hydrochloride, Thiopental sodium sterile, etc. All these products have their specific Endotoxin limit value determined by 'EU/mg'.

GRAM-NEGATIVE BACTERIA

In the method of bacterial differentiation such bacteria do not retain the crystal violet stain used in the gram-staining.

These bacteria are characterized by their cell envelopes. Their cell wall are composed of a thin peptidoglycan cell-wall sandwiched between an inner cytoplasmic cell membrane and a bacterial outer membrane.

Gram-negative bacteria are found almost everywhere. The gram-negative bacteria include the model organism *Escherichia coli*, as well as many pathogenic bacteria. Their outer membrane protects them from many antibiotics (including penicillin); detergents that would normally damage the peptidoglycans of the (inner) cell membrane; and lysozyme, an antimicrobial enzyme produced by animals that forms part of the innate immune system. Additionally, the outer leaflet of this membrane comprises a complex lipopolysaccharide (LPS) whose 'lipid A' component can cause a toxic reaction when these bacteria are lysed by immune cells. This toxic reaction can include fever, an increased respiratory rate, and low blood pressure — a life-threatening condition known as septic shock.

Some examples of Gram-negative bacteria that have demonstrated drug resistance include

- *E. coli*, which causes the majority of urinary tract infections
- *Acinetobacter baumannii*, which causes disease mainly in healthcare settings. In addition, wound infections caused by *Acinetobacter* have been found in U.S. military personnel who were deployed to Iraq and Afghanistan.

- *Pseudomonas aeruginosa*, which causes bloodstream infections and pneumonia in hospitalized patients. It is a common cause of pneumonia in patients with cystic fibrosis.
- *Klebsiella pneumoniae*, which causes many types of healthcare-associated infections, including pneumonia, urinary tract infections, and bloodstream infections
- *Neisseria gonorrhoeae*, which causes the sexually transmitted disease gonorrhea, is the second most commonly reported infectious disease in the United States.

ENDOTOXINS

The bacteria those are associated with Endotoxin grows side by side with fungus and or mold in watery environments specifically. These gram negative micro-organisms (bacteria) collectively get accumulated in the human digestive system and thus these microorganisms are responsible for the inflammation in the gut and also in different parts of the body. Endotoxin had been authenticated as inflammatory agent, onset clinical diabetes, obesity, nausea, vomiting, diarrhea, fever, disseminated intravascular coagulation, vascular collapse, and organ failure.

Endotoxins are thus very necessary to get removed eliminated out from the inject-able pharmaceutical products.

AMOEBOCYTE LYSATE

The term 'lysate' is defined as the mixture of substances which is formed by the lysis of the cells. Amoebocyte lysate is associated with the WBC of horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). Amoebocyte lysate is a lyophilized product procured from the lysate of amoebocyte from the horseshoe crab. Lysate those are obtained from '*Limulus polyphemus*' is known as '*Limulus Amoebocyte Lysate*', while lysate those are obtained from '*Tachypleus tridentatus*' is known as '*Tachypleus Amoebocyte Lysate*'. Amoebocyte lysate is needed to be dissolved in the water associated with the types of lysate e.g. for '*Limulus*

Amoebocyte Lysate' the water that is needed to reconstitute the lysate is thus '*LAL Reagent Water*' whereas for '*Tachypleus Amoebocyte Lysate*' the water that is needed to reconstitute the lysate is thus '*TAL Reagent Water*'. Lysate is lyophilized and it should be stored at 2- 8° C.

LYSATE VALIDATION

Lysate validation is a no touch technique. It directs towards the preparation of '*Control Standard Endotoxin*' (CSE). Before the preparation of '*Control Standard Endotoxin*' (CSE), first we need to check the '*Certificate of Analysis*' (CoA) and needed to match the Standard Endotoxin, LAL Reagent Water and Lysate. Thereafter the reconstitution of CSE shall be conducted. The following steps are required to be followed for the reconstitution of CSE, or in other words for the 'Lysate Validation':

1. CSE is needed to be reconstituted with the 5 ml of LAL Reagent Water (LRW).
2. It is then vortexed for about 15 min. Reconstituted CSE can be stored at 4°C for 28 days and every time when it has to be used always vortex it for 15 min before use.
3. If the given Concentration is 20 EU/ml according to the CoA, then the dilution of 1EU/ml can be prepared using the following process:

Took 100µl or 0.1ml of CSE and Mix 1.9ml of LRW
100µl CSE + 1.9ml of LRW = 1Eu/ml

4. If the given Concentration is 22EU/ml according to the CoA, then the dilution of 1EU/ml can be prepared using the following process:

In 100µl or 0.1ml of CSE and Mix 2.1ml of LRW
100µl CSE + 2.1ml of LRW = 1Eu/ml

5. After then the dilution is thus being prepared by the following dilution process.

Table-01

| Dilution Process | Identifiers | Concentration (EU/ml) | Sensitivity λ |
|------------------------------------|-------------|-----------------------|---------------|
| 0.1 ml (20 EU CSE) + 1.9 ml of LRW | A | 1 | 8 λ |
| 1 ml (A) + 1ml of LRW | B | 0.5 | 4 λ |
| 1 ml (B) + 1ml of LRW | C | 0.25 | 2 λ |
| 1 ml (C) + 1 ml of LRW | D | 0.125 | λ |
| 1 ml (D) + 1 ml of LRW | E | 0.06 | λ/2 |
| 1 ml (E) + 1 ml of LRW | F | 0.03 | λ/4 |

LYSATE RECONSTITUTION

1. While proceeding towards the Lysate reconstitution, the label of the Lysate is needed to be checked first.
2. If the label of the Lysate indicates 1.8ml, then it signifies that it is for 16 tests from now.
3. Lysate is thus being reconstituted by adding 1.8 ml of LRW. After the addition of LRW to the Lysate, it is required to '*swirl gently and not to vortex it*'.
4. If the label of the Lysate indicates 5.2ml, then it signifies that it is for 50 tests from now.
5. Lysate is thus being reconstituted by adding 5.2 ml of LRW. After the addition of LRW to the Lysate, it is required to '*swirl gently and not to vortex it*'.

Reconstituted Lysate can be stored for 24 hours at 2°C-8°C. For longer storage, reconstituted Lysate can be stored at -8°C or lesser than this temperature.

This test should be in Quadruplicate reaction tube to be conducted by Following Process:

Table-02

| Lysate Sensitivity | CSE Quantity | Lysate Quantity | Result |
|--------------------|---------------------------------|-----------------|----------|
| 2λ (4 tubes) | 0.1ml of sample C from Table-01 | 0.1ml of Lysate | +ve |
| λ (4 tubes) | 0.1ml of sample D from Table-01 | 0.1ml of Lysate | +ve /-ve |
| λ/2 (4 tubes) | 0.1ml of sample E from Table-01 | 0.1ml of Lysate | +ve /-ve |
| λ/4 (4 tubes) | 0.1ml of sample F from Table-01 | 0.1ml of Lysate | -ve |
| NC (4 tubes) | 0.1ml of LRW | 0.1ml of Lysate | -ve |

This test is also known as the 'Assay Test'.

Here, NC stands for the Negative Control.

1. After the preparation of the dilution according to the Table-02, all the respective reaction tubes are incubated for 60minutes, at 37°C, in the heating mantle.
2. After the completion of 60minutes, the incubation of the reaction tubes are over, and thus it is necessary to remove the reaction tubes one by one carefully and is inverted about 180°.
3. The tubes are then to be observed and the result of the reaction in each of the respective tubes has to be recorded. As if the clot formation takes place in the reaction tubes, then it means Endotoxin was present in that reaction tube and thus the result is considered to be positive. And if the clot formation doesn't takes place, then it means Endotoxin was absent in that reaction tube and thus the result is considered to be negative.

MAXIMUM VALID DILLUTION (MVD)

The maximum allowable dilution of a sample at which the Endotoxin Limit can be determined is known as the maximum valid dilution.

$$\text{MVD} = (\text{endotoxin limit} \times \text{concentration of sample solution}) / (\lambda)$$

ENDOTOXIN LIMIT

The endotoxin limit of parenteral drug, defined on the basis of dose, equals K/M^2 , where K is a threshold pyrogenic dose of endotoxin per kg of body weight. When the product is injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period. The endotoxin limit of the parenteral drugs is specified in the individual monograph in units such as EU/ml, EU/mg, EU/Unit of biological activity, etc.

Parenteral drugs are the sterile preparations containing one or more active ingredients intended to be administered to a patient other than the alimentary canal (gastrointestinal tract) routes.

The concentration of the sample solution:

1. mg/ml: in the case of endotoxin limit particularized by weight (EU/mg);
2. ml/ml: in the case of endotoxin limit particularized by volume (EU/ml);
3. Units/ml: in the case of endotoxin limit particularized by unit of biological activity (EU/unit);

DILLUTION PREPARATION TECHNIQUE GEL-CLOT TECHNIQUE

4. λ: the labeled sensitivity in the Gel-Clot technique (EU/ml).

Following are the few parenteral drugs raw material and their endotoxin limits:

Table-03

| S. No. | Raw Material | BET Limit (EU/mg) |
|--------|---------------------------------------|-------------------|
| 1. | Amikacin sulphate inj. | 0.33 |
| 2. | Ascorbic acid inj. | 1.2 |
| 3. | Esmoprazole sodium sterile | 0.125 |
| 4. | Methylprednisolone | 0.17 |
| 5. | Iron sucrose | 3.7 |
| 6. | Ondansetron inj. | 9.9 |
| 7. | Ranitidine hydrochloride inj. | 7.0 |
| 8. | Thiopental sodium sterile | 1.0 |
| 9. | Triamcinolone acetone inj. | 4.4 |
| 10. | Hydrocortisone sod. succinate sterile | 1.25 |
| 11. | Rabeprazole | 0.125 |
| 12. | Pantoprazole | 0.20 |
| 13. | Omeprazole | 0.125 |
| 14. | Vancomycin sodium sterile | 0.25 |
| 15. | Gentamycin sulphate sterile | 1.67 |
| 16. | Dexamethasone | 31.3 |
| 17. | Sodium chloride | 0.25 |
| 18. | Cefoperazone & sulbactam inj | 0.17 |
| 19. | Ceftriaxone inj. | 0.20 |
| 20. | Cefotaxime sodium | 0.20 |
| 21. | Ceftazidime inj. | 0.17 |
| 22. | Cefuroxime inj. | 0.1 |
| 23. | Sterile Water for Injection | 0.25 |
| 24. | Paracetamol infusion | 2.0 |
| 25. | Lignocaine | 1.1 |
| 26. | Piperacillin tazobactam | 0.08 |
| 27. | Artisunate | 1.25 |
| 28. | Meropenem | 0.125 |
| 29. | Sodium bicarbonate | 1.7 |
| 30. | Flucloxacillin sodium | 0.2 |
| 31. | Dicyclomine HCl | 17.2 |
| 32. | Ampilon | 0.15 |
| 33. | Monoasthal | 1.0 |
| 34. | Iron dextrose | 0.05 |
| 35. | Betamethasone sodium phosphate | 29.2 |
| 36. | Tobramycin sulphate | 2.0 |
| 37. | Hyoscine-N-Butylbromide | 1.94 |

Table-04

| Test for product | LRW (μl) | CSE (μl) (4λ) | Sample (μl) | Lysate (μl) | Observation |
|--------------------------|----------|---------------|-------------|-------------|-------------|
| Negative water Control | 100 | ---- | ---- | 100 | -Ve |
| Negative Product Control | 50 | ---- | 50 | 100 | -Ve |
| Positive water Control | 50 | 50 | ---- | 100 | +Ve |
| Positive Product Control | ---- | 50 | 50 | 100 | +Ve |

Table-05

| Test for SWFI | LRW (μl) | CSE (μl) (20λ) | Sample (μl) | Lysate (μl) | Observation |
|---------------------------------|----------|----------------|-------------|-------------|-------------|
| Negative water Control | 100 | ---- | ---- | 100 | -Ve |
| Negative Product Control | ---- | ---- | 100 | 100 | -Ve |
| Positive water Control | 100 | 10 | ---- | 100 | +Ve |
| Positive Product Control | ---- | 10 | 100 | 100 | +Ve |

OBSERVATION

1. All the respective reaction tubes are incubated for 60minutes, at 37°C, in the heating mantle.
2. After the completion of 60minutes, the incubation of the reaction tubes are over, and thus it is necessary to remove the reaction tubes one by one carefully and is inverted about 180°.
3. The tubes are then to be observed and the result of the reaction in each of the respective tubes has to be recorded. As if the clot formation takes place in the reaction tubes, then it means Endotoxin was present in that reaction tube and thus the result is considered to be positive. And if the clot formation doesn't takes place, then it means Endotoxin was absent in that reaction tube and thus the result is considered to be negative.

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