

AN OVERVIEW OF LIMULUS AMOEBOCYTE LYSATE (LAL) TEST

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ABSTRACT

Limulus Amoebocyte Lysate (LAL) test is in vitro test to detect and measure bacterial endotoxins. It is the most critical quality control test required by FDA for all parenteral drugs in their final stages of formulation. Endotoxins are products of metabolism associated with outer membrane of gram negative bacteria especially *Pseudomonas*, *E. coli* and *Serratia* and are most significant source of pyrogens. They are ubiquitous and cause profound physiological changes when administered parenterally. Also they play an important role in occurrence and development of many diseases. Their detection is very important in manufacturing of parental drugs, biological products and medical devices. There is specification of endotoxin limit provided in monograph of each pharmacopoeia. The conventional rabbit test has several disadvantages. LAL test is recently developed quantitative test has overcome rabbit test due to many reasons. The article will discuss about the basic principle, development, different methods, comparison with rabbit test and applications of the test.

KEYWORDS: Pyrogens, amoebocytes, endotoxin limit, lipopolysaccharides.

INTRODUCTION

Sterile products are parenteral products which are injected through the skin or mucous membranes into internal body components. They must be free from microbial contamination, from toxic components as well as should be pure. All the processes involved must be designed to eliminate contamination of all types whether physical, chemical or microbiological origin¹. Pyrogens are produced mainly by gram negative bacteria and form a part of endotoxin (osmotic antigen). All the vehicles used in parenteral are tested for pyrogens. Many medicinal agents if present interfere with the test results because of their antipyretic or other interfering effects. Hence pyrogen test is performed on those finished products which do not interfere with the test. Drugs that are to be intravenously injected must be of a pyrogen-free quality. For other parenteral drugs, given subcutaneously or intramuscularly in much smaller volumes (e.g. vaccines) a maximal acceptable endotoxin concentration has to be defined for quality-control purposes.

Water is used as the vehicle for most injections as aqueous preparations are tolerated well by the body and are safest and easiest to administer. In preparation of water for injection removal of entrained contaminants from the vapour before it is condensed by passage through an efficient baffle system eliminate pyrogens

from the product. Endotoxins are also removed by lipopolysaccharide affinity resins, two phase extractions, ultra filtration, hydrophobic interaction chromatography, ion exchange chromatography and membrane adsorbers. The injection of distilled water may cause a rise in body temperature water producing this reaction is called pyrogenic meaning producing fever. Water free from this is called apyrogenic water. Pyrogens are high molecular weight complexes², although endotoxins are related with cell wall they are continuously liberated into the environment. Release happens during growth and division or even with death of bacteria. A single *E. coli* contains about 2 million lipopolysaccharide molecules per cell. Endotoxin does not act directly against cell or organs but through activation of immune system especially through monocytes and macrophages.

Pyrogens are products of metabolism from bacteria, molds and viruses, non volatile, filterable (hence cannot be removed by bacteria proof filters) and thermostable. There are several different pyrogens with chemical structures closely related to each other. Bacteria shed small amounts of endotoxins into their surroundings while they are actively growing and large amounts when they die. Endotoxin consists of a complex of a pyrogenic lipopolysaccharide which is composed of a protein and inert lipid. (Figure1) Lipopolysaccharides (LPS) constitute the O-antigens and endotoxins of Gram-

negative bacteria whereas both the polysaccharide and lipid portion of LPS contribute to the pathogenic potential of this class of bacteria. It is the lipid component (lipid A) which determines the pyrogenicity endotoxic properties of LPS³. Since lipid is embedded in outer membrane of bacterial cells it exerts its toxic effects when released from multiplying cells or when bacteria are lysed as a result of autolysis, ingestion and killing by phagocytes or certain types of antibiotics. A polysaccharide increases its activity as it becomes more soluble. Pyrogens are more serious in large volume injections. As large volume injections are given intravenously and vehicle is aqueous, pyrogens have a rapid effect. Eliminating the pyrogens is extremely important. The apparatus and method of storage of injectables after distillation, inadequate protection from air, storage at temperature that favours bacterial growth may increase bacterial content. It may be 3800000/ml after 15 days. Also gram negative bacteria are widely used to produce recombinant DNA products such as peptides and proteins. Such products are always contaminated with endotoxins.

LIMITATIONS OF RABBIT TEST

The presence of pyrogenic substances in parenteral preparations is determined by qualitative biological method based on fever (rise in temperature) of individual or sum response of rabbits⁴. After about 1Hr pyrogenated injection causes rise in body temperature, chills, body ache, cutaneous vasoconstriction and rise in arterial blood pressure. In this method a substance is injected into the marginal ear vein of rabbit and elevation of temperature is measured within a period of three hours. Maintaining the conditions of housing, handling of rabbits and getting consistent results are the main requirements of a rabbit test. However, the rabbit pyrogen test has several limitations. The sensitivity of rabbits towards endotoxin reference preparations depends on the strain used and the experimental conditions (for example, age, gender and housing conditions^{5,6}). The rabbit strain to be used is not defined in the respective monographs of the pharmacopoeias. Even if the highest permitted volume (10ml/kg body weight) is injected, the detection limit is restricted to 50–350pg (i.e., 0.5–3.5IU) of LPS/kg; however, the human fever threshold is around 30pg/ml^{7,8} and for many drugs the volume tested is significantly smaller.

The rabbit pyrogen test gives only a pass/fail result and is not suitable for the control of endotoxin limit. Hence it is not a quantitative test, and it is less-well standardised^{9,10}. Age related changes in endotoxin sensitivity and the febrile response of new born rabbits¹¹

is another factor to be considered while performing a rabbit test. A positive result in the rabbit pyrogen test demonstrates contamination with pyrogens according to the requirements and definitions of relevant monographs and guidelines. However, there remains a gap between the observed pyrogenicity in rabbits and the expected pyrogenicity in humans. Due to species differences, some materials (for example, biologicals for human use) might cause fever in humans but not in rabbits, and vice versa. Secondly, the rabbit test is not sensitive enough to detect endotoxin in water for reconstitution (LAL reagent water) in bacterial endotoxin test intended for tests on products with a very low endotoxin limit. It is not quantitative, it is less sensitive than the LAL test, the outcome depends on the rabbit strain and housing conditions; it is not suitable for all product categories; it is expensive; and it involves the use of animals. Also radiopharmaceuticals cannot be detected in rabbit tests.

PRINCIPLE OF LAL TEST

The LAL reagent is derived from blood cells (amoebocytes) of a horse shoe crab – *Limulus Polyphemus*. Other species of a horse shoe crab used are namely *Tachypleus gigas*, *Tachypleus tridentatus* and *Carcinoscorpius Rotundicauda*¹². The oxygen-carrying pigment in horseshoe crab blood is a protein called hemocyanin. It is very similar to the hemoglobin molecule in human blood. Hemocyanin contains a copper molecule which gives a blue colour to blood. Blood is removed from the crab's body, the blood cells are separated from the serum using centrifugation and are then placed in distilled water, which causes them to swell up and burst ('lyse'). This releases the chemicals from the inside of the cell (the 'lysate'), which is then purified and freeze-dried. To test a sample for endotoxins, it is mixed with lysate and water; if coagulation occurs endotoxins are present.¹³ (Figure 2)

ENDOTOXIN LIMIT (EL)

The quantities of endotoxins are expressed in defined Endotoxin Unit (EU)

1 EU= 1 IU IU- International Unit. EL= K/M

Where,

K- Maximum No. of IU of endotoxin which the patient may receive without suffering toxic reactions (minimum pyrogenic dose)

M- Maximum dose of the drug substance per kg per hour. (Table 1)

REQUIREMENTS OF LAL TEST

LAL Test Reagent: LAL Test Reagent contains synthetic chromogenic substrate and buffered amoebocyte lysate of any of the species of a crab stabilized by monovalent and divalent cations. It is freeze

dried (LAL Powder) and reconstituted before test. It can also be reconstituted with buffer, a beta glucan inhibiting buffer, to render the assay endotoxin specific and prevent potential enhancement and false positives caused by glucans.

LAL powder can be stored at 2 to 8°C. Prolonged exposure to 25°C should be avoided. Reconstituted LAL can be stored at 2 to 8°C during intermittent use up to 24 hours or it can be stored at -20°C for two weeks after reconstitution. The reconstituted lysate is stable for 24 hours at 2–8°C or for two weeks at -20°C or colder if frozen immediately after reconstitution. Reconstituted lysate may be frozen and thawed once.

LAL Reagent Water (Non LAL active) : It is used to reconstitute LAL reagent and prepare samples, controls and endotoxin standards. It should be free from bacterial endotoxins. It is prepared by triple distillation in an apparatus with an arrangement to remove endotoxins.

The Endotoxin Reference Standard: It is a purified endotoxin of *Escherichia coli* calibrated with International standard.

Control Standard Endotoxin: It is suitably standardized against Endotoxin Reference Standard and is used for routine bacterial endotoxin testing.

LAL test conditions: The test should be carried out in aseptic environment to avoid microbial contamination. The containers used in the test should be sterilized in oven at 250°C or above for not less than 60 minutes in order to remove surface endotoxins.

GEL CLOT METHOD

Principle: This is the simplest method. In presence of endotoxin a proenzyme in amoebocyte lysate is converted into an active form. The amount of active enzyme generated is dependent upon concentration of endotoxin originally present. The active enzyme then cleaves a clotting protein, also found within the *Limulus* Amoebocyte. The cleaved fragments self aggregate to form a clot. Optimum gel clot occurs for a mixture pH 6.0 to 8.0. Tenth of a millimetre of LAL is incubated with an equal volume of sample for one hour at 37°C. After incubation period reaction tubes are inverted. If clot remains intact after inversion, the test is positive. Maximum sensitivity of the test is 0.03 EU/mL. Gel clot method is the least expensive method as no optical reader is required. It is the method of choice for opaque samples, suspensions and coloured samples. Equipment used in Gel Clot Method is inexpensive. It is a manually read test. Reagents of different sensitivity used are 0.5, 0.25, 0.125, 0.06 and 0.03 EU/mL. Since less sensitive to interference than other methods, it is used as a referee method for majority of products.(Figure 3)

Lysate sensitivity test: Since the gel clot test is a qualitative test only, it is essential to determine minimum amount of endotoxin required for clot formation under standard conditions. This minimum quantity of endotoxin is termed as lysate sensitivity. It ensures the precision and validity of the test. It is expressed in IU/ml; of the lysate solution prior to use in the test. Confirmation of Lysate sensitivity is carried out when a new batch of lysate is used or when there is any change in the experimental conditions which may affect the test. Standard solution of at least 4 concentrations equivalent to 2, 1, 0.5 and 0.25 by diluting the standard endotoxin stock solution with water for BET is prepared. Volume of Lysate solution is mixed with equal volume of one of the standard solutions (such as 0.1 ml aliquots) in each tube and is incubated at 37°C for one hour avoiding vibration. A firm gel that remains in place after inversion shows that a test is positive. The lowest concentration of standard solution should show a negative test result.

Test for interfering factors

The test preparation should not inhibit or enhance the reaction or otherwise interfere with the test. The test for interfering factors is repeated when any changes are made in experimental conditions that influence the result of the test. The water for BET is diluted and Maximum Valid Dilution ((MVD) is measured which is dependent on the concentration of the product. If the preparation being examined interferes with the test at a dilution less than MVD, test for interfering factors is repeated using a greater dilution not exceeding MVD.

TURBIDIMETRIC METHOD

This is the most sensitive method available (maximum sensitivity is 0.001 EU/mL.) It is based on development of turbidity after cleavage of an endogenous substrate¹⁵. This test is based upon measurement of optical density (photometric test) which increases after clotting reaction. Based on the test principle employed this technique is classified as being the endpoint turbidimetric test or kinetic-turbidimetric test. The end point turbidimetric test is based upon quantitative relationship between the endotoxin concentration and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period. The Kinetic-Turbidimetric test is a method to measure either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance or the rate of turbidity development. The test is carried out at incubation temperature at 37°C.

CHROMOGENIC METHOD

LAL reagent is formulated with a synthetic substrate gives yellow colour when acted upon by endotoxin activated enzyme. Reagent sample mixture is incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a microplate reader equipped with a 405nm filter with a sample lysate ratio of 1:1. The sensitivity for chromogenic assays is determined by the lowest standard concentration on the standard curve used for the assay. Software is used to construct the standard curve and calculate the endotoxin concentrations. The maximum sensitivity of this method is 0.005 EU/mL.

It is based upon the development of colour after cleavage of a synthetic peptide chromogen complex¹⁶. It is used to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the lysate. This test is classified as end point chromogenic test or kinetic chromogenic test. The end point chromogenic test is based upon quantitative relationship between the endotoxin concentration and the quantity of chromophore released at the end of incubation period. The Kinetic chromogenic test measures either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance or the rate of colour development. The test is carried out at incubation temperature¹⁷.

APPLICATIONS OF LAL TEST

1. The LAL test has been used to rapidly diagnose urinary tract infections and spinal meningitis. In these cases, the presence of endotoxin is almost always indicative of living bacteria, i.e., an infection, and the types of bacteria causing these infections are few and quite similar.
2. The LAL test has also been used to assess food spoilage (fish, milk, ground beef), air and water quality, and (in experiments) to determine the ability of new drugs to neutralize the toxic effects of endotoxin.
3. Since LAL detects endotoxin, a component of Gram-negative bacteria, the test can also be used to detect the presence of these bacteria. Advantage of LAL test is that in-process solutions, raw materials can be tested.
4. Injectables, biological products, surgical devices or medical devices and renal dialysis fluids can be tested for absence of pyrogens by LAL test.
5. LAL test can be used as in process testing and is 5 to 10 times more sensitive than rabbit testing method.
6. As it is a quantitative method, concentrations of bacterial endotoxins present in samples can be detected. LAL test is used for in process testing and selective product release testing¹⁸.

7. (1₃)- β -D-glucan, a material found in fungal cell walls, plant tissue, in some algae and in some bacteria can be detected by LAL test .
8. The exquisite sensitivity of LAL compared to other assays for endotoxin has proven extremely useful in monitoring high purity water as a prime ingredient or processing agent for all biologicals, drugs and devices.
9. LAL test is also assay of choice for researchers studying both the clinical and environmental effects of endotoxin.

LIMITATIONS OF LAL TEST

1. LAL test cannot discriminate between living and dead bacteria.
2. LAL test cannot differentiate species of bacteria-endotoxin, which cause a similar reaction with LAL.

CONCLUSION

Although rabbit test exists for the detection of endotoxin, the LAL test is the most effective because it is capable of detecting endotoxins qualitatively as well as quantitatively.(As little as one millionth of a billionth of a gram of endotoxin.) LAL test is currently recognized by several major pharmacopoeias and is used worldwide. LAL test has proven its usefulness in not only to detect harmful effects of endotoxin (as pyrogens) in pharmaceutical products but has become an indispensable tool in controlling endotoxin in process and equipment used to produce pharmaceuticals.

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Table 1: Endotoxin limit of various parental products

| TYPE OF PRODUCT | ROUTE OF ADMINISTRATION | K PER Kg |
|---|--|----------|
| All parental preparations | Intrathecal | 0.2 |
| Radiopharmaceuticals | Intravenous | 2.5 |
| All parental preparations except radiopharmaceuticals | All parental routes except intrathecal | 5.0 |

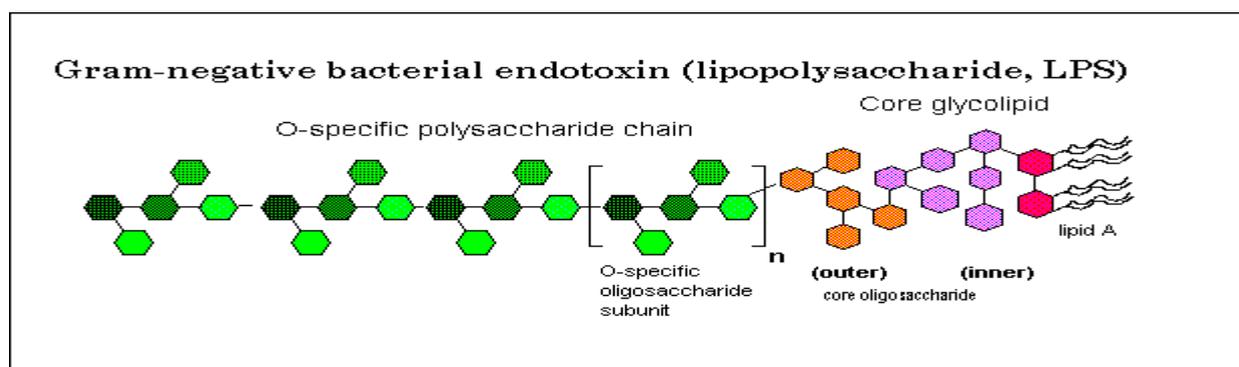


Figure 1 Structure of Endotoxin¹⁹

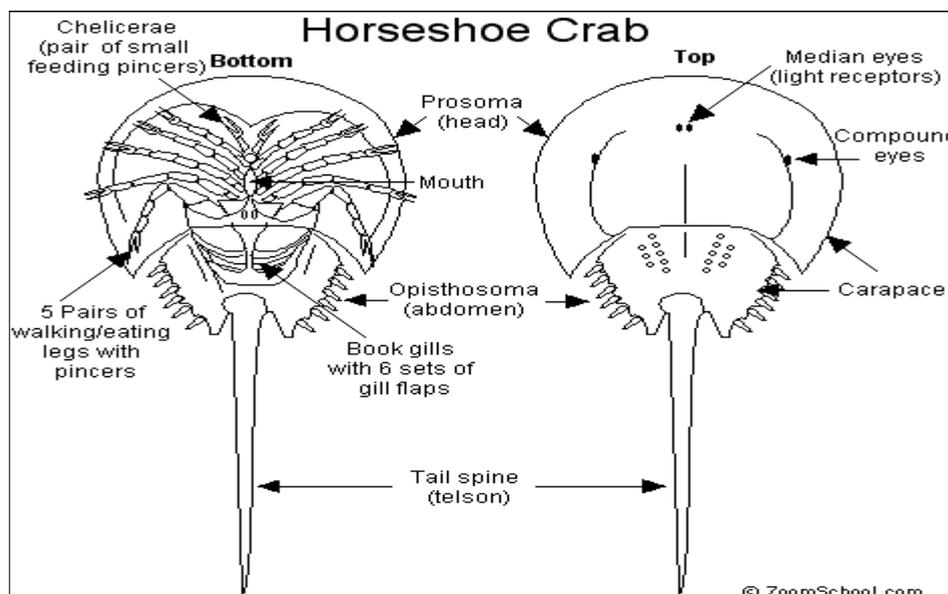


Figure2 –Morphology of horseshoe crab¹⁴

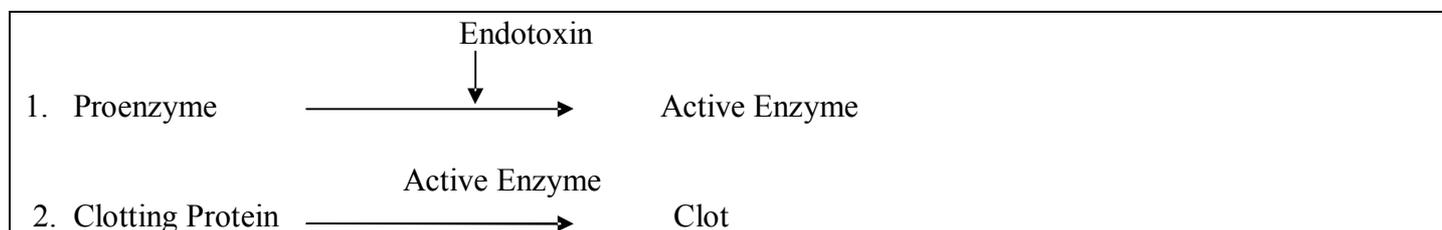


Figure 3: Reaction sequence of Gel Clot Method